

## **Resistance to TRAIL-induced apoptosis: Role of *IG20* splice variants**

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### **Summary**

Tumor necrosis factor receptor-related apoptosis-inducing ligand (TRAIL) can induce apoptosis primarily in cancer cells with little or no effect on normal cells; therefore, has the potential for use in cancer therapy. TRAIL binding to death receptors DR4 and DR5 triggers the death inducing signal complex (DISC) formation and activation of procaspase-8, which in turn activates caspase-3 leading to cell death. Like FasL, TRAIL can trigger Type 1 (caspase-8→caspase-3) or Type 2 (caspase-8→Bid cleavage→caspase-9→caspase-3) apoptotic pathways depending on the cell type. Some cancers are resistant to TRAIL treatment because most molecules in the TRAIL signaling pathway, including FLIPs and IAPs, can contribute to resistance. In addition, we have identified an essential role for splice variants of the *IG20* gene in TRAIL resistance.

The *IG20* gene was identified by subtractive hybridization of human insulinoma-glucagonoma. Through alternative splicing of exons 13L and 16, it can encode at least four different splice variants (SVs) in non-neuronal cells, namely IG20pa (IG20 pro-apoptotic), MADD (MAPK activating death domain), IG20-SV2 and DENN-SV (differentially expressed in normal and neoplastic cells). *IG20* SVs play important roles in cell survival, proliferation and apoptosis, and vesicular trafficking. MADD and DENN-SV are constitutively expressed at higher levels in cancers and cancer cells. MADD is required and sufficient for cancer cell survival, and can also confer TRAIL resistance, particularly in the absence of IG20pa, by acting as a negative regulator of caspase-8 activation. DENN-SV, co-expressed with MADD, can act like an “oncogene” and promote cell proliferation through NF- $\kappa$ B activation. When expressed, IG20pa can act as a tumor suppressor likely by functioning as a dominant negative of MADD. **The use of siRNA that targets all isoforms of *IG20* or the MADD isoform in conjunction with TRAIL to increase its efficacy is thus a distinct possibility.** Therefore, identifying key TRAIL specific resistance markers will help enhance the efficacy of TRAIL in cancer therapy.

### **Background**

#### **TRAIL and its receptors**

TRAIL is expressed as a trimeric type II transmembrane protein on mainly NK and NK-T cells. TRAIL can bind to 5 distinct receptors; Death Receptor 4 (DR4 or TRAILR-1), Death Receptor 5 (DR5 or TRAILR-2), Decoy Receptor 1 (DcR1, TRAILR-3, LIT or TRID), Decoy Receptor 2 (DcR2, TRAILR-4, TRUNDD) and Osteoprotegerin (OPG).

Among these, only DR4 and DR5 contain death domains and are able to mediate death signal, while DcR1 and DcR2 engage TRAIL, they fail to signal. OPG is a soluble receptor and its affinity for TRAIL is weak. Given the role of TRAIL in apoptosis and its expression on immune cells it is likely to contribute significantly to immune surveillance of virus infected and cancer cells. TRAIL has the ability to kill tumor cells, with little or no effect on most normal cells. Systemic administration of TRAIL can cause tumor shrinkage/elimination, without causing significant side effects (reviewed in 1). TRAIL in combination with chemotherapy and UV radiation, can be more effective. In contrast to FasL or TNF $\alpha$  based treatments that are associated with fulminant hepatitis and systemic inflammation respectively, TRAIL administration appears to lack these side effects; therefore, is an attractive candidate for cancer therapy (1-6).

TRAIL mediated apoptosis signaling in cancer cells is similar to that seen with Fas (CD95). DR4 and DR5, through their cytoplasmic death domains bind to the FADD death domain, which in turn interacts with procaspase-8 (or caspase-10) through its death effector domain. TRAIL binding causes increased recruitment of proteins to this Death Inducing Signaling Complex (DISC) leading to proximity induced caspase-8 activation. This causes subsequent caspase-3 (or caspase-7) activation resulting in degradation of inhibitor of caspase activated DNAase (ICAD). Proteolysis of ICAD releases CAD that cleaves the genomic DNA (7-8) (Figure 1).

In addition to the above Type 1 pathway, TRAIL mediated apoptosis can also recruit intrinsic or Type 2 pathway under conditions in which the caspase 8 levels are limiting and the proapoptotic molecule Bid is expressed. The intrinsic cell death pathway is regulated through a variety of Bcl2 family members which act primarily in mitochondria and endoplasmic reticulum. The anti-apoptotic members of the Bcl2 family such as Bcl2, Bcl-xL and Mcl1 contain four Bcl2 homology (BH1-BH4) domains, where as proapoptotic members such as Bax and Bak lack BH4 domain. In healthy cells, Bcl2 and Bcl-xL neutralize the activity of Bax/Bak. The equilibrium between the anti and proapoptotic Bcl2 family members is regulated by the mobilization of a third class of proapoptotic Bcl2 family members such as Bid, Bad, Puma and Noxa. In type 2 pathway, the activated caspase-8 cleaves Bid to form tBid whose BH3 domain is now exposed allowing it to interact with Bcl2/Bcl-xL and trigger intrinsic cell death pathway (9-10). A disruption in the equilibrium results in oligomerization of Bax/Bak at mitochondrial outer membrane leading to loss of mitochondrial integrity and leakage of cytochrome-c into cytosol. Cytochrome-c then interacts with Apaf1 and procaspase-9 and facilitates oligomerization and activation of caspase-9 that results in caspase-3 activation (11) (see figure 1).

### **Cellular FLICE Inhibitory Proteins (c-FLIPs) in TRAIL resistance**

Apoptotic pathways are kept in check by cell proliferation and survival signaling. For instance, c-FLIP is an inhibitor of DR signaling as it competes with caspase-8 to bind to FADD (12). Structurally, c-FLIP closely resembles caspase-8 but lacks the caspase-8 enzymatic activity due to substitution of a key cysteine with aspartate in the active site. Some of the c-FLIP isoforms, (e.g. c-FLIPL (p55), c-FLIPS (p26), c-FLIPR (p24), and p22 also activate the pro-survival transcription factor NF $\kappa$ B. The p22 form can associate with NEMO, and be a part of the IKK complex causing NF $\kappa$ B activation. In addition, c-FLIPL is an NF $\kappa$ B responsive gene and upon expression, it can inhibit Fas as well as TRAIL induced apoptosis (12-16) (figure 1). The death effector domain in the FLIPs competes with the DED of caspase 8 for binding to FADD in the DISC, thereby inhibiting caspase 8 activation. In comparison to FLIPL, FLIPS that lacks even the inactive protease domain, appears to be much more effective in caspase-8 inhibition. TRAIL resistant endometrial carcinomas express relatively high levels of c-FLIP and its down

modulation using either actinomycin D or specific siRNA renders the carcinoma highly sensitive to TRAIL killing. Herpes virus induced Kaposi's sarcoma shows elevated levels of FLIP due to the production of V-FLIP. This can increase the basal levels of NFκB activity leading to enhanced expression of pro-survival genes that contribute to higher incidence of cancers. Elevated c-FLIP levels have been linked to TRAIL resistance in human melanomas, B-cell lymphomas, Reed-Steinberg's cells of Hodgkin's lymphoma, carcinomas of the prostate, the stomach and the urinary bladder. Similarly, elevation in c-FLIP levels along with survivin is often observed in the highly fatal form of glioblastoma multiforme (17-27). A related member PED that targets caspase-8 and is highly expressed in B cell chronic lymphocytic leukemia may confer TRAIL resistance (28). Specific NFκB inhibitors can be administered to down modulate not only c-FLIPs but also IAPs to sensitize TRAIL resistance carcinomas to TRAIL mediated apoptosis (figure 2) (29).

### **Inhibitors of Apoptosis (IAPs) in TRAIL resistance**

IAP proteins block apoptosis by binding either to effector caspases-3 and -7 and/or to initiator caspase-9. They have one or three tandem baculoviral inhibitory repeat (BIR) domains. To date, six IAPs have been identified-cIAP1, cIAP2, X-IAP (X linked), NIAP (neuronal), Survivin, and BRUCE (Bir Repeat Containing Ubiquitin Conjugating Enzyme) of which XIAP is the most potent. During intrinsic apoptosis, in addition to cytochrome-c, Smac/Diablo is leaked into the cytoplasm wherein they interact with the BIR domains of IAPs and neutralize them. In type II cells, where caspase-8 activity is limiting, reduced or increased levels of Smac/Diablo or IAPs respectively can confer TRAIL resistance (reviewed in 30). Many human cancers harbor high levels of IAPs and a majority of pancreatic carcinoma cell lines are TRAIL resistant due to poor release of Smac/Diablo from mitochondria (31-32). Down modulation of XIAP in NSCLC xenografts render them highly susceptible to TRAIL treatment in mouse models (33-35). Although inhibition of IAPs with Smac peptides or with phenylurea may not be sufficient, it is very likely that in combination with TRAIL, it will significantly increase the efficacy of the treatment of cancers that are refractory to TRAIL treatment due to elevated levels of IAPs (Fig-2) (36-37).

### **Bcl2 family members in TRAIL resistance**

The Bcl2 family members can contribute to TRAIL resistance that can be reversed with BH3 mimetic Nona peptides (38-39). Surprisingly, in some prostate and colon cancer cells, TRAIL induces resistance through increased expression of the anti-apoptotic protein Bcl-xL, presumably due to activation of NFκB (40). Mcl-1, an anti-apoptotic member of the Bcl2 family is highly expressed in multiple myeloma, hepatocellular carcinoma, and liver metastasis of colorectal carcinoma (41-42) and confers resistance to various therapies including TRAIL. In pancreatic ductal carcinoma cells, expression of Bcl-xL not only confers resistance to TRAIL, it also unravels the ability of TRAIL to activate the NFκB mediated prosurvival pathway and enhance metastasis. This was elegantly shown by orthotopic transplantation of ductal carcinoma cells into SCID mouse pancreas. Just three TRAIL treatments in these mice were enough to trigger metastasis of the pancreatic cancer to liver, spleen and peritoneum without any apparent effect on the growth of the primary tumor (43). A similar observation has been described in another study using cholangiocarcinoma cells. TRAIL treatment promoted tumor cell migration, and silencing of NFκB had no discernable effect. In these cells, the TRAIL resistance was found to be due to Mcl-1, and unlike Bcl-xL, it is not regulated by NFκB (44-45). Therefore, a careful evaluation of the molecular nature of cancer is necessary before embarking on TRAIL treatment for cancers. The BH3 mimetic ABT737 which is a

potent inhibitor of both Bcl2 and Bcl-xL and others are currently in clinical trials and by themselves appear to be highly effective. However, they may not overcome the resistance offered by a related BCL2 family member Mcl1 as seen in figure 2 (44).

### **IG20 isoforms and their role in TRAIL induced signaling**

The *IG20* gene can encode at least four different splice variants namely, IG20pa, MADD/DENN, IG20-SV2 and DENN-SV (46-50). *IG20* isoforms play significant roles in cancer cell proliferation, survival and death (50-56). Most cancer cells constitutively express MADD and DENN-SV with varying levels to no expression of IG20pa and IG20-SV2 (50). Earlier studies, using anti-sense oligodeoxynucleotides, showed that knockdown of all *IG20*-SVs can result in spontaneous apoptosis of cancer cells but not normal cells *in vitro* as well as *in vivo* (55-57). Although an indispensable role for *IG20* in cancer cell survival was demonstrated, this study failed to reveal the relative importance of different *IG20*-SVs.

Our recent studies using shRNAs that specifically target either exon 15 that is expressed in all isoforms of *IG20* (Mid), or exon 13L or exon 16 that are differentially expressed in *IG20*-SVs, we were able to selectively knockdown either all or combinations of *IG20*-SVs in HeLa and PA-1 cells to determine their role in cell survival. The ovarian carcinoma PA1 cells are resistant to TRAIL induced apoptosis and express only MADD and DENN-SV indicating that either or both of these isoforms are indispensable for cell survival and/or TRAIL resistance. Knockdown of MADD, and not DENN-SV, enhanced spontaneous as well as TRAIL induced apoptosis through caspase-8 activation. Further, endogenous MADD could inhibit caspase-8 activation, at the DISC, by sequestering the DR4/DR5 through direct interactions. MADD *per se* does not interact with caspase 8 or FADD nor does it affect their interactions with DRs. In contrast, over expression of IG20pa rendered even PA1 cells highly susceptible to TRAIL through enhanced recruitment of DISC and activation of caspase-8. However, knockdown of endogenous IG20pa has no discernable effect on spontaneous or TRAIL induced apoptosis. Although loss of DENN-SV has no apparent effect on apoptosis, expression of DENN-SV can lead to increased resistance to TRAIL, most likely through enhanced production of pro-survival factors through NFκB activation (figure 1) (50, 52-54, 58-59).

Taken together, our studies demonstrate a pro-survival role for MADD, and expression of MADD especially in the absence of IG20pa appears to confer resistance to TRAIL induced apoptosis. IG20pa, when expressed, behaves like a dominant negative MADD. It is therefore very likely that the constitutively expressed isoforms, MADD and DENN-SV, contribute to TRAIL resistance by inhibiting caspase-8 and activating NFκB respectively. Our results clearly demonstrate that knockdown of all *IG20* splice variants or the MADD variant using a lentiviral vector that can express either mid or 13L siRNA respectively not only induces significant apoptosis but it also synergizes with TRAIL treatment (figure 2).

### **Clinical Translational Advances**

TRAIL appears to be an excellent choice of treatment for a variety of cancers. However, there is a distinct possibility that the treatment itself can quickly induce resistance and promote metastasis through activation of NFκB. In addition, intrinsic TRAIL resistance appears to occur more frequently than what was originally anticipated and the signaling components in the TRAIL mediated cell death pathway such as FLIP and IAP appear to play a significant role. Loss of function mutations in TRAIL receptors, over expression of decoy receptors, loss of caspase-8 expression due to gene methylation can also contribute to TRAIL resistance (60-67). Our recent studies with various splice variants of

IG20 unraveled yet another level of regulation of TRAIL induced cell death. Constitutive expression of the MADD and DENN-SV isoforms, especially in the absence of IG20pa and IG2-SV2, appears to confer TRAIL resistance. As shown in the figure, MADD appears to regulate the very proximal event in TRAIL signaling. It is possible that MADD can sequester DR4/DR5 receptors and thus negatively regulate TRAIL induced apoptosis in addition to its inhibitory effect on endogenous caspase 8 activation at the DISC. Therefore, in order to maximize the efficacy of TRAIL it is critical that we rapidly and reliably identify the potential TRAIL resistance mediators. A combinatorial approach that will target the key contributors to TRAIL resistance and includes TRAIL is likely to be a more effective therapeutic approach to cure cancer. A single combination is unlikely to be the cure all and thus a cancer-specific combinatorial therapy based on the molecular expression pattern of TRAIL resistance markers might be more effective (figure 2).

### **Acknowledgement**

This work was supported by a grant 5R01CA107506 from the National Institutes of Health, Bethesda, MD.

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## Figure Legends

**Figure 1: Molecular mechanisms of resistance to TRAIL induced apoptosis.** The diagram shows the TRAIL induced signaling, and the interplay between cell proliferation and death signals. Green and red lines indicate up-and down-regulation respectively. Thickness of lines indicates relative strengths of signals. The FLIPs, IAPs and some of the Bcl2 family members are the major contributors to TRAIL resistance. The *IG20* gene splice variants, MADD and DENN-SV, are constitutively expressed at high levels in all cancer cells and tissues tested to date and they confer resistance to TRAIL, especially in the absence of IG20pa. MADD can directly interact with death receptors (DR4 and DR5) and inhibit activation of endogenous caspase-8. MADD can also cause MAPK activation and is therefore an important early regulator of TRAIL induced cell death. On the other hand, IG20pa, when expressed, can act like a dominant negative MADD and enhance DISC formation, and be part of it. DENN-SV when co-expressed with MADD can promote the pro-survival transcription factor NF- $\kappa$ B and promote cell proliferation. As shown above, the cell survival/proliferation and death pathways regulate each other through both protein-protein interactions and transcription. Perturbations in the above pathways could contribute to TRAIL resistance. Therefore, a combination of TRAIL treatment with targeted suppression of specific proteins that contribute to resistance is likely to be a more effective treatment against cancer.

**Figure 2: Potential targets for combinatorial therapy with TRAIL:** Key molecules involved in the TRAIL induced apoptosis are shown in green and the anti-apoptotic factors that contribute to TRAIL resistance in various cancers in yellow. Potential therapeutic agents that can synergize with TRAIL and enhance its therapeutic efficacy are shown in purple. Either pan abrogation of all *IG20* isoforms or down-modulation of MADD alone can cause spontaneous apoptosis and/or enhance susceptibility to TRAIL treatment. Increased expression of the IG20pa isoform can also render cells more susceptible to TRAIL induced apoptosis, perhaps by acting as a dominant negative of MADD.

Figure-1

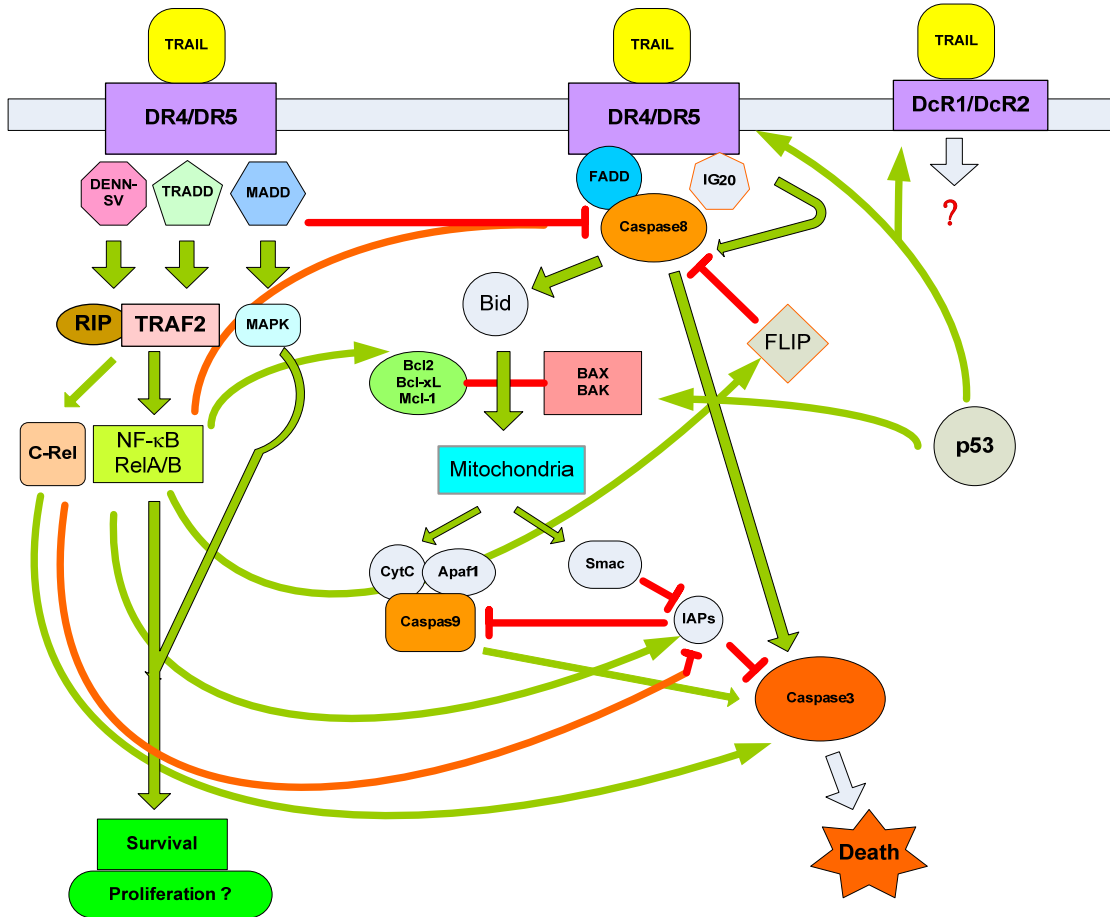


Figure-2

