

## Review Article

# Epigenetic regulation of the TRAIL/Apo2L apoptotic pathway by histone deacetylase inhibitors: an attractive approach to bypass melanoma immunotherapy resistance

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Received December 26, 2012; Accepted January 19, 2013; Epub February 27, 2013; Published March 9, 2013

**Abstract:** TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) is a major cytotoxic mechanism employed by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells to eradicate malignant cells. TRAIL/Apo2L interacts with its cognate receptors located on tumor cell surface namely, TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and osteoprotegerin (OPG). The exact function of DcR1 and DcR2 remains elusive. TRAIL/Apo2L or agonistic monoclonal antibodies directed against TRAIL/Apo2L death-inducing receptors (DR4, DR5) have become an attractive immunological therapeutic tools in clinical oncology due to their selective killing of tumors and lack of affinity towards healthy cells. Though a potent anti-cancer modality, some cancer cells exhibit inherent or acquired resistance to TRAIL/Apo2L. Postulated resistance mechanisms include up-regulation of c-FLIP, down-regulation of caspase-8, down-regulation/shedding of death receptors and an imbalanced ratio of pro- to anti-apoptotic genes due to aberrant activity of cellular survival signal transduction pathways. The development of resistance has spurred the use of combination therapy, in particular using small molecule sensitizing agents, to restore apoptosis sensitivity. A novel category of such compounds is histone deacetylase inhibitors (HDACi), which block HDACs from removing acetyl groups from histone tails thereby preventing silencing of pro-apoptotic genes and regulating the expression of non-histone proteins (i.e., apoptosis-associated genes), are effective agents in some malignancies. Some HDACi, such as Suberoylanilide Hydroxamic Acid (SAHA), have received FDA approval for cancer treatment. In various melanoma preclinical models, HDACi in conjunction with TRAIL/Apo2L, via modulation of apoptotic machinery, have proven to overcome acquired/inherent resistance to either agent. Here, we discuss recent findings on the role of TRAIL/Apo2L and its agonistic mAbs in melanoma immunotherapy with discussions on potential cellular and molecular events by which HDACi can sensitize metastatic melanoma to TRAIL/Apo2L-mediated immune-therapy, thereby, overcoming resistance.

**Keywords:** TRAIL/Apo2L, apoptosis, signal transduction, resistance, melanoma, immunotherapy, SAHA, histone deacetylase inhibitor, sensitization, adoptive cell transfer, agonistic TRAIL/Apo2L mAbs, monoclonal antibody, drozitumab, gene regulation

### Introduction: Intrinsic and extrinsic pathways of apoptosis (programmed cell death; PCD)

The process of programmed cell death (PCD, Apoptosis) occurs during development or disease states as a mean of eradicating surplus, unwanted or damaged cells [1]. Biochemically and morphologically it involves cellular shrinkage, membrane blebbing, formation of membrane bound apoptotic bodies, translocation of phosphatidyl serine (PS) to outer membrane,

nuclear condensation, formation of 180-220bp DNA fragments, and eventual complete disintegration of the cells, which are usually phagocytosed by neighboring cells. Apoptosis is the major mechanism by which anti-cancer modalities (chemotherapy, radiation, and immune-based approaches) eradicate tumor cells. Within the immune system, two major apoptotic pathways operate: extrinsic and intrinsic. The extrinsic apoptosis pathway relies upon signal transmission from cytotoxic ligands such as

tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and Fas (FasL) ligand through their cognate receptors TNF-R1, -R2, and Fas (CD95), respectively [2]. Another important apoptosis-inducing signaling ligand, TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) transmits its biological effects through multiple receptors including DR4 and DR5 as well as decoy receptors DcR1, DcR2, and OPG [3-6]. The extrinsic (death receptor) pathway initiates following receptor-ligand trimerization followed by recruitment of the adaptor protein FADD to the cytoplasmic portion (death domain; DD) of the receptors recruiting pro-caspase-8 molecules to form the death-inducing signaling complex (DISC). The formation of the multimeric DISC complex will subsequently activate caspases-8 and -10, through autocatalytic activation [7]. Activation of initiator caspases induces the cleavage and activation of caspase-3, -6, and -7 (effector caspases), culminating in DNA fragmentation and apoptosis [8]. In addition to other stimuli such as  $\gamma$ -irradiation and chemotherapeutic agents, TRAIL/Apo2L signaling through DR4 and DR5 can activate the intrinsic apoptosis pathway [9]. After DISC formation and caspase-8 activation, low levels of caspase-8 cleave the pro-apoptotic Bcl-2 family protein, Bid (tBid) which activates Bax and Bak in the mitochondria, reducing mitochondrial transmembrane potential ( $\Delta\Phi_m$ ) [10] causing the cytoplasmic release of apoptogenic molecules SMAC/DIABLO and apoptosis inducing Factor (AIF) where they competitively bind to inhibitor of apoptosis (IAP) proteins initiating caspase cascade (9, 3, 6, 7) activation and apoptosis [11]. Mitochondria also releases an additional cytotoxic molecule; Cytochrome c (Cyt c) into the cytoplasm where in an ATP-dependent manner, 7 molecules of Cyt c bind to 7 molecules of Apaf-1 and procaspase-9 to form the large multimeric apoptosome complex, resulting in autocatalytic cleavage and activation of caspase-9 [12]. Active caspase-9 uses caspase-3 as substrate and apoptosis ensues.

### **Molecular, pathological, clinical manifestations and therapeutic options for metastatic melanoma**

Melanoma is an aggressive form of skin cancer that originates in the melanocytes, specialized pigment-producing cells predominantly located in the basal layer of the epidermis and the eyes [13]. Normally, melanocytes synthesize mel-

anin, the pigment responsible for skin and hair color, which serves as an excellent photoprotectant. In response to ultraviolet (UV) radiation, melanocytes are stimulated by epidermal keratinocytes to increase melanin production, resulting in the tanning of the skin. Hence, melanocytes play a key role in protecting the skin from the damaging effects of UV radiation and in preventing skin cancer [14]. Consequently, people with fair skin or those who lack functional melanocytes especially in pigmentary disorders such as vitiligo and albinism, are at greater risk of developing skin cancer upon UV radiation exposure [15]. It may seem contradictory then that melanocytes are the precursors of the most deadly form of skin cancer.

Skin cancer is the most common form of all human malignancies and its global incidence is rising at an alarming rate. Basal cell carcinoma and squamous cell carcinoma make up the vast majority of cases while melanoma is the least common. There are an estimated 2-3 million global cases of skin cancer each year, and melanoma accounts for about 132,000 of these cases [16]. Although melanoma constitutes only 4% of all cases, it is the most dangerous form, being responsible for nearly 79% of skin cancer-related deaths [17]. The incidence of melanoma is rising steadily in western populations especially in Caucasian males, and the number of worldwide cases has doubled in the last 20 years [18]. Looking at the number of cases and deaths in the United States in 2009 alone, an estimated 68,720 new cases were diagnosed with 8,650 deaths [19].

Typically, melanoma development progresses through five distinct stages. The formation of pigmented lesions called benign nevi is the first stage. This is followed by conversion to dysplastic nevi, which is characterized by an increased level of structural and architectural atypia. The third stage is the first recognizable malignant stage defined as the radial growth phase (RGP), which grows laterally and is mostly confined to the epidermis. Growth is primarily local, lacking metastatic capacity. If left untreated, melanoma can progress to the vertical growth phase (VGP), which is associated with invasion of the dermis by large clusters of melanoma cells and the acquisition of metastatic potential. The final stage in the progression process involves metastasis to distant organs followed by an

overgrowth of metastatic melanoma at these sites [20]. Though, not all melanomas follow each individual stage, RGP or VGP can both develop directly from isolated nevi or melanocytes, or they may progress directly to metastatic malignant melanoma [21].

The success of systemic therapy of metastatic melanoma has been minimal at best. Despite an epic number of clinical trials, advanced melanoma is still incurable. If melanoma is diagnosed at the very early stages before the malignant melanocytes become invasive, it can be cured by surgical resection of the primary tumor with > 95% success rate [22]. Unfortunately, melanoma lesions commonly go unnoticed or are asymptomatic for extended periods of time, challenging even well-trained dermatologists [23]. Upon acquisition of metastatic potential, malignant melanoma cells tend to disseminate to multiple organs [24], including brain, lungs, liver or bone, rendering surgical excision useless. The patient is left with a median survival rate of only 6 months and a 5 year survival rate of <5% [18]. Conventional chemotherapy treatments utilizing drugs such as decarbazine (DTIC) or its derivative temozolomide (TMZ), exhibit the best single agent activity, though they confer complete remissions in only 5-10% of patients [25].

In more recent years, there has been growing enthusiasm to harness the immune system for cancer therapy. Initial attempts at immunotherapeutic strategies demonstrated promising results for its time with response rates in the order of 5-15%, though these responses were frequently durable and resulted in clinical benefit in a subset of patients [26, 27]. Interferon- $\alpha$  (IFN- $\alpha$ ) was the first exogenous cytokine to demonstrate antitumor activity against advanced melanoma. IFN- $\alpha$ 2b is a highly pleiotropic cytokine with immunoregulatory, antiproliferative and antiangiogenic properties in multiple malignancies, leading to its approval for adjuvant treatment of stage IIB/III melanoma by the Food and Drug Administration (FDA) in 1995 [28]. It remains the only approved adjuvant therapy for patients with high risk for recurrence and death, though tolerability is an issue due to flu-like symptoms, fatigue, anorexia and depression [29].

The second promising exogenous cytokine to demonstrate antitumor activity, the T-cell

growth factor IL-2, received FAD approval in 1998 for treatment of adults with advanced metastatic melanoma. IL-2 is a key player in immune regulation and T-cell proliferation. High-dose bolus intravenous IL-2 activates endogenous tumor-reactive T cells *in vivo* and causes regression of some human solid tumors. Retrospective long-term analysis of phase II studies demonstrated an objective response rate of 16% with a durable response rate of 4% [30]. Although IL-2 administration may induce toxicity owing to a capillary leak syndrome, treatment-related mortalities are less than 1% [31].

Active immunization is another immunotherapeutic approach, which utilizes either whole cells, proteins, peptides or other immunizing vectors that either increase immune recognition of tumor cells or enhances lymphocyte activation [32]. Vaccines contained a single antigen specific to the target, or utilized a mixture of antigens such as Canvaxin, which contained over 20 tumor antigens [33]. Although up to 30% of circulating melanoma-reactive CD8+ T cells could be induced by immunization, tumors continued to progress. However, Canvaxin vaccine may induce significant immunosuppression, which demonstrates the double-edged sword nature of complex vaccines [34].

Pioneering work by several groups in recent years have overcome low response rate associated with non-specific immunomodulation and active immunization approaches by using adoptive transfer T cell therapy (ACT) [27, 35]. These investigators have demonstrated that antigen-specific T cells reactive to infectious pathogens and tumor antigens can be generated *in vitro* and adoptively transferred to patients providing a clinical benefit. Investigators at the National Cancer Institute (NCI) Surgery Branch led by Steven Rosenberg and our group at the University of California, Los Angeles (UCLA) have utilized MART-1 TCR engineered  $\alpha$  and  $\beta$  genes with high affinity for the melanoma tumor antigen MART-1<sub>27-35</sub> presented in the context of HLA A\*0201. The transfer of TCR genes is necessary and sufficient to endow recipient T cells with the specificity of donor cells. TCR genetically modified T cells respond to target Ag recognition through the transgenic TCR both *in vitro* and *in vivo*, leading to effective immune responses to viral and tumor challenges in

murine adoptive transfer models. T cells redirected by TCR gene transfer are fully functional after transfer into mice, expand dramatically (over three logs) after encounter with their cognate antigen *in vivo*, conferring new antigen specificity and functional activity to TILs. In addition, MART-1<sub>27-35</sub> pulsed dendritic cells are infused to ensure a more robust and sustained T cell activation [27, 35].

To improve the efficacy of genetically modified T cells, conditioning regimen (lymphodepletion) with cyclophosphamide-fludarabine is also being incorporated into the protocol. Murine models have demonstrated that CD4+CD25+FOX3+ regulatory T cells (Treg) hamper the efficacy of ACT, therefore, selective depletion of Treg cells would be beneficial. In addition, functionality of tumor infiltrating lymphocytes (TIL) may be improved by utilizing antibodies or genetic approaches that block potential inhibitory signals from CTLA-4, PD-1 and TGF- $\beta$ . Administration of helper cytokines (e.g., IL-2, IFN- $\gamma$ ) as well as activation of host APCs with Toll-like receptor agonists are other potential beneficial parameters. Response rates up to 50% have been achieved with an optimal combination of *ex vivo* clonally expanded TILs, lymphodepletion and helper cytokine administration [35].

### **TRAIL/Apo2L apoptotic signal transduction pathway in melanoma and potential mechanisms of resistance**

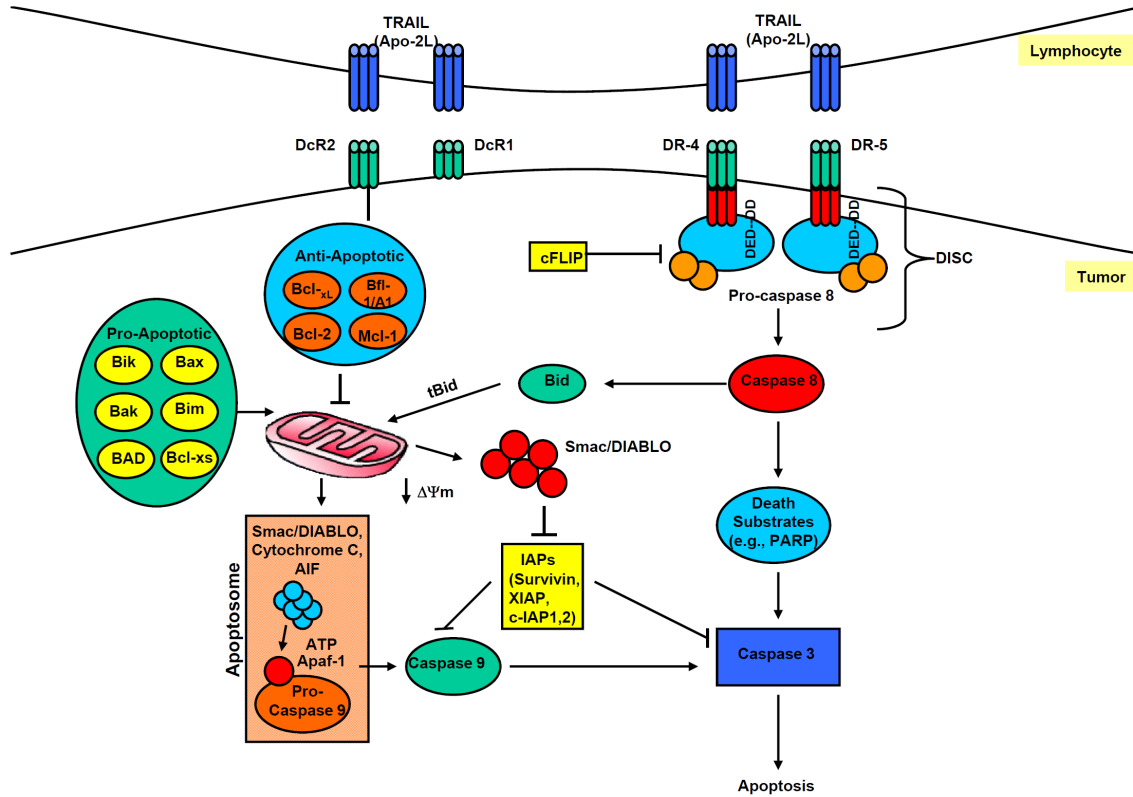
Cytotoxic T lymphocytes (CTLs) trigger two major apoptotic pathways to eliminate tumor cells: the death receptor-induced pathway and the granule-exocytosis pathway. Cytotoxic ligands generally belong to the tumor necrosis factor (TNF) family of ligands and include TNF- $\alpha$ , Fas ligand (FasL, CD95) and TRAIL/Apo2L. They transmit the death signal upon ligation to their cognate receptors. The type II transmembrane protein, TRAIL/Apo2L, is a potent apoptosis inducer in tumors while sparing untransformed normal cells [36, 37]. TRAIL/Apo2L can engage two death-inducing receptors namely, TRAIL-R1 (DR4) and TRAIL-R2 (DR5) [38]. Along with DR4 and DR5, at the same time TRAIL/Apo2L can bind two decoy receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2). The role of these decoy receptors is not fully understood but it is hypothesized that they play a role in negative regulation of TRAIL/Apo2L signaling pathway by

competing with DR4 and DR5 in binding to TRAIL/Apo2L and may contribute to TRAIL/Apo2L resistance. DcR1 and DcR2 also lack the cytoplasmic signaling components required for the transmission of the apoptotic death signal [39]. Upon interaction of the trimerized TRAIL/Apo2L with DR4/DR5 a series of intracellular events will initiate: the death receptors undergo trimerization, the adaptor protein Fas-associated death domain (FADD), and initiator caspases (procaspase-8 and -10) [cysteine proteases with aspartic acid specificity] will be recruited to a specific region of the cytoplasmic domain of the receptor (called the death domain; DD) [40]. Cellular FADD-like IL-1 beta-converting enzyme inhibitory protein (c-FLIP) can bind to both FADD and procaspase-8 and inactivate them, thus, acting as a negative regulator of the TRAIL/Apo2L pathway [41]. This serves as a regulatory step in TRAIL/Apo2L pathway (**Figure 1**). Recruitment of FADD and procaspase-8 leads to the formation of the multimeric large protein complex called death inducing signaling complex (DISC). After DISC formation, two procaspase-8 molecules are required for full activation of caspase-8 which occurs via autocatalytic cleavage of each procaspase-8 molecule. Two distinct yet overlapping apoptotic pathways are identified operating in various cells; in type I cells polymerization of the death receptors activates caspase-8, which directly activates effector caspases such as caspase-3 causing DNA fragmentation (extrinsic pathway). In the event that the cells lack sufficient levels of caspase-8 to directly activate caspase-3 (type II cells), low levels of caspase-8 cleave proapoptotic Bid; truncated Bid can destabilize mitochondria releasing apoptotic molecules (AIF, Cyt-c and Smac/DIABLO). Anti-apoptotic Bcl-2 members prevent the collapse of the mitochondria while proapoptotic molecules such as Bax, Bid, Bad, Bik and Bcl<sub>xs</sub> promote depolarization (intrinsic pathway) [41-44]. Cytochrome-c and Apaf-1 form of the apoptosome complex and activate caspase-9, which will use of caspase-3, -6 and -7, to cleave death substrate and induce apoptosis (**Figure 1**).

The vast majority of immunotherapeutic strategies, in particular ACT, exert their anti-tumor effects by induction of apoptosis mediated via death receptor [41-44]. There are presently six known death receptors; TNF-R1, Fas, DR3, DR4, DR5 and DR6 [45-49]. In particular, the



## HDACi sensitizes resistant melanomas to immunotherapy

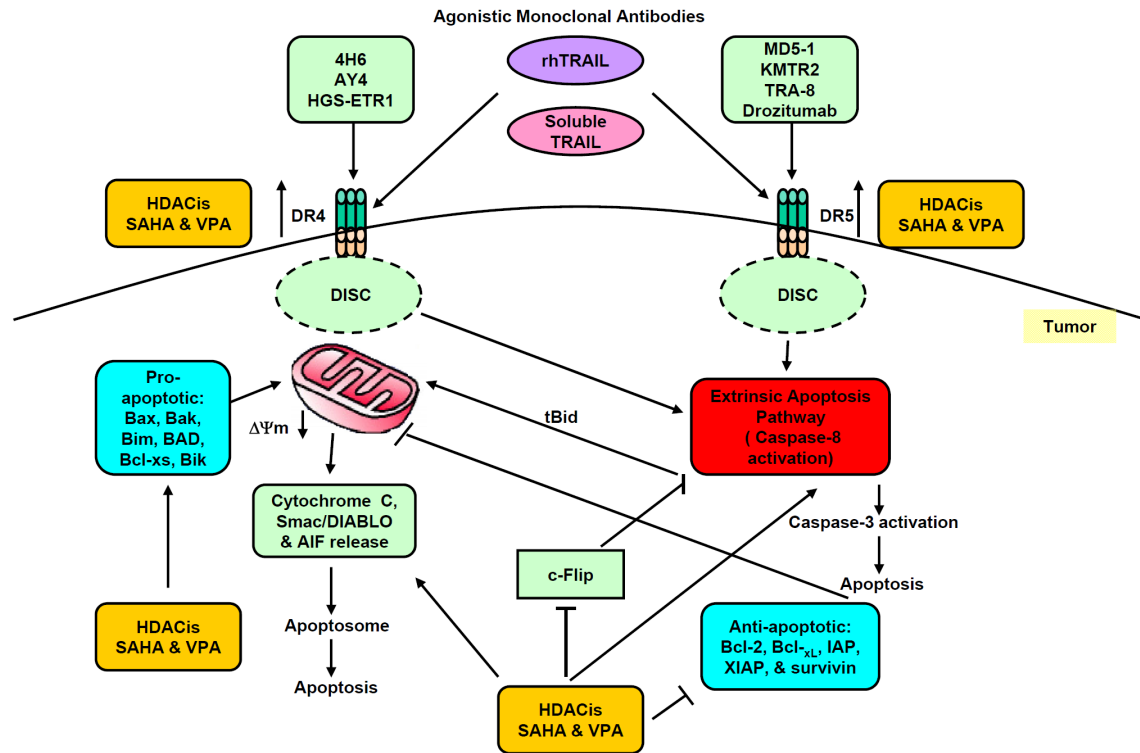


**Figure 1.** TRAIL/Apo2L Apoptosis Signal Transduction Pathway: TRAIL/Apo2L is a major apoptotic pathway initiated by activation of CTLs and NK cells in killing tumor cells. The pathway is triggered by binding of TRAIL/Apo2L to its cognate death inducing receptors (DR4, DR5) and subsequent induction of apoptosis. The execution of apoptosis is mediated by cysteine proteases known as caspases, which become activated by autocatalytic processing, and are divided into initiators and effectors. Based on their activation pattern, two types of cells have been characterized: in Type I cells, caspase cascade is triggered upon the oligomerization of DR4 and/or DR5 and undergoes a sequential activation. Depending on the amount of caspase-8, stimuli directly induces initiator caspase-8 auto-processing leading to caspase-3 activation and cleavage of death substrates (PARP) and apoptosis. Alternatively, when cells lack sufficient amounts of caspase-8, apoptotic stimuli (e.g., TRAIL/ Apo2L and chemotherapeutic drugs) utilize type II pathway involving mitochondria and Bcl-2 members. Caspase-8 cleaves the pro-apoptotic Bid (tBid). tBid will then migrate to and reside in the mitochondrial outer membrane, where in association with other pro-apoptotic molecules induce the formation of mitochondrial permeability transition pore leading to decrease in mitochondrial transmembrane potential ( $\Delta\Psi_m$ ). Mitochondrial integrity is maintained by the protective effects of Bcl-2, Bcl-xL, Bfl-1/A1, Mcl-1, which is antagonized by pro-apoptotic Bax, Bid, Bad, Bik, Bcl-xs. Mitochondrial collapse facilitates the cytosolic release of cytochrome c, AIF and Smac/DIABLO. Cytochrome c, in the presence of Apaf-1, dATP/ATP and caspase-9 will form the apoptosome complex expediting caspase-9 activation. Smac/DIABLO physically associates with c-IAPs removing the inhibitors of caspase activation. Through autocatalytic processing, caspase-9 becomes activated and uses executioner caspases as substrates leading to the cleavage of death substrates and apoptosis. This is the converging point of type I and II apoptotic pathways.

potential utilization of the TRAIL/Apo2L apoptotic pathway has been extensively studied in various cancer models including melanoma [45-49]. The fact that the threshold of cancerous cells to TRAIL/Apo2L-mediated apoptosis is significantly lower than that of untransformed cells causing tumor cell death while sparing normal tissues, makes TRAIL/Apo2L system an attractive approach [50] which is currently being tested in phase I and II clinical trials [51].

Nonetheless, the underlying mechanisms of differential sensitivity of tumor and normal cells are largely unknown. Differential expression of death-inducing versus decoy receptors as well as higher density of DR4 and DR5 on cancer cell surface (whose expression levels directly correlates with TRAIL/Apo2L-mediated cell death) might, in part, explain the sensitivity of tumor cells to immunotherapeutic strategies involving TRAIL/Apo2L [50-55].

## HDACi sensitizes resistant melanomas to immunotherapy



**Figure 2.** DR4 and DR5 Agonistic mAb-induced Apoptosis: Regulation of TRAIL/Apo2L Resistance by HDACi. Anti-DR4 and -DR5 agonistic mAbs induce apoptosis by binding to and trimerizing death receptors in place of TRAIL/Apo2L. Recombinant human TRAIL (rhTRAIL) can also be used, however, mAbs are more effective due to longer half-life *in vivo*. Ligation of anti-DR4 (4H6, AY4, HGS-ETR1) and anti-DR5 (such as MD5-1, KMTR2, TRA-8, and Drozitumab) agonistic mAbs to their cognate receptors (DR4 and DR5, respectively) leads to the formation of the death-inducing signaling complex (DISC) and apoptosis (as detailed in **Figure 1**). HDACi such as SAHA and VPA, enhance death receptor mediated apoptotic signaling by decreasing cellular levels of anti-apoptotic molecules including c-Flip, reducing the expression levels of Bcl-2 and c-IAP family members, and increasing levels pro-apoptotic Bcl-2 family members (BAK, BAX, BID, BAD, BIK). SAHA and VPA also increase surface expression of death receptors DR4 and DR5. By altering the dynamics of intracellular signaling pathways and changing the ratio of anti/proapoptotic molecules, HDACi favor the formation of a proapoptotic milieu and increase the efficiency of mAbs as anti-cancer agents, thus, overcoming resistance.

Resistance to apoptosis is one of the potential limitations in successful treatment of many cancers including melanoma [56-58]. Unfortunately, the exact mechanism of resistance is unknown as *ex vivo* studies have shown tumor infiltrating lymphocytes (TIL) are fully functional and capable of killing tumor cells yet no significant clinical responses are achieved. Lack of objective responses can be explained by the development of resistance mechanisms including loss of antigen presentation, decreased release of cytokines by T cells, T cell exhaustion, or induction of anergy, peripheral/central tolerance or induction of T regulatory cells [59]. On the tumor side, these resistance mechanisms are mainly due to simultaneous activation of anti-apoptotic and down-regulation of pro-apoptotic mechanisms [60]. For

instance, studies in melanoma have shown up-regulation of NF- $\kappa$ B by TRAIL/Apo2L [62]. It is well-established that various signal transduction pathways such as NF- $\kappa$ B, PI3K/AKT/mTOR, ERK1/2 MAPK (including activating BRAF<sup>V600E</sup> mutation) are deregulated in melanoma, all of which are directly linked to apoptosis-resistance. Thus, combination strategies using immunotherapies (such as TRAIL/Apo2L) with agents that specifically target the components of aberrant signaling pathways may be a valid treatment strategy. Studies have shown that specific inhibition of ERK1/2 and AKT pathway can sensitize melanomas to TRAIL/Apo2L-mediated apoptosis [62].

One of the most frequently observed obstacles regarding resistance to TRAIL/Apo2L or antago-

nistic DR4 or DR5 mAb associated therapeutics is down-regulation or loss of death receptors' expression on cancer cells. This could result from chromosomal deletion (8p22-21) or possibly due to epigenetic events [63, 64]. Over expression of c-FLIP [which has three isoforms c-FLIP<sub>L</sub>, c-FLIP<sub>R</sub> and c-FLIP<sub>S</sub>], which blocks caspase-8 activation at the DISC level, is another potential mechanism of TRAIL/Apo2L-resistance [65]. Studies are underway to develop strategies to target c-FLIP isoforms.

### **Histone modifications and implications in regulation of gene expression**

The most fundamental mechanism of regulation and processing huge amounts of genetic information involves the condensation of DNA within the nucleus into a higher order structure called chromatin. Individual structural units of chromatin, nucleosomes, consist of 146 base pairs of DNA wrapped around eight core histone proteins [66]. The amino (N)-terminal tail of histones is highly susceptible to post-translational epigenetic modifications including acetylation, methylation, ubiquitination, and SUMOylation. These modifications can alter the expression of corresponding genes by changing their accessibility to transcription initiation complexes, RNA polymerase II, activators and co-activators and transcription factors [67]. Methylation of cytosine residues reduces the rate of gene expression by preventing the binding of transcription factors and recruiting methyl-CpG-binding proteins to silence genes. Gene silencing is further facilitated by recruitment of ATP-dependent chromatin remodeling complexes. Gene regulation by ubiquitination and SUMOylation involves the modification of lysines on histones by small proteins called ubiquitin and ubiquitin-related modifiers (SUMO). Acetylation increases gene expression by the addition of an acetyl group to the lysine (K) residue located at the amino (N)-terminal tail of the core histone protein. Acetylation involves replacement of a hydrogen atom on the lysine residue with an acetyl group and thereby neutralizing the positive charge of the lysine, thus, reducing its affinity to negative phosphate groups located within DNA backbone. By weakening this interaction the nucleosome becomes less condensed and is more open to interactions with transcription factors (open configuration). Acetylation can also physically prevent the attachment of proteins which

act as transcriptional barriers or act as scaffolding for transcriptional protein attachment. Thus, acetylation leads to higher expression of genes [67]. The enzyme histone acetyltransferase (HAT) increases gene expression by transferring acetyl groups to the lysine residue of the (N)-terminal tail of core histones causing chromatin remodeling, whereas histone deacetylases (HDAC) decrease gene expression by removing acetyl groups from these residues [68].

### **Histone deacetylase inhibitors as anti-cancer therapeutic agents**

In general, the expression levels of HDACs are abundant in cancer cells, causing deacetylation of pro-apoptotic genes or silencing of tumor suppressors (e.g., PTEN) leading to aberrant apoptosis pathways which will eventually impose an apoptosis-resistant phenotype to tumors [69], highlighting the potential usefulness of histone deacetylase inhibitors (HDACi) in clinical oncology. Thus far, there are four classes of HDACi categorized based on their structure and differing affinities for specific HDACs, and are short-chain fatty acids, hydroxamates, benzamides and cyclic tetrapeptides [70]. Butyrate, trichostatin A (TSA), Sirtinol, tenovin, valproic acid (VPA), romidepsin and suberoylanilide hydroxamic acid (SAHA, Zolinza®, Vorinostat) are few HDACi currently being used in various clinical trials. However, only SAHA, romidepsin and VPA have received FDA approval. Approved in 2006, SAHA is used for the treatment of Cutaneous T-Cell Lymphoma (CTCL) [71]. Since then its usage has been expanded to a wide array of cancers including melanoma. Comparison of cell viability of human lung, breast and skin cancer cells to depsipeptide and trichostatin A (TSA) revealed a dose-dependent decrease in cell viability of all cell lines with melanoma cell lines (HCC1500, HCC1806, and HCC1954) being more sensitive to the cytostatic effects of HDACi beginning at 10nM TSA and >1nM depsipeptide [72]. In another study various human melanoma cells (WM115; primary, WM266; metastatic, A375; amelanocytic and SK-Mel28: melanocytic) were treated with VPA (1 mM-one week) or with TSA (100 ng/ml) or SAHA (4μM) for 72 hours. While VPA only inhibited proliferation in the WM266 and A375 cell lines, SAHA and TSA inhibited the proliferation of all cell lines treated. This reduced proliferation is

thought to be a combined result of apoptotic death of the cells (evidenced by high number of cells accumulated at the sub-G1 phase; >2N DNA content) following treatment as well as those arrested at G1 phase [73]. These and many other preclinical studies suggest that HDACi can be effectively used in melanoma.

### **Combinatorial therapeutic approaches to overcome inherent or acquired resistance**

Although TRAIL/Apo2L appears to be a promising cytotoxic ligand for selective killing of cancer cells, there exists the major problem of resistance. Resistance may be inherent, due to abnormal expression of resistant factors, failure of the cells to carry out signaling or insufficient expression of signaling molecules, or it can be acquired following successful initial treatment. Up-regulation of c-FLIP in resistant cancer cells, which competes with procaspase-8 for binding to the DISC, prevents caspase-8 activation and hampers the initiation of caspase cascade [74]. c-Flip also activates the NF- $\kappa$ B signaling pathway, which in turn activates the transcription of IAPs and other anti-apoptotic factors [75, 76]. Triggering the TRAIL/Apo2L apoptotic pathway can also lead to the activation of NF- $\kappa$ B through a secondary pathway [77]. Induction or over-expression of c-FLIP and NF- $\kappa$ B activation and subsequent anti-apoptotic gene expression contribute to the development of resistance to TRAIL/Apo2L [78].

Combination of the chromatin remodeling drugs such as SAHA with rhTRAIL has shown promise for reversing TRAIL/Apo2L resistance. SAHA sensitizes human hepatocellular carcinoma cells to TRAIL/Apo2L induced apoptosis by increasing DR5 expression and down regulating c-Flip while exhibiting minimal toxicity towards primary human hepatocytes. Activation of caspase-8 as well as cleavage of NF- $\kappa$ B by caspase-3 can impart enhanced apoptosis in combination therapy [79]. Also, simultaneous SAHA and TRAIL/Apo2L treatment caused anoikis of breast cancer cells (bound cell detachment and subsequent apoptosis) while each drug used as single agents were incapable of killing tumor cells [80].

Studies conducted by Lakshmikanthan and colleagues showed that SAHA and TRAIL/Apo2L independently induce apoptosis in prostate

cancer cell lines, but that the level of killing increased twofold with the lowest concentration of SAHA plus TRAIL/Apo2L and increased to 7 times at higher doses. SAHA did not cause an increase in intracellular caspase-3 and TRAIL/Apo2L yielded a moderate increase, however, in combination they resulted in substantial increase in caspase-3 processing and activation. Also, significant decrease in the levels of I $\kappa$ B $\alpha$  and IKK $\alpha$ /IKK $\gamma$  were observed by combination treatment (SAHA+TRAIL/Apo2L). Finally, levels of another DISC component, RIP, which is responsible for recruitment of IKK to activate NF- $\kappa$ B, were decreased by combination treatment [81]. These results indicate that combination therapy using SAHA and TRAIL/Apo2L is far more superior at initiating caspase cascade activation and apoptosis induction. Combination of TRAIL/Apo2L and SAHA potentiates apoptosis of melanoma cells by increased caspase activation. SAHA increased the levels of the cyclin-dependant kinase inhibitor (CDKi) WAF1/p21 arresting melanoma cell cycle progression [82]. SAHA was previously shown to upregulate the expression of death-receptors DR4 and DR5 potentially resulting in more efficient TRAIL signaling in resistant cells [83, 84]. These studies were confirmed by Lillehammer and colleagues, where they reported 56% increase in surface expression of DR4 by SAHA whereas no significant change in DR5 expression was noted [82]. Induction of death receptors would explain a possible mechanism by which SAHA enhances TRAIL/Apo2L-mediated apoptosis of resistant melanomas. Expression of anti-apoptosis Mcl-1 and Bcl-xL was not remarkably affected by single agent treatment with SAHA, however the levels of these factors were drastically decreased upon combination therapy. Based on the aforementioned, components of the intrinsic apoptosis signaling pathway play a fundamental role in SAHA-mediated sensitization of resistant melanomas to TRAIL/Apo2L [82]. These results also suggest that combination of SAHA plus TRAIL/Apo2L treatment may be an effective new approach to bypass resistance of melanoma and other cancers to each agent alone.

Advances in technology such as array-based high-throughput gene expression analysis in understanding the specific genes involved, the signal transduction pathways, and the comparative gene expression patterns of responsive and non-responsive melanoma have provided



unique opportunities to examine this deadly disease in greater detail. In Particular, these advances have presented opportunities to an improved understanding of the gene expression patterns involved with melanoma progression despite successful initial therapy. Irreversible changes in the DNA sequence, including chromosomal abnormalities and gene mutations, are implicated in melanoma progression. However, growing attention is focused on understanding the implications of 'epigenetic' events. Epigenetic events do not alter DNA sequence, but potentially lead to stable inherited changes in gene expression patterns. Epigenetic events leading to abnormal gene expression in melanoma are usually due to post-translational histone modifications (methylation, ubiquitination, phosphorylation, sumoylation, acetylation), methylation of gene regulatory (enhancer, promoter) regions, and methylation of CpG islands or CpG dinucleotides (gene silencing). Thus, epigenetic mechanisms have emerged as playing a key role in gene regulation of human melanoma, including the identification of several putative tumor suppressor genes and oncogenes. Studies are under way which will lead to the development of novel therapeutics that will likely target and alter such epigenetic changes [67-74].

Adoptive cell therapy (ACT) of metastatic melanoma patients with T cell receptor (TCR $\alpha/\beta$ )-engineered T lymphocytes results in dramatic clinical responses in a significant percentage of the patients; in recent clinical trials conducted at UCLA and Caltech by the Program in Engineered Immunity (PEI), most patients showed tumor regression, many quite dramatic, but all recurred within months. These clinical observations raise the obvious question what are the underlying molecular mechanisms of resistance and are there approaches to reverse the acquired/inherent resistance and to "sensitize" melanoma cells to T lymphocyte-delivered apoptotic death signals?

Our group has approached this question using multiple metastatic melanoma cell lines that are MART-1 positive and express high levels of surface HLA A\*0201: these metastatic melanoma cell lines are exquisitely sensitive to MART-1 F5 TCR-engineered T lymphocytes (F5 CTL)-mediated apoptosis. Serial exposure of these lines over several months to step-wise increasing numbers of highly avid and specific

F5 CTLs has yielded multiple completely resistant lines. We have made the novel preliminary observation that resistant cells are completely resistant to killing by F5 CTL and rhTRAIL/Apo2L, suggesting that both rhTRAIL/Apo2L and F5 CTLs share a common apoptotic pathway in killing the tumor cells. Using specific pharmacological inhibitors we identified that F5 CTLs primarily use the TRAIL/Apo2L pathway, in particular via DR5 death receptor, in killing melanoma targets. This notion was further reinforced by the observation that the expression pattern of a wide array of apoptosis gene products are being modified in F5 CTL-resistant lines. A regulatory role of the HDACis on the expression pattern of apoptotic genes rendering the cells more susceptible to apoptotic stimuli and in overcoming TRAIL/Apo2L resistance is reported [68-74]. Thus, it is logical to assume that treatment of immune-resistant melanomas with physiologically relevant and clinically achievable concentrations of HDACi, through modulation of apoptotic machinery, could potentially reverse the acquired resistance. To corroborate our hypothesis, we used two different class I HDACis: LBH5089 and SAHA. Both of these agents are known to modify gene expression profile, in particular those involved in cellular signal transduction pathways, proliferation and apoptosis. Pretreatment of immune-resistant melanomas with low-dose and clinically achievable concentrations of HDACi rendered the cells sensitive to the cytotoxic effects of TCR engineered F5 CTLs (unpublished data). Preliminary focused microarray data suggests that reversal of resistance is due to regulation of the expression levels of apoptotic genes. In particular, HDACi negatively regulates the transcript levels of anti-apoptosis genes and simultaneously, positively regulates the expression of positive regulators of apoptosis (e.g., caspases, death domain proteins, TRAIL, TNF superfamily members), DNA damage molecules, apoptosis inducers. This suggests that HDACi mediates its sensitizing effect via combinatorial cooperation among several groups of apoptotic genes (simultaneous reduction of anti-apoptotic and induction of proapoptotic genes). However, cell fate is ultimately determined by an imbalanced ratio of pro- and anti-apoptotic proteins. Apparently, HDACi favors the generation of an intracellular proapoptotic environment that predestines melanomas to undergo apoptosis upon receiving

apoptotic death signal delivered by F5 CTLs. Thus, epigenetic modifiers (chromatin remodeling drugs) represent as new therapeutic modalities in the treatment of advanced and resistant melanomas which can be used as adjuvants to immunotherapy (Figure 2).

### Monoclonal antibody-mediated triggering of TRAIL/Apo2L apoptosis signaling pathway: potential applications in clinical oncology

Triggering receptor-mediated apoptosis signaling pathway(s) to selectively kill tumor cells without affecting normal cells will potentially yield promising results in cancer treatment. Activation of death receptors to induce apoptosis of cancer cells for therapeutic purposes is accomplished either through engagement with their natural ligands or via agnostic monoclonal antibodies (mAb). An early demonstration of mAbs as cancer therapeutics was observed in 1989 with the discovery of an Anti-Apo-1 IgG3/ $\kappa$  mAb which halted cell growth and induced apoptosis in lymphocytes and primary leukemia samples [85]. Since then, a wide array of mAbs have been developed; some have received their FDA approval while others are in various stages of clinical testing. Monoclonal antibodies are advantageous over endogenous ligands as they display a longer half-life during treatment, requiring lower therapeutic dosages [86]. Being a potent and selective apoptosis inducer in tumor cells, various agonistic mAbs have been developed against TRAIL DR4 and DR5. Below is a historical summary of the development and efficacy of these mAbs in various tumor models.

#### Agonistic Abs directed against TRAIL DR4

##### 4H6

In 2001, Chuntharapai and colleagues developed an anti-DR4 mAb, 4H6, which caused *in vivo* tumor regression in a murine colon cancer model. The mAb was generated by injecting nude BALB/c mice 11 times at 3-4 day intervals with 0.5  $\mu$ g human DR4-Fc fusion protein, creating hybridomas from the popliteal lymph node cells; mAbs which selectively bind to human DR4 were isolated using capture ELISA. An IgG1 mAb, 4H6, used intraperitoneally (IP) (1.25 mg/kg three times a week), induced complete regression of Colo 205 human colon carcinoma tumor cell line in mice. This IgG1 mAb

was far more effective than another IgG2a anti-DR4 mAb, 4G7, which only caused 60% tumor regression at a dose of 2.5 mg/kg three times a week. Further analysis of multiple other isolated mAbs revealed that all IgG1 type Abs were more effective anti-cancer agents than IgG2a mAbs. Although IgG2a mAbs were more effective at inducing apoptosis *in vitro* when used with crosslinking agents, such as secondary antibodies or human C1q, IgG1 mAbs were far more effective at causing tumor regression *in vivo* without crosslinking agents, likely due to endogenous murine complement acting as a crosslinking agent [87]. The 4H6 mAb was most effective and exhibited a binding site on DR4 receptor which overlaps the TRAIL epitope. This was the first example of an agonistic mAb triggering the TRAIL signaling pathway capable of inducing apoptosis *in vitro* and *in vivo*.

##### AY4

Subsequently, in 2009, Sung and colleagues discovered another anti-DR4 mAb called AY4 and investigated its anti-cancer properties. AY4 was generated by injecting peptide fragments of the extracellular domain of human DR4 into BALB/c mice at 2 week intervals, creating hybridomas by fusing splenocytes from the mice with murine myeloma cells, and screening the hybridoma supernatants for DR4 agonistic activity using ELISA. An IgG1/ $\kappa$  mAb was isolated, AY4, which exhibited tumoricidal activity and was selected from the synthesized mAbs for further evaluation. Multiple TRAIL-sensitive cancer cell types, such as colorectal carcinoma, non-small cell lung carcinoma (NSCLC), acute T-cell leukemia, and Burkitt's lymphoma (BL), were treated with TRAIL (0.001-0.5  $\mu$ g/mL) or AY4 (0.001-10  $\mu$ g/mL). The same dosage was used to treat the TRAIL-resistant astrocyte glioma and hepatocellular carcinoma lines. As expected, TRAIL induced cell death in the sensitive but not the resistant cell lines. AY4 also induced apoptosis in all except one (acute promyelocytic leukemia) of the TRAIL-sensitive cell lines and neither of the TRAIL-resistant lines. Competitive ELISA assay revealed that the AY4 binding epitope is distinct from endogenous TRAIL. Investigations on the toxicity of AY4 on non-cancerous hepatocytes was conducted by incubating cells with 10  $\mu$ g/mL AY4 for 24 hours where only < 10% of these cells were killed using a colorimetric MTT-based cell growth assay. This was slightly less than the same

treatment with 5 µg/mL TRAIL which resulted in approximately 12% killing. Western blot also indicated that apoptotic signaling molecules such as procaspases-8, -3, -9, and Bid were cleaved as a result of AY4 treatment, providing more evidence that AY4 induces apoptosis through the death receptor mediated, caspase-dependant cascade. These results indicate that AY4 is an effective DR4-agonist mAb which may be used to selectively induce apoptosis in cancer cells [88].

These investigators humanized the murine AY4 (mAY4) mAb in order to decrease immunogenicity while maintaining its binding specificity. They grafted the complementarity-determining regions (CDRs) of the mAY4 into a human framework (FR) to create a humanized AY4 (hAY4) mAb, which retained the binding specificity of the original antibody. However, DR4 binding affinity of hAY4 was 10-fold lower compared to the original mAY4. To increase binding affinity, 8 Vernier zone residues—framework regions beneath CDRs—were replaced with murine residues. This modification restored binding affinity to levels similar to those of the original mAY4 antibody. Single hAY4 mAbs were ineffective at inducing tumor death, so dimeric leucine zippers (LZ) and trimeric isoleucine zippers (ILZ) were used to link hAY4 mAbs to form bi- and trivalent forms, respectively. MTT assays revealed that LZ-hAY4 and ILZ-hAY4 caused dose-dependent apoptosis in multiple cancer cell lines previously shown to be sensitive to the parental mAY4 mAb [89, 90]. Some cell lines (H460 and HCT116) exhibited significantly higher cell death when treated with ILZ-hAY4 compared to LZ-hAY4. Pretreatment with caspase inhibitors blocked nearly all cell killing, and western blot revealed that treatment with both effective forms of hAY4 (LZ and ILZ) resulted in cleavage of procaspases-8, -3, -9, Bid, and PARP. This reveals that hAY4 killing is mediated via activation of both the extrinsic and intrinsic apoptosis pathways; the same mechanisms used by natural TRAIL signaling through death receptors [91].

These investigators further tested the effects of the original mAY4 mAb on human leukemia cell lines when used in combination with histone deacetylase inhibitors (HDACi). All except one of the leukemia cell lines were highly resistant to 10 µg/ml of AY4 (< 15% cell death). TRAIL induced only moderately more killing in a

few cell lines. However, AY4 or TRAIL combined with two HDACis, SAHA or VPA, induced significantly more cell death in the CEM-CM3 and CCRF-CEM cell lines. SAHA, but not VPA, also enhanced killing in a third cell line, K562. Western blot revealed that 12 hour AY4 or TRAIL treatment combined with SAHA or VPA significantly increased activation of caspases-8, -3, -9, Bid, and PARP in CEM-CM3 and CCRF-CEM, compared with single treatment with AY4 or TRAIL. Combination treatment also resulted in downregulation of c-FLIP, a competitive inhibitor of caspase-8 activation which may contribute to TRAIL resistance. Furthermore, combination treatment for 24 hours caused down-regulation of anti-apoptotic proteins, Bcl-2, Bcl<sub>XL</sub>, XIAP and survivin, which prevent intrinsic apoptosis pathway. These results indicate that SAHA and VPA sensitize some leukemia lines to both pathways of apoptosis induced by AY4 as an effective strategy for overcoming resistance to AY4 [90]. Lee and colleagues confirmed the results with AY4 *in vitro* and *in vivo* with multiple cell lines [89].

### *HGS-ETR1 (mapatumumab)*

In 2005, Pukac and colleagues developed a fully humanized anti-DR4 agnostic mAb called HGS-ETR1 (Mapatumumab). Human phage display libraries were used to isolate mAbs which bind to the extracellular domain of DR4. Candidate antibodies were amplified using NSO mouse myeloma cell line and assayed for binding specificity and affinity. Mapatumumab yielded specific binding for DR4 and caused death of SW480 colon cancer cells in an *in vitro* experiment. Subsequent experiments revealed significant dose-dependent apoptosis induction following activation of caspases-3 and -7 in multiple cancer cell lines upon 48 hour treatment with mapatumumab (0.9–66.7 nM). Western blot analysis of SW480 colon carcinoma and ST486 Burkitt's lymphoma cell lines revealed that cleavage of procaspases-8, -9, -3, Bid and PARP beginning 4 hours post mapatumumab treatment. These molecular effects reveal that it effectively engages the extrinsic and intrinsic apoptosis pathways. *In vivo* experiments on murine xenograft models of three cancer cell lines exhibited 97% reduction in tumor size after 25 days of weekly treatments of 10 mg/kg mapatumumab. This mAb also showed promising results when used in con-

junction with various chemotherapeutic agents (carboplatin, camptothecin, cisplatin and 5-fluorouracil). These studies suggest mapatumumab as a promising novel mAb for therapeutic use [91].

Hotte and colleagues conducted phase 1 clinical studies on the safety and immunogenicity of mapatumumab in 2008. Patients with untreated, malignant tumors of various origins were given intravenous infusions of mapatumumab (0.01 to 20 mg/kg). Toxicity of mapatumumab seemed to be quite low; no maximum tolerated dose was discovered. Grades 1 and 2 side effects included: fatigue, nausea, hypotension, and fever in a minority of patients. A few isolated cases of grade 3 side effects such as hypomagnesaemia, hypertension, fatigue, thrombocytopenia, and vomiting were observed. However, no objective responses were obtained at the dosage administered. There was no consistent tumor regression and seven of the patients died. This study revealed that doses of mapatumumab up to 20 mg/kg are non-toxic and safe for use in humans [92].

In 2010, Younes and colleagues conducted a phase 1b/2 trial on forty patients with non-Hodgkin's lymphoma. Eight patients were treated with 3 mg/kg mapatumumab every 21 days and thirty-two were treated with 10 mg/kg every 21 days. Two patients (one from the low-dose group and one from the high-dose group) with follicular lymphoma (FL) experienced a complete response (CR) and another patient exhibited a partial response (PR). However, the rest of the patients did not exhibit clinical responses, and the treatment did not result in permanent tumor regression. Mild, grades 1 and 2, side effects included nausea, fatigue, diarrhea, anorexia, and pyrexia, and there was one case of grade 3 pyrexia. Laboratory data revealed that there was no significant hepatic or renal toxicity. This was the first study which demonstrates clinical responsiveness to mapatumumab monotherapy in FL patients, and it confirmed the previous findings that mapatumumab is a non-toxic therapeutic agent [93].

### **Agonistic Abs directed against TRAIL DR5**

#### *MD5-1*

Similar to DR4 Abs discussed above, anti-DR5 mAbs have been potent and selective agents in

killing cancer cells. In some cases, they are more effective than DR4 agonists *in vivo* due to their interaction with cells of the innate immune system. In 2004, Takeda and colleagues discovered a novel DR5 agonist mAb called MD5-1 by immunizing a hamster with a murine DR5-Ig fusion protein and isolating resulting mAbs which bound to murine DR5-transfected baby hamster kidney cells. All TRAIL-sensitive mouse tumor cell lines were sensitive to MD5-1. However, in order to exhibit cytotoxicity, MD5-1 had to be crosslinked by biotinylation and linking to streptavidin, crosslinking with anti-hamster Ig antibodies, or crosslinking with Fc-receptors on murine P815 cells. However, binding of MD5-1 to DR5 did not inhibit TRAIL signaling, suggesting that they share non-overlapping epitopes. Caspase cascades were implicated in the MD5-1 killing mechanism as the pan-caspase inhibitor z-VAD-fmk and c-FLIP transfected cells were resistant to MD5-1 and TRAIL cytotoxicity. MD5-1 was also effective in mediating antibody-dependent cellular cytotoxicity (ADCC). Macrophages were determined to be more effective at promoting MD5-1 cytotoxicity than NK cells, and in both cases cytotoxicity was dramatically decreased by anti-FcR mAbs. Perforin knockout mice exhibited equivalent MD5-1 cytotoxicity as normal mice indicating that FcR-expressing effector cell help is only required to crosslink MD5-1 antibodies. However, freshly isolated effector cells did exhibit slightly higher cytotoxicity than fixed cells grown in flasks, revealing that MD5-1 crosslinking of FcRs also induced some ADCC by the effector cells. Intraperitoneal administration of MD5-1 significantly inhibited 4T1 murine mammary carcinoma tumor growth as well as lung and liver metastases. The discovery of this novel anti-DR5 mAb yielded promising results due to enhanced tumoricidal activity resulting from its interactions with innate effector cells in these *in vivo* experiments [94].

The same research team analyzed the specific nature of effector cells involved in promoting MD5-1 cytotoxicity [95]. They chose murine colon adenocarcinoma cell line MC38 for *in vivo* experiments due to its high sensitivity to death receptor-mediated apoptosis. MD5-1 treatment caused MC38 tumor regression *in vivo*, even complete tumor regression in some cases, starting as little as 24 hours post-treatment. Depletion of NK cells and T cells had no



effect on the tumoricidal action of MD5-1. However, the tumoricidal activity of MD5-1 was completely abrogated in B-cell deficient mice. Further, dendritic cell (DC)-deficient mice exhibited significant decrease in MD5-1 efficacy. MD5-1 induced apoptosis was decreased significantly at 8 hours after treatment in B-cell deficient but not DC deficient mice. However, 24 hours post-treatment, DC deficient mice also exhibited reduced apoptosis compared to wild type. These results indicated that although B cells may be the essential effector cells required to initiate MD5-1 mediated apoptosis, DCs are also required to sustain tumoricidal activity [95].

### KMTR2

Nagane and colleagues at the Kyorin University Hospital used mAbs E11, H48, and KMTR2 against DR5 and mAb B12 against DR4 on twelve human glioma lines [96]. DR5 mAbs E11 and H48 were successful at inducing apoptosis at  $IC_{50} < 0.1 \mu\text{g/mL}$  in 8/12 cell lines when administered with crosslinking anti-human IgG antibodies. This  $IC_{50}$  value was similar to results obtained with soluble FLAG-TRAIL. KMTR2 (a human Ab developed by Motoki and colleagues) killed three cancer cell lines without requiring crosslinking Abs [97]. *In vivo* experiments on BALB/c mice with tumor xenografts revealed the superior efficacy of KMTR2 over E11 at suppressing tumor growth. Mice with intracerebral human glioma xenografts exhibited slightly better survival rates when treated with E11 while KMTR2 treatment completely prevented tumor growth. This was likely due to the fact that KMTR2 does not require crosslinking antibodies and can function regardless of crosslinker presence at the tumor site. The expression levels of c-Flip were lower in the TRAIL sensitive cell lines, contributing to greater efficacy of DR5 agonistic mAbs. These findings present DR5 agonist KMTR2 as a highly effective tumoricidal agent [96].

### TRA-8

In 2001, Ichikawa and colleagues generated a novel DR5-agonist IgG1/k mAb by immunizing BALB/c mice with a human DR5/IgG1 Fc region fusion protein. A resultant mAb, TRA-8, bound exclusively to human DR5; it competed with TRAIL for DR5 binding, revealing that its binding site overlaps with the TRAIL epitope. TRA-8

exhibited high tumoricidal activity against Jurkat cells. Western blot revealed cleavage and activation of caspases as early as 30 minutes after treatment, and caspase inhibitors blocked its tumoricidal activity, implicating the extrinsic apoptosis signaling pathway. Treatment with 100  $\mu\text{g}$  intravenous (IV) TRA-8 prevented tumor formation in mice inoculated with human astrocytoma or leukemic cells and decreased pre-existing tumor weight by at least four-fold. TRA-8 was shown not to be toxic to somatic cells such as hepatocytes. This study establishes TRA-8 as a highly effective anti-cancer agent [98].

Buchsbaum and colleagues evaluated TRA-8 in conjunction with chemotherapy and radiation for treatment of breast cancer in 2003. They found that only four out of nine breast cancer cell lines tested *in vitro* were sensitive to TRA-8 killing with  $IC_{50}$  values between 17 and 299 ng/ml. Combination therapy with TRA-8 and the chemotherapeutic agent Adriamycin (ADR) (Doxorubicin) yielded synergistic killing in four out of the nine cell lines. Murine xenograft breast cancer models exhibited inhibition of tumor growth when treated with TRA-8 alone, and TRA-8 and Adriamycin co-treatment resulted in synergistic inhibition of tumor growth. When  $^{60}\text{Co}$  radiation therapy was added to combined TRA-8 and Adriamycin treatment, six out of eight mice exhibited complete tumor regression and four of the mice remained tumor-free for the next 180 days. This reveals the effectiveness of TRA-8 as a therapeutic agent when combined with chemotherapy and radiation [99]. In 2006, the efficacy to TRA-8 treatment alone and in combination with chemotherapy and radiation for cervical cancer was evaluated [100]. *In vitro*, TRA-8 single treatment caused cell death in only three cervical cancer cell lines tested, and the other three cell lines were resistant. TRA-8 administered in conjunction with the cisplatin (CDDP) and/or  $^{60}\text{Co}$  radiation resulted in synergistic killing. TRA-8 also exhibited tumoricidal effects in mice with cervical cancer xenografts. Co-treatment with CDDP and  $^{60}\text{Co}$  increased effectiveness [100].

In 2011, these investigators investigated the efficacy of TRA-8 against basal-like breast cancer stem cells BrCSC [101]. They enriched multiple BrCSC lines and found that all except one were extremely sensitive to TRA-8 killing, with  $IC_{50}$  less than 100 ng/ml. They then incubated

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BrCSC with TRA-8 or a control IgG for 2 hours and found caspases-8 and -3 activation in those exposed to TRA-8 but not the control, indicating that TRA-8 was successfully inducing apoptosis in a caspase-dependant manner. BrCSC can normally develop into tumor cells in immuno-compromised mice. To test the effect of TRA-8 on tumor formation, BrCSCs were treated with TRA-8, an anti-DR4 mAb, or an IgG control for 3 hours prior to injection into the mouse. All 5 mice treated with anti-DR4 mAb or the IgG control developed tumors. However, TRA-8 treated cells only developed small tumors with impaired growth in 2 out of the 5 mice after 50 days. Extending the experiment with another BrCSC line, no tumors developed in TRA-8 treated mice after 105 days, but all of the anti-DR4 mAb and IgG control treated mice developed tumors. TRA-8 inhibited tumor growth over 50 times more effectively than TRAIL or anti-DR4 administered at the same concentrations. This study reveals that TRA-8 can effectively prevent BrCSC tumorigenicity [101].

### *Drozitumab*

In 2008, scientists at Genentech led by Avi Ashkenazi used a human antibody phage display library to develop a novel IgG1/ $\gamma$ 3 monoclonal antibody that specifically binds to DR5 and triggers apoptosis. X-ray crystallography revealed that this antibody, named Apomab, binds to a DR5 epitope which partially overlaps the TRAIL binding sites. Exposure of NSCLC cells to Apomab caused DR5 clustering followed by DISC formation and caspase-8 activation. Caspase-8 activation was potentiated by the addition of anti-Fc crosslinking antibodies. Apomab induced dose-dependent killing of NSCLC and COLO205 colorectal cancer cells that was enhanced by the addition of anti-Fc crosslinking Ab. However, human hepatocytes were resistant to Apomab. Treatments of 3 and 10 mg/kg Apomab caused complete tumor regression *in vivo* in 7/9 mice with subcutaneous COLO205 tumors. Similarly, mice with H2122 NSCLC xenografts exhibited complete tumor regression in response to the same dose of Apomab and remained tumor free for 32 days. Apomab also caused complete regression of Mia-PaCa-2 pancreatic tumor xenografts at a dose of 10 mg/kg [102]. Zinonos and colleagues investigated the use of Apomab for breast cancer treatment. Apomab (200 ng/

mL and anti-Fc Ab) caused 40-90% apoptosis in several breast cancer cell lines, but it had no effect on any non-cancerous cells. Sensitive cell lines expressed higher levels of DR5, DR4, caspase-8, caspase-10, and FADD. In an *in vivo* model of athymic nude mice with breast cancer xenografts, mice injected with 10 mg/kg Apomab intraperitoneally once a week beginning prior to xenograft injection did not develop any tumors for 90 days. Weekly doses of 3 and 10 mg/kg Apomab caused complete and sustained tumor regression [103], showing the safety and efficacy of Apomab as an anti-cancer therapeutic agent.

In 2010, Camidge and colleagues conducted a Phase I clinical study on the safety and efficacy of Apomab (renamed PRO95780) in cancer patients. 50 patients with a variety of advanced cancer were treated with an escalating dose of 1-20 mg/kg PRO95780 every 14 days. Eight percent of patients experienced adverse side effects greater than grade 2. No significant tumor regression was observed, 49% of patients exhibited stable disease and 51% experienced progressive disease symptoms. However, this study revealed that PRO95780 is safe at doses up to 20 mg/kg and pharmacokinetic analysis revealed the optimum dose to be 10-15 mg/kg every 2 to 3 weeks [104].

Wilson and colleagues at Genentech conducted a study with PRO95780 (again renamed Drozitumab) to identify the mechanism by which it mediates apoptosis. *Ex vivo* analysis of colon carcinoma cells from xenografts treated *in vivo* with Drozitumab revealed recruitment of caspase-8 to the DISC, followed by caspase-3 activation. Colo205 colon carcinoma co-incubated with leukocytes expressing Fc $\gamma$ R exhibited caspase-3 activation upon incubation with Drozitumab; cells exposed to Fc $\gamma$ R-blocking antibodies had significantly reduced levels of activated caspase-3. This reveals that Fc $\gamma$ R on tumor associated cells may assist in Drozitumab's clustering of DR5 and subsequent induction of apoptosis [105]. These results indicate that the expression of the Fc $\gamma$ R by immune system leukocytes is essential for the proper function of Drozitumab-mediated DR5 engagement and apoptosis induction.

Kang and colleagues investigated Drozitumab *in vitro* and *in vivo* for the treatment of Rhabdomyo-sarcoma (RMS). Eleven RMS cell

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lines were analyzed and 6 of them displayed high sensitivity to Drozitumab and anti-Fc crosslinking Ab as early as 2 hours following treatment. Drozitumab-resistant cell lines contained lower levels of intracellular caspase-8. Successful Drozitumab-induced cell death was marked by association of FADD with caspase-8 (to form the DISC complex), as expected. One resistant RMS cell line transfected with a caspase-8/GFP gene exhibited increased sensitivity to Drozitumab-mediated killing. An *in vitro* study using Drozitumab-sensitive RMS lines (R18) revealed that Drozitumab prevented xenograft establishment in half of the experimental mice after four months [106]. These results indicate that the DR5 agonist Drozitumab is a promising new cancer treatment that has the potential to selectively induce apoptosis of a variety of cancer cell types.

### Concluding remarks and future perspectives

Since its discovery by Avi Ashkenazi and colleagues at Genentech over two decades ago, our knowledge of the TRAIL/Apo2L cytotoxic pathway has significantly increased. Ample experimental evidence supports the hypothesis that TRAIL/Apo2L apoptotic signal transduction pathway employed by immune effector cells plays a prominent role in regulating uncontrolled growth of tumors and metastasis. TRAIL/Apo2L interacts with DR4 and DR5 (death inducing), and DcR1 and DcR2 (decoy) receptors. Due to its selective cancer killing ability, TRAIL/Apo2L or agonistic anti-DR4, -DR5 mAbs have received special attention as novel therapeutic tools in modern clinical oncology. Selective triggering of this pathway may initiate key apoptotic cascades important in killing melanomas. However, some melanomas are inherently resistant to TRAIL/Apo2L or develop resistance following initial successful treatment through up-regulation of c-FLIP, down-regulation of caspase-8, DR-4 and/or DR-5 down-regulation, aberrant signaling pathways and imbalanced ratio of pro- to anti-apoptotic gene products. A novel class of anti-cancer agents, namely, HDACi, has shown promise in cancer treatment by preventing silencing of tumor suppressors and pro-apoptotic genes, and up-regulating anti-apoptosis genes. Studies conducted on preclinical models of metastatic melanoma as well as cell lines have shown that treatment of resistant tumors with

combination of HDACi and TRAIL/Apo2L (or agonistic mAbs) overcomes acquired/inherent resistance to either agent. Thus, epigenetic modifiers (chromatin remodeling drugs) represent as new therapeutic modalities in the treatment of advanced and resistant melanomas which can be used as adjuvants to immunotherapy.

Although our knowledge of the mechanisms for TRAIL/Apo2L signaling has improved, a number of major issues still remain unclear. For instance, what are the main resistance mechanisms? What is the exact role of decoy receptors? Also, there may be as of yet other (pro-survival) signaling pathways activated by TRAIL/Apo2L. Also our understanding of the exact mechanisms of some of the key players in this signaling module remains largely hypothetical. Further research is warranted to shed more light on the intricacies of this apoptosis signaling pathway.

### Acknowledgements

This work was supported by the National Center for Research Resources and the National Cancer Institute (NCI) of the National Institutes of Health through Grant Numbers NIH 1R21 CA149938-01A1 and PO1 21B-1088934. The authors wish to acknowledge Peter Wenn for assistance with the figures.

### Conflict of interest statement

The authors claim no conflicts of interest.

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