2017

# Therapeutic Potential of rhTRAIL for Malignant Melanoma

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# THERAPEUTIC POTENTIAL OF RHTRAIL FOR MALIGNANT MELANOMA

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**Cleveland State University**

**December 2011**

**Submitted in partial fulfillment of the requirements**

**For the degree**

**DOCTOR OF PHILOSOPHY IN CLINICAL-BIOANALYTICAL CHEMISTRY**

**At the**

**CLEVELAND STATE UNIVERSITY**

**May 2017**

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*To my loving and supportive family*

**ACKNOWLEDGEMENTS**

I would first like to thank Dr. Kalafatis. I cannot thank you enough for giving me the opportunity as an undergraduate to work in your lab. Because of you I now have my PhD. Thank you for allowing me to grow as independent scientist. Allowing me to make mistakes and succeed all on my own. Thank you to all my committee members. Dr. Lindner for always taking the time to talk and listen to me. Dr. Anderson for introducing me to clinical chemistry and helping me get my fellowship at Mayo. Dr. Weyman for being such a strong female role model and teaching me to speak up and question everything. Dr. Zhou for always being supportive and interested in my success. Finally, Dr. Plow for his thoughtful input on my research.

I would like to thank everyone from the Kalafatis lab, especially Jasmine. Without her I do not think I would have made it through my PhD. I will miss our lab teamwork. Seema, for being such a caring friend and coffee date. Joe, for teaching me the basics of research. Jamila, for teaching me to be tough and confident. Thank you to all the people who have helped me along the way. Yvonne, thank you for being my friend and teaching me how to handle the mice. Michael, thank you for teaching me how to use to the flow cytometry. Because of your education I can now teach others. Dale and Barb, thank you for sharing your words of wisdom in my times of need.

In the end, I would like to dedicate my dissertation to my loving family. Thank you mom and dad for always believing in me and giving me the opportunities in life to succeed and

make you proud. You scarified everything to give me a good life and get an education. To my sweet Jimmy. I love you so much. You were always there to listen and give me the courage to take on any task and never give up. Finally, thank you Patty for your grammar skills and proofreading pretty much everything I wrote. However, even though I have my PhD I am still not 100% percent sure how to use commas.

Thank you everyone for your love and support, for without you all I would not been able to achieve my dreams.

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**KATHERINE A. TURNER**

## ABSTRACT

The application of recombinant human Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhTRAIL) for the treatment of cancer holds great promise due to its ability to selectively induce apoptosis in cancer cells while not harming normal healthy cells. This is evident by the robust levels of apoptosis induced in malignant melanoma cells while no event of apoptosis was observed in the non-transformed counterpart of melanomas, melanocytes. However, the clinical utility of rhTRAIL is limited due to the heterogeneity seen in rhTRAIL-sensitivity among cancers. rhTRAIL-resistance is especially prevalent in cases of malignant melanoma. Melanoma rhTRAIL-resistance can be attributed to a number of different causations including low expression of rhTRAIL-binding receptors (death receptors (DRs)) and overexpression of anti-apoptotic proteins. Most noteworthy is the correlation between rhTRAIL-sensitivity and the membrane expression of rhTRAIL receptors DR4 and DR5. The membrane expression of DR4 and DR5 may be potential markers for predicting a patient's sensitivity to rhTRAIL. We propose the development of an *in vitro* assay to measure the membrane expression of DR4 and DR5 to determine a patient's suitability for rhTRAIL-treatment. Additionally, rhTRAIL-resistance can be circumvented by combining rhTRAIL with the "Mother Nature"-derived compound quercetin. Quercetin possesses the ability to modulate some of the cellular components that confer rhTRAIL-resistance. Resistant malignant melanomas are sensitized to the effects of rhTRAIL by the quercetin-mediated

upregulation of DR4 and DR5 and the downregulation of the anti-apoptotic protein FLIP. Overall, these data show the potential of rhTRAIL to act as a potent anti-cancer therapeutic and methods to overcome rhTRAIL-resistance.

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# CHAPTER I

# INTRODUCTION

## 1.1 Malignant Melanoma.

Malignant melanoma is the most deadly of the skin cancers with increasing incidence and mortality rates worldwide. Characterized by high rates of metastasis and chemo-resistance, malignant melanoma is associated with a lifetime risk of 1 in 52. In the U.S., 76,380 people are expected to be diagnosed with malignant melanoma resulting in 10,130 deaths and $3.3 billion spent in treatments for 2016. In its early stage, melanoma is easily cured, but the prognosis associated with metastatic malignant melanoma remains very poor. For localized melanoma contained to the epidermis, the 5-year survival rate is 98%. When detected early, the melanoma cells can be removed in most cases by one of several methods such as surgical excision. Whereas advanced cases of metastatic malignant melanoma has an extremely low median survival rate. Once the cancer invades the basement membrane and moves to the lymph nodes to form regional melanoma and finally metastasizes to other organs to form distant melanoma the 5-year survival rate decreases to 63% and 17%, respectively (1). Despite decades of clinical trials, a standard first-line treatment for metastatic malignant melanoma has not been established and remains one of the most treatment-refractory malignancies. Melanomas with deep tissue

invasion or that have metastasized may be treated with surgery, targeted therapy, immunotherapy, chemotherapy or radiation therapy. However, treatments with chemotherapeutic agents, Dacarbazine and Tamoxifen, or immunotherapies with Interleukin-2 (IL-2) or Interferon-α (INF- α) have not resulted in responses of long-lasting remissions. Extensive research on the epidemiology of melanoma has resulted in more effective therapies such as targeted treatments, Vernurafenib and Darbrafenib, and immunotherapies, anti-CTLA-4 and anti-PD1 (2–4). Unfortunately, these newer therapies have been linked with numerous negative side effects, slow effectiveness and only transient anti-tumor activity due to acquired resistance with the emergence of resistant cells and tumor recurrence (5,6). New strategies are needed to improve the treatment outcome and survival of these patients

**1.2 Dysregulation of Apoptosis and Cancer.**

Cancer is a disease characterized by irregular proliferation, inappropriate cell survival, decreased apoptosis, cell immortalization, invasion of surrounding tissue and metastasis. In particular, apoptosis or programmed cell death is a tightly-controlled physiological process of cell elimination that is essential in the maintenance of tissue homeostasis. Dysregulation in the apoptotic pathway or resistance to apoptotic stimuli is instrumental in the initiation and progression of cancer. Mechanisms of resistance include overexpression of oncogenes, inactivation of tumor suppressor genes, imbalance of pro- and anti-apoptotic proteins and inactivation of the intrinsic and extrinsic apoptotic pathways. Many current cancer therapies such as conventional chemotherapy and radiotherapy aim to eliminate cancer cells through induction of apoptosis. However,

induction of apoptosis only occurs secondarily as a result of causing severe cell damage in a p53-dependent manner. The tumor suppressor gene, p53, is an important mediator between sensors of cellular damage and the intrinsic pathway of apoptosis. However, in over 50% of all cancers, p53 is mutated and resistance to standard p53-dependent cancer therapies often occurs. Additionally, these traditional therapies are non-specific for cancer cells and target all cells, cancerous and non-cancerous, for cell death. The lack of specificity often results in systemic toxicity that effects the patients overall quality of life and prevents optimal drug-dosing (7). The development of more selective and specific therapies that can circumvent the resistance of chemotherapy and radiotherapy is vital. A direct and selective therapy is the application of pro-apoptotic receptor agonists that can induce apoptosis through the p53-independent extrinsic pathway (8).

**1.3 Pathways of Apoptosis.**

Apoptosis or type I programmed cell death is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, destruction of the cytoskeleton, blebbing of the cellular membrane and the formation of apoptotic bodies. The apoptotic bodies are then recognized and phagocytized by macrophages without an inflammatory response (9). The process of apoptosis can be triggered by two distinct but convergent pathways: the death-receptor-mediated extrinsic pathway and the mitochondrial-dependent intrinsic pathway (Fig. 1.1), both of which offer therapeutic manipulation. The extrinsic pathway of apoptosis is triggered by the binding of pro-apoptotic death-ligands, such as TRAIL, to receptors located on the extracellular member that possess cytoplasmic death domains (DD). Ligand-receptor binding results in the trimerization of the receptors leading to the

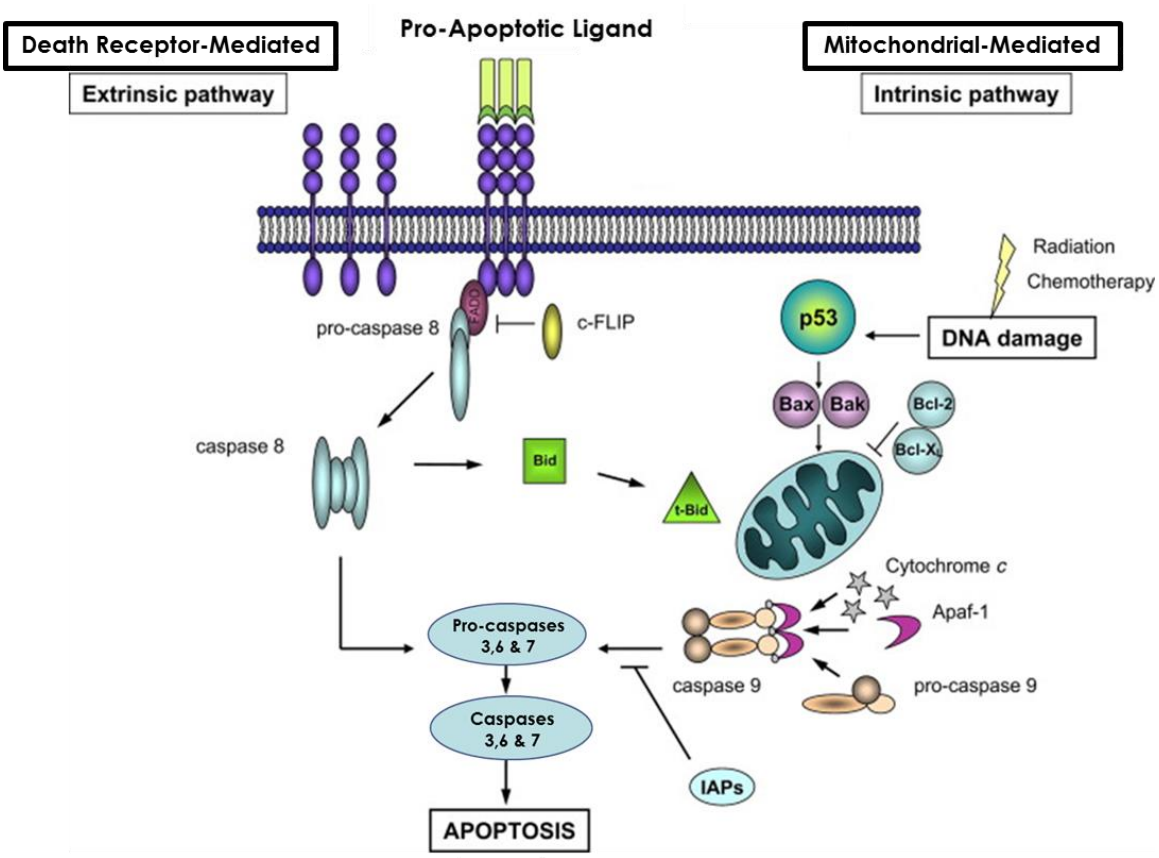


**Figure 1.1: Schematic of the extrinsic and intrinsic pathways of apoptosis.** (Modified from Duiker *et al*. 2006)

assembly of the intracellular death-inducing signaling complex (DISC). At the DISC, the adaptor protein, Fas-associated death domain (FADD), acts as a bridge between the death-receptor complex and the pro-domain of the initiator caspase, procaspase 8. Induced proximity results in the autoproteolytic cleavage of procaspase 8 into its active form, caspase 8. Caspase 8 possesses the ability to activate downstream effector or executioner caspases such as procaspases 3, 6 and 7 to caspases 3, 6 and 7, which ultimately execute the hallmark events of apoptosis. Moreover, tfhe mitochondrial-dependent intrinsic pathway is initiated after DNA or microtuble damage by such agents or environmental changes as chemotherapy, radiotherapy, hypoxia, starvation and other kinds of severe cellular stress. This pathway is dependent on p53 and the ratio between pro-apoptotic and anti-apoptotic members of the Bcl-2 family. When the intrinsic pathway is activated, pro-apoptotic members of the Bcl-2 family, Bax and Bak, will translocate to the mitochondria causing a decrease in its membrane potential and the release of cytochrome c. Cytosolic cytochrome c binds to the adaptor protein, apoptotic protease activating factor-1 (Apaf-1), and procaspase 9 in the presence of dATP, forming the apoptosome signaling complex. Formation of the apoptosome complex results in the activation of procaspase 9 to caspase 9. Activated caspase 9 can then activate the executioner procaspases 3, 6 and 7 to their active form. Cross-talk between the extrinsic pathway and the intrinsic pathway can occur and is mediated through Bid, a BH3-only protein of the Bcl-2 family. Caspase 8 can activate Bid through proteolytic cleavage to truncated Bid (tBid), which then translocates to the mitochondria, activating the intrinsic pathway. This mitochondrial amplification loop intensifies the pro-apoptotic signal initiated by the death-receptor-mediated extrinsic pathway and provides a mechanism to activate the intrinsic pathway independent of p53.

Of additional importance are proteins that inhibit the pathways of apoptosis, such as anti-apoptotic members of the Bcl-2 family and inhibitors of apoptosis proteins (IAPs). Bcl-2 and Bcl-$x_L$, members of the Bcl-2 family, prevent the activation of the intrinsic pathway by preventing the release of cytochrome c from the mitochondria. IAPs such FLIP, XIAP, cIAP and survivin act as anti-apoptotic proteins by inhibiting the activation of caspases from their inactive precursors, specifically procaspase 8 and 9 (10).

**1.4 TRAIL.**

A member of the TNF-gene superfamily, TRAIL was discovered through sequence homology to Fas Lignad (FasL). TRAIL is expressed as a type II transmembrane protein possessing a highly-conserved extracellular C-terminus. The extracellular domain of the membrane-bound TRAIL can be cleaved by metalloproteases to form soluble TRAIL (sTRAIL) (11). Both full-length membrane-bound TRAIL and sTRAIL can induce apoptosis in a wide variety of human cancers, ranging from colon, lung, breast, kidney, brain, pancreas, prostate, skin, leukemia, multiple myeloma, lymphoma and non-Hodgkin's lymphoma (NHL), independent of p53 status and with minimal toxicity toward normal tissues both *in vitro* and *in vivo* (10). Other members of the TNF-family such as FasL, TNFα and CD40L are also able to induce apoptosis, but their ability to be utilized as effective cancer therapeutics is hindered by their systemic toxicity. For example, administration of FasL causes severe liver toxicity and systemic use induces a sepsis-like syndrome. Whereas, TRAIL shows the ability to selectively induce apoptosis in cancer cells with no influence on normal cells or signs of systemic toxicity (12). One study shows the selectivity of TRAIL by testing early-passage primary human umbilical vein

endothelial cells, lung fibroblasts, mammary, renal or prostatic epithelial cells, colon smooth muscle cells and astrocytes with high dosages of TRAIL (1 μg/ml). These healthy noncancerous cells were found to be unaffected by the TRAIL with no morphological evidence of apoptosis (13). For this reason, TRAIL represents a valuable candidate as a pro-apoptotic cancer therapy. Located on chromosome 3 at position 3q26, TRAIL mRNA is found in a variety of cells and tissues, particularly in lymphoid system, spleen, prostate, and lung (14). This contrasts FasL whose transcripts are largely restricted to stimulated T cells. The ubiquitous distribution implies that TRAIL must not be toxic to most tissues. Like other members of the TNF-family, TRAIL is involved in apoptotic signaling and in the function of the immune system. TRAIL is found to be upregulated upon lymphocyte activation and is expressed on the surface of natural-killer cells and cytotoxic T cells. Additionally, sTRAIL is found to be generated by activated monocytes and neutrophils and seems to play a role in the elimination of virus-infected or malignant cells by these immune effector cells (11,15). The role of TRAIL in tumor immune-surveillance is displayed in TRAIL neutralization experiments. In these experiments, carcinogen methylcholanthrene (MCA)-mediated tumor formation was found be to more prevalent in mice where TRAIL was neutralized and also in $TRAIL^{-/-}$ mice compared to wild-type (WT) mice with functional TRAIL expression (14). Overall, it is believed that the true function of TRAIL is in immune-surveillance functioning in innate immune responses against both tumors and virus-infected cells (16).

**1.5 TRAIL Structure.**

Crystallographic studies show that TRAIL has a homotrimeric structure, consisting of three TRAIL monomers coordinated by an internal zinc atom (Fig 1.2) (15). The zinc atom is buried at the center of the trimer, coordinated by a single cysteine residue on each monomer located at position 230 and is required for the structure and function of TRAIL. The metal-mediated trimerization of a ligand represents a unique stscarofructure and stability that differentiates TRAIL from all other members of the TNF-family. With a stoichiometry of approximately one metal ion per trimer, zinc is required for maintaining the structure and stability of the trimer and hence the overall biological activity of TRAIL. The functional importance of the zinc-binding site is demonstrated by the observation that alanine or serine substitutions of cysteine 230 results in 20- and 70-fold reduction in the apoptotic activity of TRAIL, respectively. Additionally, removal of zinc from WT TRAIL by dialysis against chelating agents results in a significant decrease in the receptor-binding affinity of TRAIL and a 90-fold decrease in apoptotic activity. Zinc depletion results in the destabilization of TRAIL marked by large conformational changes in the cysteine

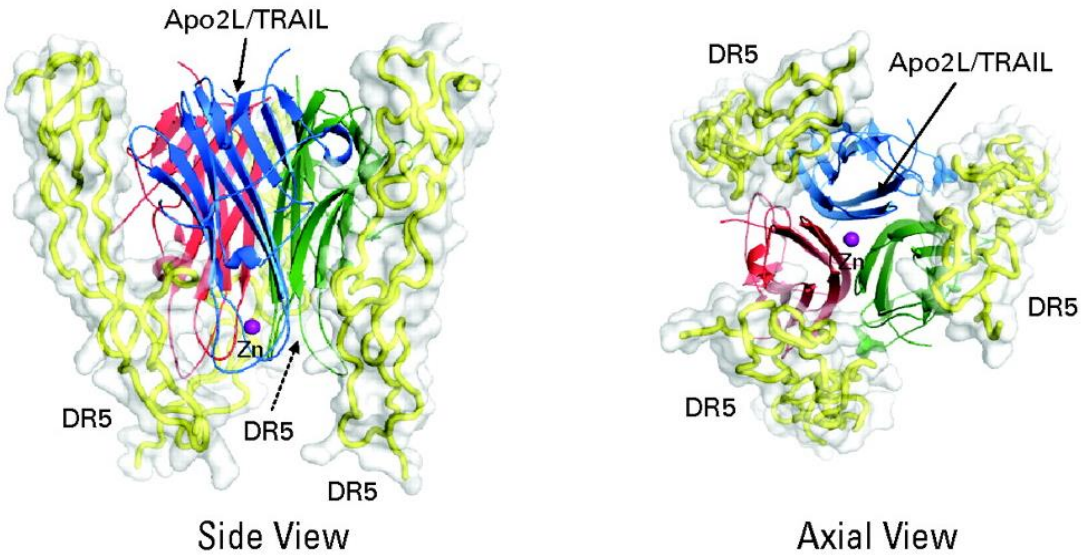


**Figure 1.2: The molecular protein structure of TRAIL.** (Ashkenzi *et al*. 2008)

230 region and a 25°C decrease in the melting point compared to the WT TRAIL. Removal of zinc makes the cysteines more prone to oxidation and to the formation of poorly active monomers or disulfide-linked dimers of TRAIL (12).

**1.6 TRAIL Receptors.**

TRAIL can bind five different receptors: four membrane-bound and one soluble (Fig 1.3). Two of the receptors, death receptor 4 (DR4) and death receptor 5 (DR5), act as functional pro-apoptotic receptors, containing a cytoplasmic DD through which TRAIL can initiate the extrinsic apoptotic pathway. The two other membrane-receptors, decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2), also bind TRAIL but act as antagonistic receptors due to their lack of a DD. Therefore, these receptors cannot transmit an apoptotic signal upon ligand binding. DcR1 is glycosylphosphatidylinositol (GPI)-linked to the cell and is absent of any transmembrane or cytoplasmic domains capable of transmitting an intracellular signal. DcR2 contains a truncated DD and also cannot transmit an apoptotic signal. In addition to the four membrane-bound receptors, a fifth soluble antagonistic receptor, osteoprotegerin (OPG) exists. When TRAIL binds a functional receptor, DR4 or DR5, then the extrinsic apoptotic cascade will be triggered, but if TRAIL binds a decoy receptor, then no signal will be transmitted. TRAIL binds as a homotrimer stabilized by a zinc molecule to the cysteine-rich pseudorepeats in the extracellular domain of these receptors causing trimerization of the receptors. The crystal structure of the ligand-receptor complex between TRAIL and DR4 and/or DR5 shows that the trimeric ligand engages with three monomeric receptors, interacting at the interfaces between the monomers of TRAIL

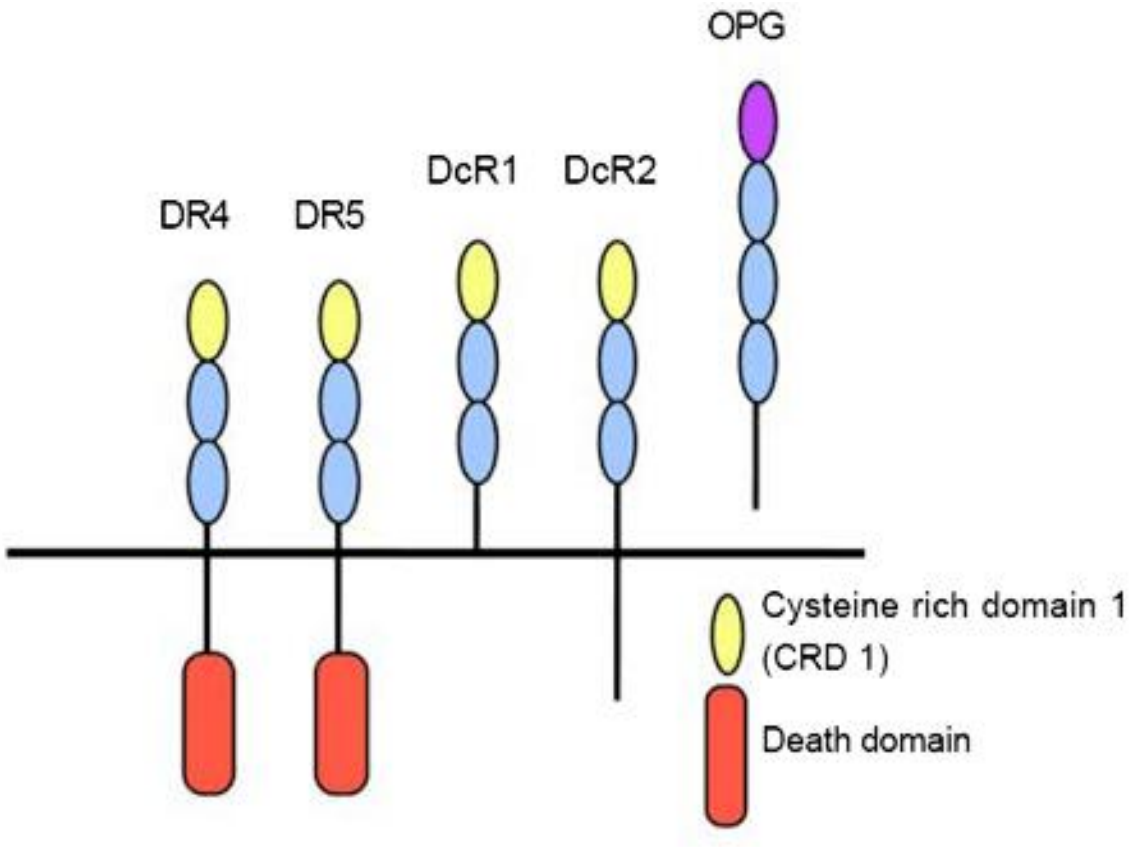


**Figure 1.3: Schematic of the five TRAIL receptors.** (Kimberley F *et al*. 2004)

(Fig 1.2). This phenomenon led to the formation of the "ligand trimerization model" in which the incoming trimeric TRAIL recruits three receptor molecules, triggering an intracellular signaling cascade (16).

**1.7 TRAIL preparations.**

A recombinant version of TRAIL is utilized here, recombinant human TRAIL (rhTRAIL). rhTRAIL is the optimized form of the endogenous apoptosis-inducing ligand, consisting of the extracellular C-domain, amino acids 114-281, and lacks any exogenous sequence tags. rhTRAIL, like its naturally occurring counterpart, can induce apoptosis in a broad range of cancer cells by binding to DR4 and/or DR5. Interestingly, rhTRAIL can induce apoptosis in cancer cells while not activating apoptosis in normal cells, including epithelial, endothelial, fibroblastic, smooth muscle, astrocytic and hematopoietic stem cells. Although the mechanism in which rhTRAIL is able to selectively induce apoptosis in cancer cells while sparing normal cells is not yet been established, this characteristic makes rhTRAIL a prime candidate as a pro-apoptotic cancer therapy (15). However, the true potential for rhTRAIL as a therapeutic is marred by a controversial debate over its potential toxicity to human hepatocytes (16). Certain non-optimized versions of rhTRAIL that contain exogenous sequence tags have been reported to induce apoptosis in cultured hepatocytes. Specifically, a polyhistidine-tagged preparation of rhTRAIL (rhTRAIL.His) was found to induce apoptosis in human hepatocytes. Whereas the nontagged version of rhTRAIL, even at a 1000-fold higher concentration, did not induce apoptosis in the human hepatocytes. It was later shown that rhTRAIL.His contained low amounts of zinc and was unstable in solution forming insoluble aggregates upon incubation. The nontagged

rhTRAIL contained near-stoichiometric zinc levels and remained homogenous as 99% trimers (17). The safety of the nontagged rhTRAIL was also proven *in vivo* through intravenous (I.V.) injections into severe combined immunodeficient (SCID) mice harboring human hepatocytes. Injection of the nontagged rhTRAIL showed no effects to the human hepatocytes and the overall safety of the animal model was maintained (15). The rhTRAIL presented is an optimized preparation consisting of amino acid residues 114-281 and contains no exogenous sequence tags.

**1.8 Preclinical and Clinical Trials of rhTRAIL.**

RhTRAIL, an optimized and nontagged version, has been used in clinical studies led by Genentech, Inc. Beforehand, a number of preclinical studies were performed to determine the pharmacokinetics and safety of rhTRAIL. The pharmacokinetic properties of rhTRAIL were investigated in a number of diverse animal models. The half-life of rhTRAIL was found to be 3-5 minutes in rodents and 23-32 minutes in non-human primates, and its clearance directly correlated with glomerular filtration rate. The half-life was not affected by multiple dosages, and there was no evidence of drug accumulation or antibody formation (18). Additionally, preclinical safety studies in non-human primates such as cynomologus monkeys and chimpanzees showed that systemic application of rhTRAIL was unlikely to cause major toxicity in human patients. Even at dosages as high as 100 mg/kg given for seven consecutive days were well tolerated with no signs of clinical pathology to any major organs. Most noteworthy, is that there were no detectable indications of liver toxicity or changes in liver enzyme activity (13,17).

The preclinical pharmacokinetic studies of rhTRAIL led to the design of an optimized dose and schedule for rhTRAIL to be used in clinical trials. To achieve maximum drug efficacy, one-hour I.V. infusion of rhTRAIL for five consecutive days on a 21-day cycle was applied. Firstly, the safety and pharmacokinetics were evaluated in a phase Ia trial in patients with advanced cancer and lymphoma and a phase I dose-escalation study. The phase Ia trial in patients with advanced cancer and lymphoma consisted of 39 patients who received rhTRAIL at dosages ranging from 0.5-15 mg/kg I.V. for five days on a 21-day cycle. Even dosages as high as 15 mg/kg for 120 days resulted in no drug accumulation or antibody formation (19). The dose-escalation study consisted of 71 patients with advanced cancers who were treated with I.V. infusions of Dulanermin (rhTRAIL) at doses between 0.5-35 mg/kg for five days on a 21-day cycle. Evaluation of the maximum-tolerated dose and dose-limiting toxicities was done to determine the overall safety of receiving multiple doses of rhTRAIL. Adverse effects included fatigue, nausea and vomiting but overall the effects were mild and well managed and others were consistent with disease progression. It was also found that rhTRAIL doses up to 35 mg/kg were well tolerated and safe with no antibody formation or hepatotoxicity. The half-life of rhTRAIL was found to be 0.56-1.02 hours after treatment, and there was no drug accumulation after multiple doses. Overall, 33 (46%) of patients had stable disease or better at the end of cycle 2. Eight patients had stable disease for >4 months but <6 months and two patients (3%) with chondrosacroma had confirmed partial responses at >6 months (20). Additionally, rhTRAIL was evaluated in combination with Rituximab in patients with non-Hodgkin's lymphoma and also in combination with Paclitaxel, Carboplatin and Bevacizumab (PCB) in patients with advanced non-squamous non-small-cell lung cancer

(NSCLC). In the clinical trial of rhTRAIL in combination with Rituximab, seven patients were treated with one-hour I.V. infusion of 4 or 8 mg/kg rhTRAIL for five days on a 21-day cycle and Rituximab at 375 mg/m$^2$ weekly. There were no dose-limiting toxicities or adverse effects to report. Two patients had a complete response, one had a partial response and two had a stable disease response (21). Moreover, in the clinical trial of rhTRAIL in combination with PCB, the standard treatment for non-squamous NSCLC, 24 patients were treated with PCB on day one of the 21-day cycle and Dulanermin (rhTRAIL) at 4 or 8 mg/kg for five days or 15 or 20 mg/kg for 2 days. There were no dose-limiting toxicities and a maximum-tolerated dose was not reached. The study concluded with an overall response rate of 58%, with one complete response, 13 partial responses and nine patients with a stable disease response. The median progression-free survival was 7.2 months and one year after the trial, 10 out of 24 patients were in long-term follow-up for survival and 11 of 24 patients had died (22). In the end, the clinical trials found that only a small cohort of patients actually responded to rhTRAIL-based therapies. Combination studies showed that the addition of rhTRAIL to established anti-cancer therapeutics did not result in a clinical benefit. As a result, the clinical use of rhTRAIL was terminated. However, clinical trials still continue with monoclonal antibodies against DR4 or DR5 (23).

**1.9 rhTRAIL Resistance.**

Although rhTRAIL shows the ability to induce apoptosis in a broad range of human cancer cells, the sensitivity to rhTRAIL is heterogeneous. Some cell lines display resistance to rhTRAIL-induced apoptosis while others can acquire resistance after repeated exsposure. For example, a study of eight human melanoma cell lines treated with

increasing concentrations of rhTRAIL found that five of the lines (WM 9, WM 35, WM 98-1, WM 793 and WM 1205 Ln) were sensitive to the rhTRAIL, while three lines (WM 164, WM 1791-C and WM 3211) and normal human melanocytes were resistant (24). Originally the varying expression of pro-apoptotic rhTRAIL receptors versus decoy receptors was thought to be the cause for the difference in sensitivity to rhTRAIL-induced apoptosis. However, studies have shown that receptor distribution between pro- and anti-apoptotic receptors does not correlate with sensitivity (15). Current literature describes a myriad of ways in which sensitivity to rhTRAIL may be controlled, which is often cell-type dependent. One mechanism of rhTRAIL-resistance is the increased expression of IAPs such as FLIP, XIAP, cIAP and survivin. FLIP inhibits the activation caspase 8, stopping the extrinsic pathway at the most apical point. Evidence shows at FLIP levels are the highest in rhTRAIL-resistant cells while being low or undetectable in rhTRAIL-sensitive cell lines. Actinomycin D-mediated inhibition of FLIP resulted in the sensitization of rhTRAIL-resistant cells (24). Additionally, the synergistic effects of 5'FU with rhTRAIL in killing cancer cells was attributed to the downregulation of FLIP (16) . In a study using siRNA to downregulate specific IAPs, it was found that inhibition of XIAP and survivin were the most effective in sensitizing cells to rhTRAIL-induced apoptosis compared to other IAPs (25). Moreover, the equilibrium between pro- and anti-apoptotic members of the Bcl-2 family plays an important role in rhTRAIL-sensitivity. Overexpression of anti-apoptotic proteins, Bcl-2 and Bcl-$x_L$, correlates highly with rhTRAIL-resistance. Transfection with vectors for increased Bcl-2 and Bcl-$x_L$ expression resulted in rhTRAIL-resistance in rhTRAIL-sensitive cell lines (26,27). Furthermore, inactivation of the pro-apoptotic Bcl-2 proteins, Bax and Bak, along with the Bcl-$x_L$-

mediated sequestering of tBID, renders cells resistant to rhTRAIL (27,28). Finally, rhTRAIL-resistance may occur at the receptor level such as lack of pro-apoptotic receptor expression or mutations of the functional death-receptors. Low levels of DR4 and DR5 on the cancer cell membrane are highly associated with rhTRAIL-resistance (29). In one case, high levels of death-receptors on the cell surface was correlated with a lack of rhTRAIL-sensitivity. This insensitivity was later explained by the presence of a polymorphism in the DD of DR4, leading to an inhibition of signaling (16). Methods to overcome rhTRAIL-resistance is thus essential for the successful clinical application of rhTRAIL.

**1.10 rhTRAIL Synergism.**

Studies suggest that an increased potency against cancer cells is achieved when rhTRAIL is administered as a combination therapy with preexisting anti-cancer drugs. An increase in rhTRAIL-induced apoptosis was seen in co-treatments with chemotherapy, radiotherapy and even with nontraditional therapies such as proteasome inhibitors, histone deacetylase inhibitors and tyrosine kinase inhibitors *in vitro* and *in vivo*. The enhancement of rhTRAIL through co-treatments was seen in human cancers such as hepatocellular, colon, NSCLC, prostate, pancreatic, breast, sarcoma and B-cell NHL. The augmentation of rhTRAIL was most attributed to an increase in DR5 levels, which can be upregulated in response to DNA damage (13,16). Of special interest is the use of compounds derived from "Mother Nature" as co-treatments for rhTRAIL. For centuries, natural products have been used to treat various diseases due to their efficacy, safety and inexpensiveness, compared to traditional chemotherapeutic agents that are expensive and associated with severe negative side effects as a result of their non-specificity. Natural compounds are

pertinent sources for rhTRAIL co-treatments due to their potent anti-cancer activity. Firstly, a number of natural compounds are known to upregulate pro-apoptotic rhTRAIL-binding receptors DR4 and DR5. Additionally, these compounds are able to downregulate cell survival proteins and pathways. As single agents, natural compounds have the ability to activate cancer cell apoptosis and potentiate rhTRAIL-induced apoptosis. Lastly, the use of natural compounds as co-treatments for rhTRAIL would be successful due to their multi-targeted anti-cancer mechanisms. When mono-targeted therapies are used, they are less effective and resistance can occur. However, multi-targeted compounds are substantially more effective and less likely to cause resistance (30,31). One natural compound of significance is the application of quercetin as a co-treatment for rhTRAIL-resistant malignant melanomas.

**1.11 Quercetin.**

Quercetin is a polyphenol classified as a flavonol based on the presence of an oxygen-containing ring between the two benzene rings (Fig. 1.4). Quercetin is found in wide variety of plant-derived sources ranging from onions and apples to red wine (Fig. 1.5). Dietary quercetin is ingested in the form of glycosides which are metabolized by intestinal microflora to form free quercetin which is absorbed through the gastrointestinal tract (32). Numerous *in vitro* studies have shown the potent effects of quercetin on a wide range of cancer types ranging from breast, lung and melanoma to leukemia and lymphomas. The anti-cancer mechanisms of quercetin are multi-targeted and include inhibition of proliferation signal transduction pathways, reduction of inflammatory metabolites, interaction with type II estrogen binding sites, inhibition of glycolysis, cell

cycle arrest, upregulation of tumor suppressor genes, inhibition of heat shock proteins and induction of apoptosis (33). Here we are interested in the ability of quercetin to sensitize rhTRAIL-resistant cancer cells to undergo apoptosis. The cellular targets quercetin interacts with make it an excellent co-treatment candidate. Firstly, quercetin has the ability to upregulate rhTRAIL-binding receptors, DR4 and DR5 on the cancer cell membrane. One study with colon cancer cells shows that quercetin can upregulate both DR4 and DR5 on the cancer cell membrane (34). However other studies in prostate, lung, liver and ovarian cancers show that quercetin only upregulates DR5 through increased transcription. Additionally, these studies find that quercetin can directly active transcription factors CHOP and Sp1, promoting the transcription of the DR5 gene (35–38). Moreover, several studies have demonstrated that quercetin is able to downregulate a number of

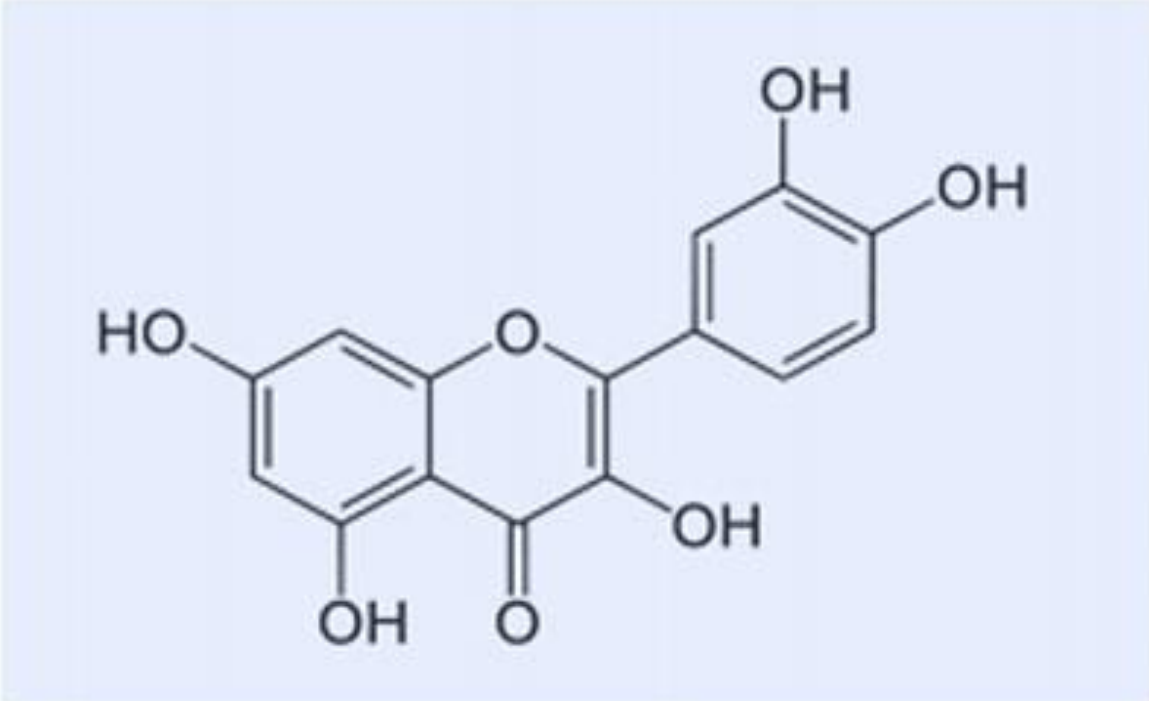


**Figure 1.4: Structure of Quercetin.** (Miles *et al*. 2014)

| Ingredient | Quercetin (mg/100 g) |
|---|---|
| Red wine | 0.84 |
| Canned or jar cherries | 3.20 |
| Blueberries | 3.31 |
| Raw celery | 3.50 |
| Canned and pureed tomatoes | 4.12 |
| Apples with skin | 4.42 |
| Raw chives | 4.77 |
| Raw yellow onions | 13.27 |
| Raw cranberries | 14.02 |
| Raw green chili peppers | 16.80 |
| Cooked yellow onions | 19.36 |
| Unsweetened cocoa | 20.13 |
| Raw fennel | 48.80 |
| Raw yellow chili peppers | 50.63 |
| Dill | 55.15 |
| Canned capers | 180.77 |

[a] Data from the USDA Database for the Flavonoid Content of Selected Foods, Release 3.1 (December 2013)[1].

**Figure 1.5: Content of Quercetin in Selected Foods.** (Miles *et al*. 2014)

anti-apoptotic proteins. Firstly, quercetin has been shown to promote the proteasome-mediated degradation of the caspase 8 inhibitor, FLIP (38,39). Quercetin can also downregulate the anti-apoptotic protein survivin mediated by Akt dephosphorylation (37,40). Overall, quercetin shows significant potential to act as a sensitizer of rhTRAIL-resistant malignant melanomas.

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# CHAPTER II

# RECOMBINANT HUMAN TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND SELECTIVELY INDUCES APOPTOSIS IN MALIGNANT MELANOMA

## 2.1 Abstract.

Skin cancer is among the most commonly-diagnosed cancers with malignant melanoma being associated with the highest rate of metastasis and death. In its early stage, melanoma is easily cured, but the prognosis associated with metastatic malignant melanoma remains very poor and is one of the most treatment-refractory malignancies. This work was undertaken to assess the effectiveness and safety of recombinant human Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhTRAIL) as a potential therapeutic for malignant melanoma. rhTRAIL is the optimized version of the naturally-occurring death-ligand TRAIL. TRAIL shows cancer cell specificity through its innate ability to induce apoptosis in a broad range of transformed human histologies while

showing no toxicity toward normal healthy cells. Utilizing malignant melanoma A375 cells and normal human melanocytes, the efficacy and safety of rhTRAIL was determined *in vitro* and *in vivo* through nude mice A375 xenografts. rhTRAIL induced significant levels of apoptosis in malignant melanoma cells *in vitro* and at the same time did not induce apoptosis in non-transformed melanocytes. rhTRAIL showed remarkable *in vivo* potency and was able to inhibit the growth of established melanoma tumors while showing no toxicity towards the mice model. These data suggest that rhTRAIL is a valid candidate for the treatment of malignant melanoma, displaying significant anti-tumor activity with sustainably less negative side effects than traditional therapies.

## 2.2 Introduction.

Malignant melanoma is the most deadly form of skin cancer with increasing incidence and mortality worldwide. In its early stage, melanoma has an excellent prognosis, but advanced metastatic melanoma correlates with therapeutic resistance and low survival rates (1,2). Extensive research has resulted in the generation of targeted treatments and immunotherapies for malignant melanoma. However, their utilization is linked with numerous negative side effects, slow effectiveness and only transient effects due to acquired resistance. A standard first-line therapy for malignant melanoma has not yet been established and remains one of the most treatment-refractory malignancies (3,4). New therapeutic strategies are needed to improve the treatment outcome and survival of malignant melanoma patients.

Several members of the Tumor Necrosis Factor (TNF) family, including FasL, TNF-α and TNF-Related Apoptosis-Inducing Ligand (TRAIL), robustly induce apoptosis in transformed cancer cells (5). However, the therapeutic potential of FasL and TNF-α is hindered by their toxicity upon systemic administration. In contrast, TRAIL selectively induces apoptosis in cancer cells. Even at supraphysiological concentrations, TRAIL shows minimal toxicity to healthy non-transformed cells (6,7). TRAIL acts as a pro-apoptotic ligand through its interactions with extracellular death receptors (DR) DR4 and DR5 (8). Binding of TRAIL to DR4 and/or DR5 initiates the extrinsic pathway of apoptosis characterized by the cleavage of procaspase 8 to caspase 8 followed by the activation of downstream executioner caspases 3, 6 and 7. TRAIL can also indirectly activate the intrinsic apoptotic pathway through the caspase 8-mediated cleavage of Bid to truncated Bid (tBid). tBid then stimulates the mitochondrial release of cytochrome c resulting in activation of caspase 9 followed caspases 3, 6 and 7 and the hallmark events of apoptosis (9,10).

In the present study we test a recombinant version of TRAIL, recombinant human TRAIL (rhTRAIL). An optimized form of the apoptosis-inducing portion of the protein, rhTRAIL consists of the extracellular C-domain amino acids 114-281 and lacks any exogenous sequence tags. Clinical trials performed with rhTRAIL demonstrate the safety and tolerability of rhTRAIL. Adverse effects included fatigue, nausea and vomiting, but overall, the effects were mild and well tolerated. The half-life of rhTRAIL was 0.56-1.02 hours with no drug accumulation, antibody formation or hepatotoxicity after receiving multiple doses. The anti-tumorigenic effects of rhTRAIL as a single agent were limited

when given to patients with advanced cancer. Few patients showed partial tumor regression while most patients experienced disease progression (11,12). However, when rhTRAIL was given in combination with other anti-cancer therapies the majority of patients responded with complete tumor regression (13,14).

Herein we assessed the potential of rhTRAIL for the treatment of malignant melanoma. With no standard protocol for the treatment of malignant melanoma, rhTRAIL is an excellent candidate due to its selective toxicity towards cancerous cells only. Thus treatment with rhTRAIL will result in maximum anti-tumor effects with decreased negative impact on patients. We aimed to determine the effectiveness of rhTRAIL to act as a cancer cell-specific pro-apoptotic molecule *in vitro* utilizing malignant melanoma A375 cells and human melanocytes. A375 xenografts were employed to test the *in vivo* efficacy of rhTRAIL to inhibit the growth of established tumors while showing no toxicity towards the mice model. Overall, these data show the potential of rhTRAIL as a viable candidate for the treatment of malignant melanoma.

**2.3 Methods.**

**A. Drugs and Chemicals.** Recombinant human Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhTRAIL) was produced according to well defined and previously detailed protocols (40,41,42). Briefly, rhTRAIL was produced in E.Coli using an optimized cDNA for the particular strain used. Following induction for 22h, the cell paste was harvested and rhTRAIL was purified stepwise by FPLC

using previously described methods. rhTRAIL was purified to homogeneity and analyzed at each step by SDS-PAGE following staining with Coomassie. The final product was found to be > 99% pure as demonstrated by HPLC and mass spectrometry.

**B. Cell Culture.** Human adult primary epidermal melanocytes (ATCC PCS-200-013) were maintained in Dermal Cell Basal Medium (ATCC PCS-200-030) supplemented with Adult Melanocyte Growth kit (ATCC PCS-200-042) and 1% Antibiotic/Antimycotic Solution (10,000 IU/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin). A375 cells were maintained in DMEM, supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic/Antimycotic Solution. Cells were incubated in a 90% humidified atmosphere with 5% $CO_2$ at $37^0$C.

**C. Apoptosis Assay.** Treated cells were trypsinized, harvested, washed twice with cold PBS and resuspended in 100 μl of Annexin-V binding buffer at a concentration of $1x10^3$ cells/μl. According to manufacturer's protocol, cells were incubated with 5 μl of FITC-Annexin-V and Propidium Iodide (PI) for 15 minutes at room temperature in the dark (FITC-Annexin-V Kit Apoptosis Detection Kit I, BD Pharminogen). Stained cells were analyzed on BDFACS Canto II using Diva software. Single color controls (Annexin-V or PI only) were used to set up compensation and quadrants for FACS.

**D. Western Blot Analysis.** Total cell lysates were prepared using RIPA lysis buffer (Sigma) containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris, pH 8.0 plus a 1x cocktail of protease inhibitors (Protease Inhibitor Cocktail Set I, Calbiochem).

Cells were lysed for 30 minutes at 4°C followed by centrifugation for 10 minutes at 10,000 rpm at 4°C. Protein concentrations were determined using BCA protein assay (Pierce). A 35 μg protein aliquot was mixed with 4x Laemmli's SDS sample buffer (0.02% Bromophenol Blue (BPB), 8% Beta-mercaptoethanol (BME), 8% SDS, 40% glycerol and 250 mM Tris-HCl, pH 6.8). Cell lysates were heated for five minutes at 100°C, resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk or 5% BSA for ≥1 hour and incubated with primary antibodies for PARP, caspase 8, caspase 3, Bid, caspase 9, caspase 6, caspase 7 (Cell Signaling). After incubation, the membrane was incubated with secondary anti-rabbit or mouse horseradish peroxidase (HRP)-conjugated antibodies (Biorad). Proteins were visualized through development by enhanced chemiluminescence (ECL 2 Western Blotting Substrate, Pierce) and exposure on X-ray film. The blots were reprobed for β-actin to confirm equal protein loading.

**E. Cytochrome c Release.** Cells were resuspended in permeabilization buffer (400 μg/mL digitonin, 75 mM KCl, 1mM $NaH_2PO_4$, 8 mM $Na_2HPO_4$ and 250 mM sucrose) plus protease inhibitors. Samples were incubated for 10 minutes at 4°C, centrifuged at 16,000 g for five minutes at 4°C and the supernatants were kept as the cytosolic fraction. Samples were quantified as described above and 60 μg was resolved on a 15% gel. As described above, the gel was transferred to PVDF, blocked and incubated with anti-cytochrome c (Cell Signaling) and visualized by chemiluminescence.

**F. Death Receptor Membrane Expression**. Treated cells were collected with enzyme-free PBS-based cell dissociation buffer (Gibco Life Technologies) and stained with mouse anti-human DR4 and DR5 conjugated to phycoerythrin (PE) (eBioscience). Briefly, $0.25x10^6$ cells were incubated in 100 µl of staining buffer (2% FBS, 0.02% sodium azide in PBS) and 5 µl anti-DR4 or anti-DR5 for one hour on ice in the dark. As a negative control, cells were stained with mouse IgG1κ isotype antibody under the same conditions. After incubation, cells were washed twice with staining buffer and resuspended in 500 µl of staining buffer and analyzed on BD FACS Canto II using FACS Diva software.

**G. Xenografts**. Animal experiments were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic Foundation. Female athymic nu/nu mice (Taconic Farms, Inc.) supplement with 100 mg/l $ZnCl_2$ water were inoculated with $1.6x10^6$ A375 cells subcutaneously in both flanks. Tumor volume was monitored until tumors reached approximately 200 $mm^3$. Tumor dimensions were measured using calipers and tumor volume was calculated using the formula for a prolate spheroid ($V=4/3\pi a2b$, where a=minor radius and b=major radius of the tumor) three times a week. Upon formation of established tumors, mice were randomized into groups (n=8). rhTRAIL-treated mice received 32 mg/kg for five days on a 21-day cycle and vehicle-treated controls received 250 µL PBS on the same cycle intraperitoneally (ip). The physical status of the mice, weight and activity, were visually monitored and treatments continued until the mice reached their end point and were sacrificed in accordance with IACUC.

**H. Immunohistochemistry.** Tumors were subjected to TUNEL Staining (ApopTag Plus Florescein *In Situ* Apoptosis Detection Kit, Millipore) according to manufacturer's protocol. Briefly, tumors were fixed in 10% formalin, treated with protein kinase K, washed twice with PBS, incubated in equilibration buffer for 10 minutes and incubated with terminal deoxynucleotidyl transferase for 60 minutes at 37°C. Cells were washed with stop buffer for 10 minutes and then incubated with antidigoxigenin conjugate for 30 minutes at 25°C. After washing in PBS, cells were mounted with DAPI and viewed on a fluorescent microscope.

**I. Xenograft Histology.** Following euthanasia, liver, kidney, spleen, and tumors were harvested and fixed in 10% neutral buffered formalin. Sections of tumors and organs were stained with H&E and viewed microscopically.

**J. Statistical Analysis.** Student t-test was used to determined significance. P values less than 0.05 were deemed significant.

## 2.4 Results.

***rhTRAIL sensitivity in vitro.*** Malignant melanoma A375 cells and adult human melanocytes were tested for their sensitivity to rhTRAIL-induced apoptosis *in vitro*. Cells were treated with increasing concentrations of rhTRAIL ranging from 5 ng/ml to 1 µg/ml for 72 hours in full medium. Levels of rhTRAIL-induced apoptosis were determined by FITC-Annexin-V and PI staining followed by FACS analysis. A375 cells showed a dose-dependent induction of apoptosis in response to rhTRAIL marked by the increasing formation of Annexin-$V^+$ and/or Annexin-$V^+$ and $PI^+$ cells (Fig. 1A&B). rhTRAIL was able to induce apoptosis even at the lowest tested rhTRAIL concentration of 5 ng/ml with

9.7±1.0% apoptotic cells ($P<0.02$), increasing to 44.6±0.5% apoptotic cells ($P<0.02$) in the highest treated group of 1 µg/ml. In contrast, human melanocytes were completely insensitive to rhTRAIL-induced apoptosis (Fig. 1A&C). Treatment with rhTRAIL, even at the highest tested treatment concentration of 1 µg/ml of rhTRAIL, did not result in the formation of apoptotic cells as indicated by the lack of Annexin-$V^+$ and/or Annexin-$V^+$ and $PI^+$ cells as compared to the control ($P>0.05$).

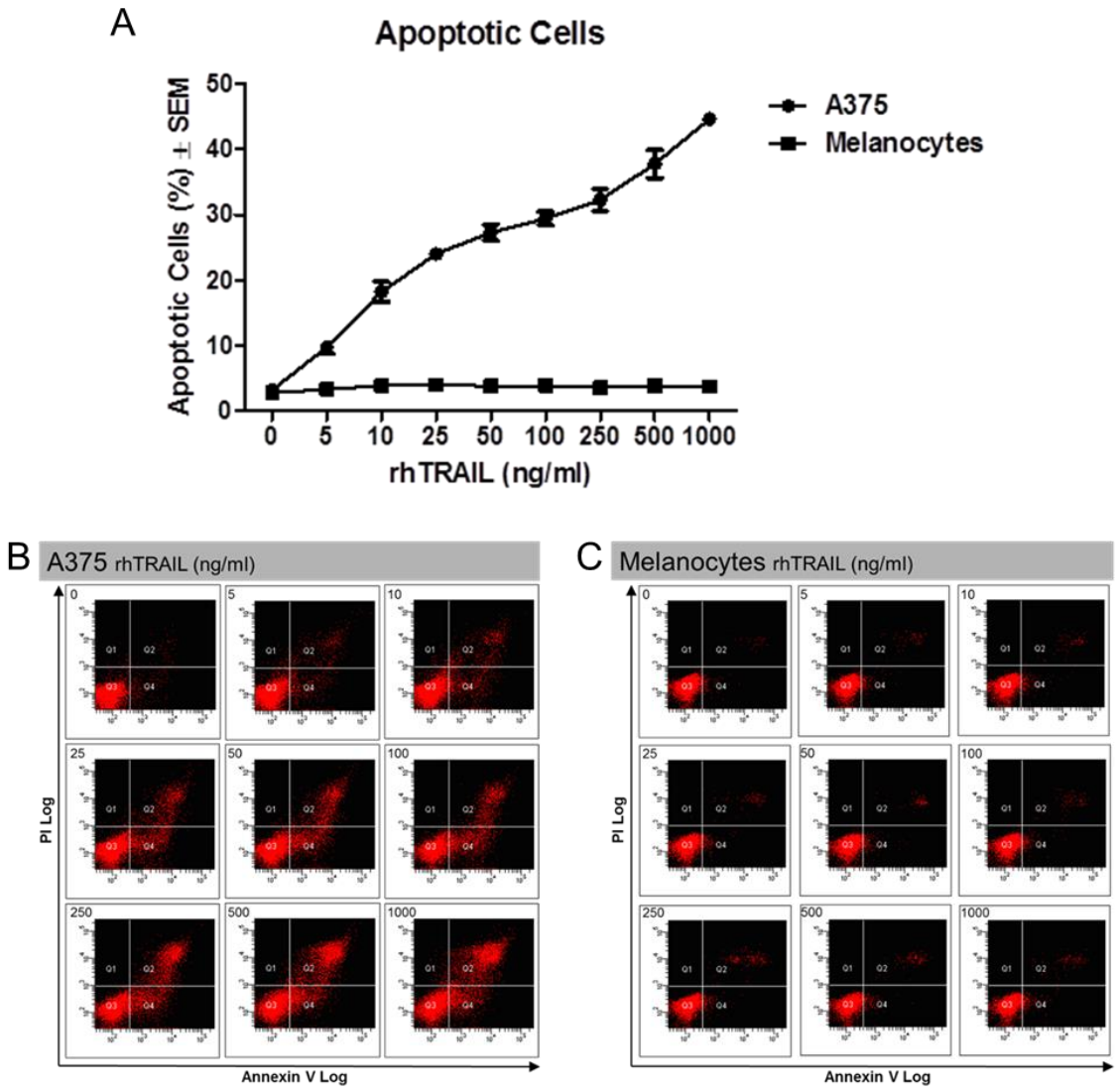


**Figure 2.1. rhTRAIL sensitivity *in vitro*.** Sensitivity of A375 and melanocytes to rhTRAIL-induced apoptosis. A) Average of three independent assays ± SEM. B) Representative histogram of A375. C) Representative histogram of melanocytes. Lower left quadrant: Viable cells (Annexin$^-$/PI$^-$), Lower right quadrant: Early apoptotic cells (Annexin$^+$/PI$^-$), Upper right quadrant: Late apoptotic cells (Annexin$^+$/PI$^+$).

***Mechanism of rhTRAIL-initiated apoptosis.*** To further examine the pathway of apoptosis induced by rhTRAIL western blotting was employed using antibodies to various components of the apoptosis cascade. As described above A375 cells and human melanocytes were treated with increasing concentrations of rhTRAIL for 72 hours, collected and whole cell lysates were revolved by SDS-PAGE and subjected to western blotting. rhTRAIL significantly induced apoptosis in A375 noted by the fragmentation of the DNA repair enzyme Poly-(ADP) Ribose Polymerase (PARP), starting at 10 ng/ml rhTRAIL (Fig. 2A). Treatment with rhTRAIL was able to effectively initiate the extrinsic pathway of apoptosis in A375 as indicated by the cleavage of procaspase 8 to caspase 8. Additionally, rhTRAIL was able to indirectly initiate the intrinsic (mitochondrial) pathway of apoptosis through the caspase 8-mediated cleavage of Bid, as demonstrated by the release of cytochrome c from the mitochondria into the cytosol and the activation of caspase 9. Lastly, executioner caspase 3 was activated, whereas, other executioner caspases 6 and 7 were not activated through treatment with rhTRAIL. Adult human melanocytes were completely resistant to rhTRAIL-induced apoptosis and did not show PARP cleavage or activation of any other proteins in the apoptotic pathways (Fig. 2B).

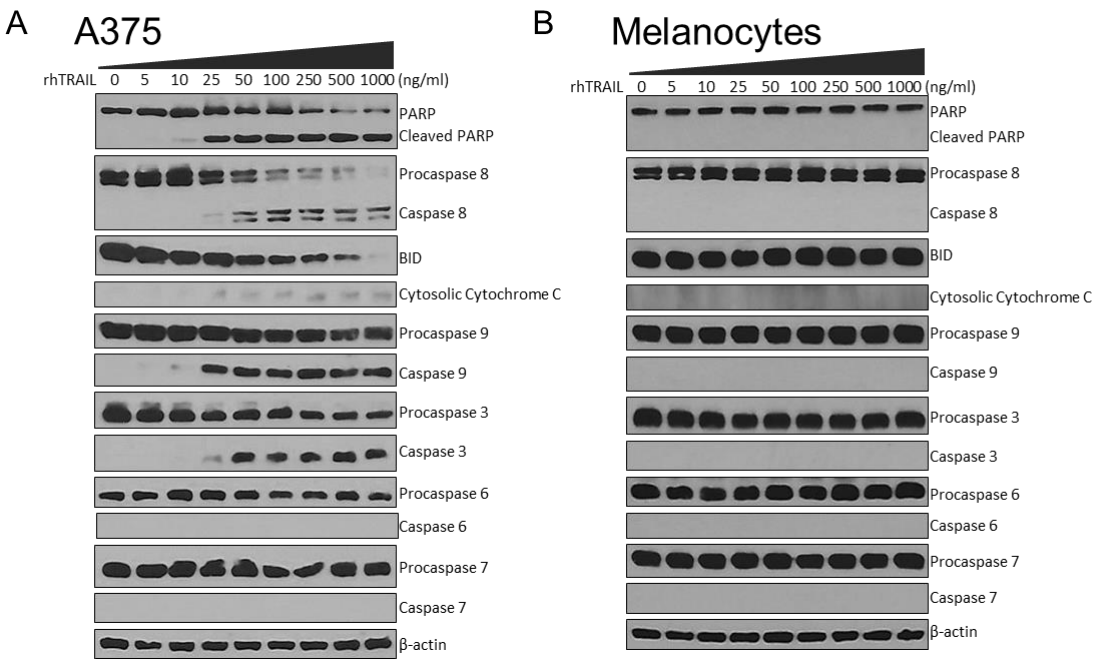


**Figure 2.2. Western blot analysis of apoptosis-related proteins.** A) A375 and B) Melanocytes ± rhTRAIL subjected to western blot analysis and probed with anti-PARP, caspase 8, Bid, cytochrome c, caspase 9, caspase 3, caspase 6 and caspase 7. β-actin was used as a loading control for each membrane. Representative β-actin is depicted. Western blots were done in duplicates.

***Death Receptor Expression.*** To explain the mechanism of rhTRAIL-selectivity, the membrane expression of DR4 and DR5 were measured on A375 cells and melanocytes through staining with fluorescent antibodies to DR4 and DR5 followed by FACS analysis. Overall, malignant A375 cells had significantly higher expression of both DR4 and DR5 (Fig. 3). Comparing the membrane expression of DR4, A375 cells had significantly higher levels with a mean fluorescence intensity (MFI) of 70.3.8±7.3 compared to melanocytes with a MFI of 33.3±1.5 ($P<0.01$) (Fig. 3A). For DR5, A375 cells had a higher level of membrane expression with a MFI of 1591.3±66.9 compared with melanocytes with a MFI of 758.0±7.5 ($P<0.01$) (Fig. 3B).

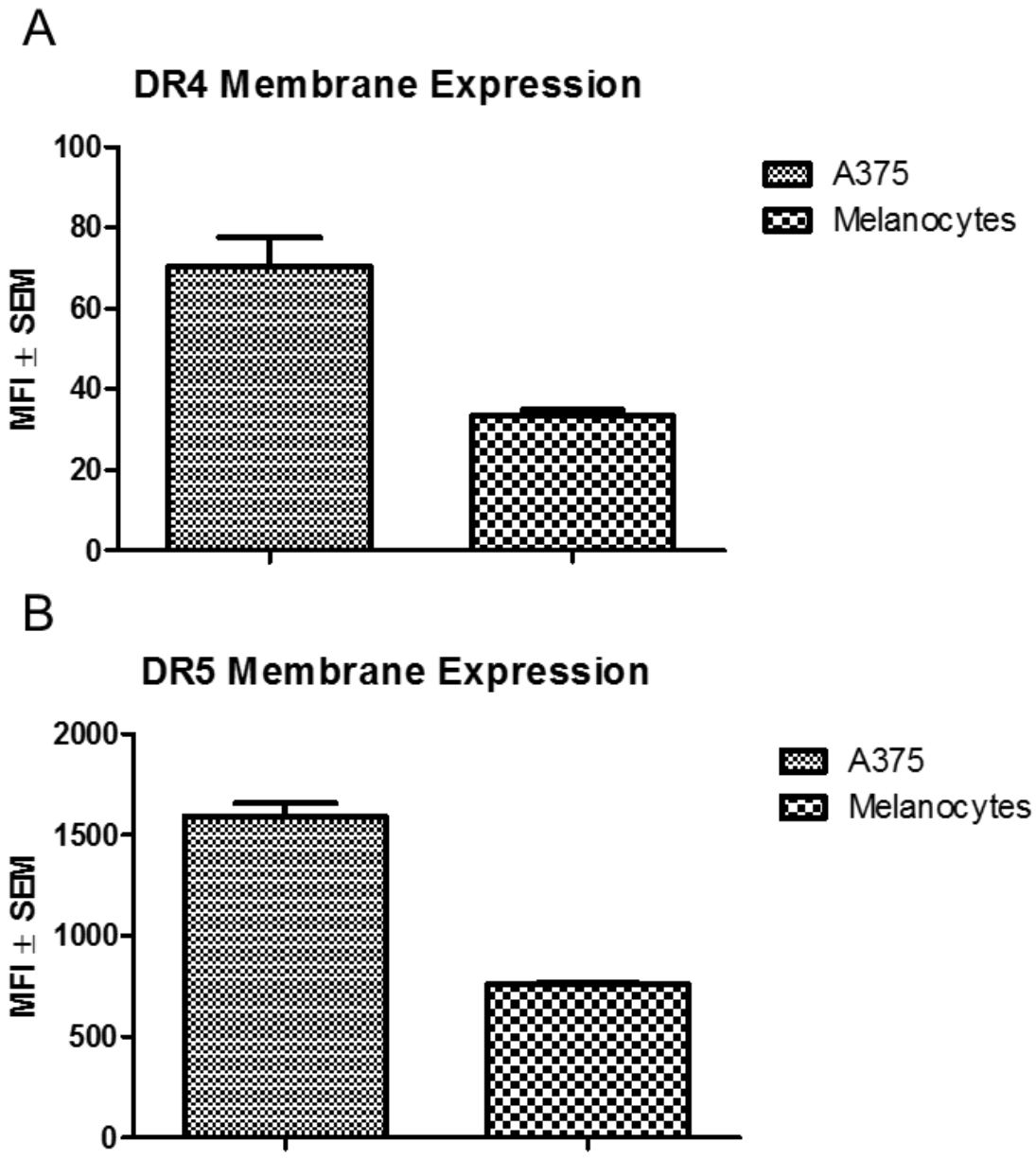


**Figure 2.3. Death Receptor Expression.** Membrane expression of rhTRAIL-binding receptors DR4 and DR5. Mean Fluorescent Intensity (MFI) ± SEM. Average of three independent assays. A) A375 B) Melanocytes.

***Anti-tumorigenic effects of rhTRAIL in vivo.*** To test the *in vivo* efficacy of rhTRAIL we further employed nude mice xenografts. Female athymic nu/nu mice were inoculated with $1.6x10^6$ A375 cells subcutaneously in both flanks and allowed to grow established tumors (~200 $mm^3$). Treatment began on day 18 with the rhTRAIL-treated group (n=8) receiving 32 mg/kg rhTRAIL for five days on a 21-day cycle. Control mice (n=8) received the vehicle of PBS on the same schedule. All mice were supplemented with 100 mg/l $ZnCl_2$ in the water daily and tumor volume was measured three times a week. At the death of the first mouse in the control group at day 29, mice treated with rhTRAIL had an average tumor size of 585.9±232.6 $mm^3$ while the control mice had an average tumor size of 2055.3±476.8 $mm^3$ (Fig. 4A). rhTRAIL-treated mice had 71.5% less tumor growth than the vehicle-treated controls ($P<0.02$) (Fig. 4B). Additionally, the survival rate of the mice was significantly enhanced following treatment with rhTRAIL compared to the control (Fig. 4C). For the control group, all mice had to be sacrificed by day 35 due to extremely large tumor size; however, in the rhTRAIL group 100% of the mice survived to day 43, 75% to day 57 and one mouse lived until day 178. As compared to the control, rhTRAIL-treated mice maintained their weight, activity and exhibited no adverse side effects as determined by visual inspection.

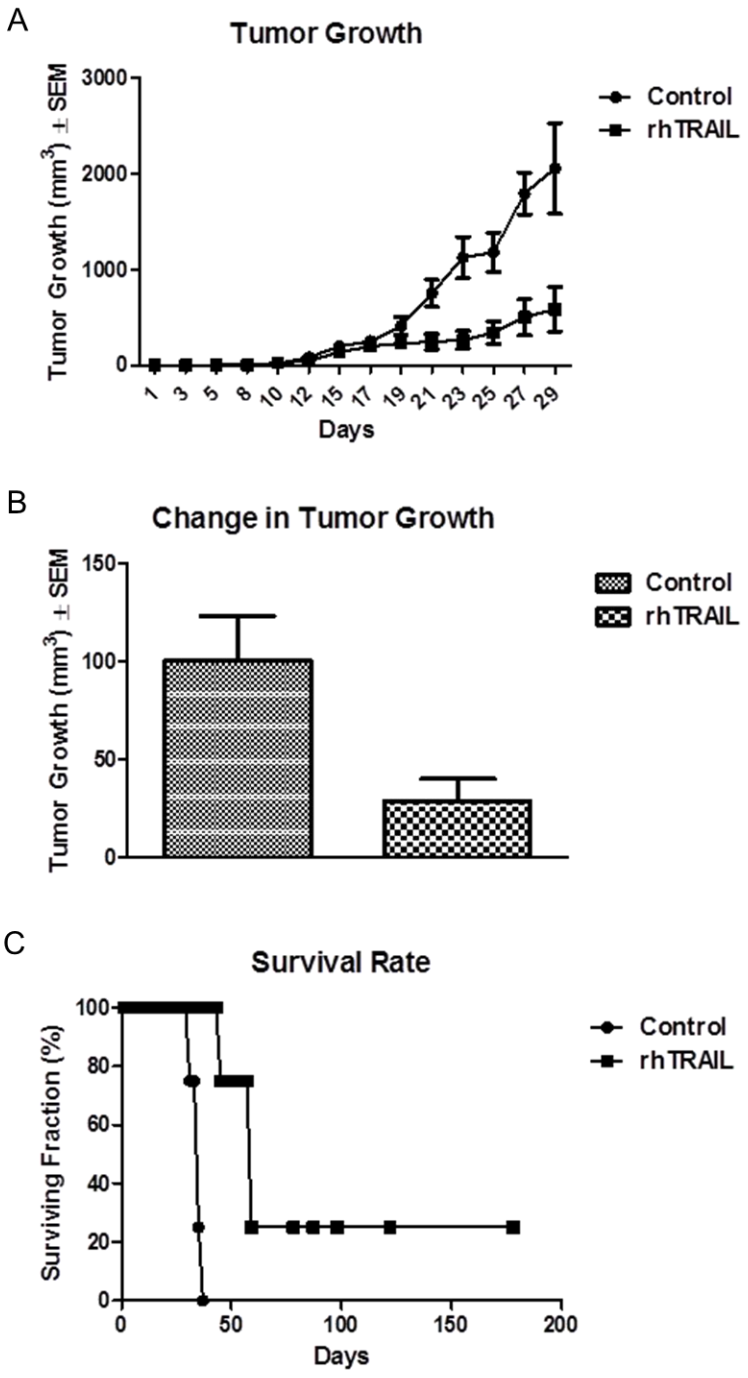


**Figure 2.4. Anti-tumor activity of rhTRAIL.** Sensitivity of A375 to rhTRAIL *in vivo*. Treatment began day 18. rhTRAIL-mice received 32 mg/kg and control mice received PBS ip for five days on a 21-day cycle. A) Tumor growth curve. B) End point tumor volume. C) Survival rate.

***Xenograft histology.*** Tumors and major organs of sacrificed mice were collected and analyzed. TUNEL staining of the tumors revealed significant induction of apoptosis in the rhTRAIL-treated tumors compared to the control mice (Fig. 5A&B). Control tumors had 0.3±0.1% TUNEL positive cells relative to DAPI stained cells compared to the rhTRAIL-treated tumors which had 52.3±5.7% TUNEL positive cells relative to DAPI stained cells ($P<0.00001$). H&E staining was also performed on the harvested tumors (Fig. 5C). rhTRAIL-treated tumors showed signs of apoptosis evidenced by the presence of irregularly shaped and condensed nuclei compared to the control tumors possessing nicely defined nuclei. Finally, major organs, liver, kidney and spleen, were harvested and analyzed by H&E staining (Fig. 5C). Compared to the control, the rhTRAIL-treated organs were healthy and showed no signs of any rhTRAIL-induced toxicity.

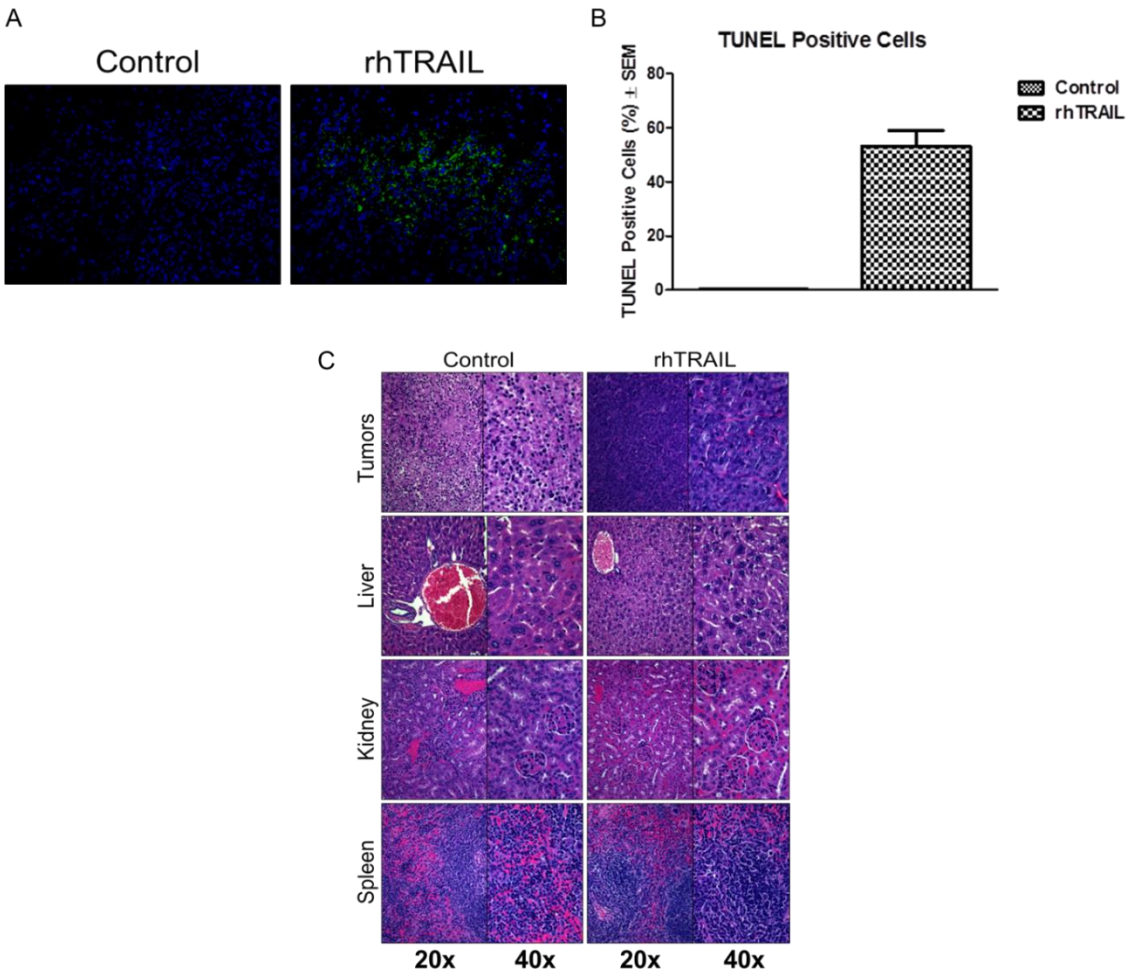


**Figure 2.5. Xenograft tumor and organ analysis.** A) TUNEL staining of harvested tumors. Representative picture, 20x. B) Percent TUNEL positive cells relative to DAPI positive cells ± SEM. Values represent the average of three tumors. C) H&E staining of tumors and major organs. Representative pictures, 20x and 40x

## 2.5 Discussion.

rhTRAIL shows great promise as a pro-apoptotic anti-cancer therapeutic, possessing the ability to selectively target and induce apoptosis in a wide variety of human cancers ranging from solid tumors such as colon, lung and breast to hematological cancers such as leukemia and lymphoma. At the same time, rhTRAIL does not activate apoptosis in normal cells including endothelial, astrocytes and hematopoietic stem cells (15,16). Currently, advanced cases of malignant melanoma are one of the leading causes of death worldwide with no effective treatment option. Here we analyze rhTRAIL as a potential anti-melanoma therapeutic. These data shows that rhTRAIL is highly efficient in selectively killing malignant melanoma cells while demonstrating no toxicity towards non-transformed melanocytes. The hallmark events of apoptosis, generation of apoptotic cells (Annexin-$V^+$) (17) and the fragmentation of the DNA repair enzyme PARP (18), occurred in A375 cells but not in normal melanocytes. rhTRAIL initiates the extrinsic pathway of apoptosis through the binding of DR4 and DR5 (19). We show that rhTRAIL is extremely effective in directly activating the extrinsic pathway of apoptosis apparent by the robust activation of caspase 8 in melanoma cells but not in normal melanocytes. At the highest dose of rhTRAIL nearly all procaspase 8 is converted to caspase 8, revealing the high affinity of rhTRAIL for DR4 and DR5 in melanoma cells. The difference in rhTRAIL-sensitivity between cancer cells and normal cells may lie in their membrane expression of DRs (20,21). Examination of the membrane expression of DR4 and DR5 showed that melanocytes have 2-fold less membrane expression of both DR4 and DR5 as compared to malignant melanoma A375 cells. The decreased expression of DRs may explain why

rhTRAIL does not harm normal cells and is well suited and safe for systemic administration as an anti-cancer therapeutic with low side effects for patients.

There is crosstalk between the extrinsic and intrinsic pathways of apoptosis mediated by the cleavage of Bid to tBid. tBid translocates to the mitochondria and initiates the intrinsic pathway by promoting the release of cytochrome c into the cytosol. This mitochondrial amplification loop intensifies the pro-apoptotic signal initiated by the rhTRAIL-activated extrinsic pathway, resulting in enhanced apoptosis compared to either pathway alone (22). Initiation of the intrinsic pathway typically occurs through activation of p53 in response to cellular stresses such as DNA damage. This is the mechanism by which many chemotherapeutic agents and ionizing radiation kill malignant cells. However, in over 50% of all cancers, p53 is mutated and resistance to standard p53-dependent cancer therapies often occurs (23). As a result of defective p53 in cancerous cells, traditional therapies often result in severe systemic toxicity by destroying normal cells possessing the functional form of p53, causing numerous side effects that negatively influence the patient's quality of life and prevent optimal drug-dosing (24). However, we show that rhTRAIL can indirectly activate the intrinsic pathway of apoptosis and bypass the need for p53. Therefore, the application of rhTRAIL for the treatment of cancer can be used for any tumor type regardless of its p53 status. Additionally, the anti-tumorigenic effects of rhTRAIL are superior over traditional therapies due to the potential activation of both the extrinsic and intrinsic pathways of apoptosis with significantly less negative impact on the patient.

In clinical trials, patients with advanced cancers were treated with rhTRAIL alone or in combination with other therapies such as Rituximab in non-Hodgkin's lymphoma or in combination with Paclitaxel, Carboplatin and Bevacizumab (PCB) in advanced non-squamous non-small-cell lung cancer (NSCLC). rhTRAIL was given at doses ranging from 0.5-35.0 mg/kg I.V. for five days on a 21-day cycle (11,12,13,14). To test the *in vivo* efficacy of rhTRAIL, nude mice A375 xenografts modeled after clinical trials were employed and mice were treated with 32 mg/kg rhTRAIL ip for five days on a 21-day cycle. Crystallographic studies show that TRAIL has a homotrimeric structure, consisting of three TRAIL monomers coordinated by an internal zinc atom. The zinc atom is buried at the center of the trimer, coordinated by a single cysteine residue on each monomer and is required for maintaining the structure, stability and the overall biological activity of TRAIL. It has been repetitively demonstrated that zinc is necessary and required for the optimal physiological function of TRAIL. Reports indicate that preparations of rhTRAIL lacking adequate zinc concentrations are associated with decreased apoptotic activity (25,26). To enhance the bioactivity of rhTRAIL *in vivo*, $ZnCl_2$ was continuously provided in the drinking water of the mice. Presented here is a novel methodology of adding of $ZnCl_2$ to the drinking water of mice in a pre-clinical *in vivo* rhTRAIL experiment. Under these conditions, rhTRAIL showed great *in vivo* efficacy and was able to inhibit the growth of established tumors while being safe and nontoxic to the mice model. As compared to the controls, rhTRAIL-treated mice had 71.5% less tumor growth. Upon starting rhTRAIL treatment, tumor growth leveled off; whereas, tumors of control mice grew exponentially over time. The *in vivo* anti-tumor effects of rhTRAIL where confirmed in a second A375 xenograft experiment in which the drinking water of the mice was also supplemented with

$ZnCl_2$. At the death of the first control mouse, rhTRAIL-treated mice had 68.0% less tumor growth than the control mice ($P<0.01$) (data not shown). Additionally, rhTRAIL was able to significantly prolong the survival of the cancer-bearing mice. Specifically, by day 35 all the control mice had to be sacrificed due to extremely large tumor volume. In contrast, 100% of the mice in the rhTRAIL group survived to day 43 and one mouse lived until day 178. The fact that a mouse lived until day 178 without any further treatment is quite significant because it had large tumors that were eliminated during rhTRAIL-treatment and did not regrow after the treatment had ended.

The mechanism of rhTRAIL-mediated tumor growth inhibition was established in the mice. Intra-tumor apoptosis was confirmed through TUNEL staining which identifies apoptotic cells (27). TUNEL staining revealed significantly high levels of apoptotic cells ($TUNEL^+$) in the rhTRAIL-treated tumors compared to the controls. Additionally, H&E staining of the tumors showed irregularly shaped and condensed nuclei in the rhTRAIL-treated tumors, indicative of induction of apoptosis (28). Together, the H&E and TUNEL staining show that inhibition of tumor growth within the rhTRAIL-treated A375 xenografts was due to rhTRAIL-induced apoptosis. Lastly, upon sacrifice of the mice, major organs, liver, kidney and spleen, were harvested and analyzed by H&E staining. Compared to the control, rhTRAIL-treated mice showed no signs of systemic toxicity to any of the major organs analyzed. In addition, rhTRAIL-treated mice were active, alert and they maintained their weight as compared to the control. The histological analysis together with the physical appearance of the rhTRAIL-treated mice confirm that although rhTRAIL is

extremely effective at inhibiting tumor growth it is still safe and well tolerated upon systemic administration.

The only previously reported rhTRAIL-treated A375 xenograft shows that when treating mice at 50 mg/kg every other day tumor growth was not inhibited as compared the control (29). However, we show that rhTRAIL is able to significantly suppress tumor growth demonstrating of the superiority of our optimized rhTRAIL molecule. The previous xenograft experiment began their treatment two days after the A375 cells were inoculated and before the establishment of a substantial tumor. Whereas in our experiment, rhTRAIL-treatment began on day 18 when established tumors, approximately 200 $mm^3$ in size, were formed. rhTRAIL was given to mice with established tumors to best model clinical settings and to see if rhTRAIL can eliminate established tumors. In the previous experiment, even at much higher concentrations of rhTRAIL, tumor growth was not impeded and was unable to stop the formation of A375 xenograft tumors. With our rhTRAIL, we were able to stop the growth of already growing tumors and significantly eliminate the tumor load on the cancer-bearing mice. Additionally, extra zinc was not provided in the drinking water of the mice in the previous experiment. The addition of $ZnCl_2$ may account for the success of the presented A375 xenograft compared to the previous study, since saturating amounts of zinc are needed for the optimal activity of rhTRAIL (25,26). This may reveal the need to supplement patients receiving rhTRAIL with extra zinc to increase the *in vivo* efficiency of rhTRAIL. Lastly, in the previous experiment, rhTRAIL was given every other day and caused no impact on tumor growth. However, we administered rhTRAIL every day for five days on a 21-day cycle and tumor

growth was successfully hindered. Although rhTRAIL only has a half-life of about an hour, it is still able to substantially inhibit tumor growth in rhTRAIL-sensitive cancer cells. The success of our experiment shows the importance of treating everyday with rhTRAIL for it to be most effective.

Although rhTRAIL shows the ability to induce apoptosis in a broad range of human cancers, rhTRAIL-sensitivity is heterogeneous. Some cell lines display resistance to rhTRAIL-induced apoptosis while others can acquire resistance after repeated exposure (30). For example, a study of eight human melanoma cell lines treated with increasing concentrations of rhTRAIL found that five of the lines were sensitive to rhTRAIL, while three lines were resistant (31). Current literature describes a myriad of ways in which sensitivity to rhTRAIL may be controlled, which is often cell-dependent. One mechanism of rhTRAIL-resistance is the increased expression of inhibitor of apoptosis proteins (IAPs) such as FLIP, XIAP, cIAP and survivin (32). Moreover, the equilibrium between pro- and anti-apoptotic members of the Bcl-2 family plays an important role in rhTRAIL-sensitivity. Overexpression of anti-apoptotic proteins Bcl-2 and Bcl-xL correlate with rhTRAIL-resistance along with downregulation of pro-apoptotic proteins Bax and Bak (33). Finally, rhTRAIL-resistance may occur because of the lack of an optimum concentration of rhTRAIL-binding receptors. Low expression levels or mutations in the rhTRAIL-binding region of the DRs have been associated with rhTRAIL-resistance (6). Studies suggest that an increased potency against cancer cells is achieved when rhTRAIL is administered as a combination therapy. An increase in rhTRAIL-induced apoptosis was seen in co-treatments with chemotherapy, radiotherapy, irradiation and even with nontraditional

therapies such as proteasome inhibitors, histone deacetylase inhibitors and the tyrosine kinase inhibitor *in vitro* and *in vivo* (16). A successful co-treatment is one in which anti-apoptotic proteins such as FLIP and XIAP are downregulated or DRs can be upregulated on the membrane of the cancer cell (34,35). By combining rhTRAIL with compounds that are able to reverse the cellular mechanisms of rhTRAIL-resistance, cancer cells that were once resistant to rhTRAIL-induced apoptosis can now be sensitized (36).

rhTRAIL remains an attractive candidate as an anti-cancer therapy because it is able to selectively induce apoptosis in cancer cells while not harming normal healthy cells (37). In the present work we show that rhTRAIL induces apoptosis *in vitro* and *in vivo* in malignant melanoma A375 cells. Our data demonstrates that rhTRAIL can induce high levels of apoptosis through direct activation of the extrinsic pathway and the indirect activation of the intrinsic pathway. We have validated the specificity and safety of rhTRAIL towards normal cells. rhTRAIL was able to induce robust levels of apoptosis malignant melanoma cells but not in normal adult human melanocytes. These data demonstrate the specificity of rhTRAIL for transformed cells. The ability of rhTRAIL to exhibit significant anti-tumor activity without harming normal cells contrasts traditional chemotherapies where all cells, normal and cancerous, are targeted for cell death (38,39). Therefore, development of anti-cancer agents such as rhTRAIL that specifically target cancer cells is essential to enhance the treatment and overall quality of life for cancer patients. In conclusion, these data show that rhTRAIL would be an effective treatment for malignant melanoma with little potential for negative side effects. Thus, treatment with rhTRAIL would fulfill the need for a potent and safe treatment option for malignant

melanoma resulting in maximum anti-tumor effects with decreased negative impact on patients' lives.

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# CHAPTER III

# DEATH RECEPTORS AS MARKERS FOR RHTRAIL-SENSITIVITY

## 3.1 Abstract.

Personalized cancer treatments can be applied to the clinical use of recombinant human Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhTRAIL). rhTRAIL holds great promise due to its selectivity for cancer cells. However, rhTRAIL clinical trials were conducted without the screening of patients' tumors for rhTRAIL-binding receptors DR4 and DR5 and the unselected treatment resulted in a lack of clinical benefit. Here we propose an *in vitro* test to analyze tumor cells isolated from patients for the membrane expression of DRs to determine their suitability for rhTRAIL-treatment. Utilizing a panel of advanced malignant melanoma cell lines, the correlation between DR membrane expression and rhTRAIL-sensitivity was evaluated. The membrane expression of DR4 and DR5 was examined through staining with anti-DR4 and DR5 followed by FACS. rhTRAIL-sensitivity was determine through Annexin-V and PI staining and western blotting post rhTRAIL-treatment. Here we show a direct correlation between the membrane expression of DRs and rhTRAIL-sensitivity. rhTRAIL-sensitive melanoma lines on average had nearly four-fold more DR4 and over two-fold more DR5 than

rhTRAIL-resistant lines. For a cancer cell to display rhTRAIL-sensitivity the optimum expression of DRs is essential. To overcome the apoptotic threshold cancer cells must express DRs over two-fold higher than their benign counterpart. These data show the potential of this flow cytometry-based assay for the analysis of isolated tumor cells for DR membrane expression. By first determining a patient's susceptibility to rhTRAIL-based treatments, they can be more appropriately placed in rhTRAIL-clinical trials and improve rhTRAIL as an anti-cancer therapeutic.

### 3.2 Introduction.

The molecular characterization of tumors allows for the application of appropriate anti-cancer treatments tailored to a particular patient. Personalized cancer treatments are critical to customize therapies to individuals resulting in increased effectiveness while minimizing negative side effects (1). Individualized cancer plans can be applied to the clinical utilization of recombinant human Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhTRAIL) as an anti-cancer therapeutic. The development of a test to identify patients that would benefit from rhTRAIL-based treatments is key to the successful clinical application of rhTRAIL.

rhTRAIL is the optimized form of the cytokine TRAIL consisting of only the biologically active C-domain (2). TRAIL is expressed by a number of immune effector cells such as monocytes and natural killer cells and is essential in regulating homeostasis through induction of apoptosis of aberrant cells (3). The application of rhTRAIL as an

anti-cancer therapy shows great promise due to its ability to selectively induce apoptosis in a broad range of tumor types while showing minimal toxicity to normal non-transformed cells. The mechanism of cancer cell specific rhTRAIL-induced apoptosis is through the binding of two pro-apoptotic death receptors (DRs), DR4 and DR5 that are more abundantly expressed on cancer cells compared to normal healthy cells (4–6). Binding of rhTRAIL to DR4 and/or DR5 initiates the extrinsic pathway of apoptosis characterized by an intracellular caspase cascade involving the proteolytic cleavage of pro-enzymes into their activate form. At the end of the cascade, executioner caspases are activated and carry out the hallmark events of apoptosis including DNA fragmentation, cell shrinkage and cytoplasmic budding into apoptotic bodies (7,8).

The application of rhTRAIL as an anti-cancer therapeutic has been a rocky "trail". Pre-clinical studies were successful, showing the potent pro-apoptotic activity of rhTRAIL to a wide variety of human cancers both solid and hematological *in vitro* and *in vivo* (9). The human pharmacokinetics and tolerability of rhTRAIL were evaluated in phase Ia trials and displayed the safety of rhTRAIL with no toxicity to normal healthy cells with a half-life of approximately one hour (10,11). However, when rhTRAIL entered phase Ib/II trials, alone and in combination with established cancer therapeutics, the anti-tumorigenic effects of rhTRAIL were low and only a small cohort of patients responded to rhTRAIL-therapy while the majority of patients were resistant and experienced disease progression. As a result of the low efficacy and short half-life, the clinical use of rhTRAIL has been discontinued (12).

rhTRAIL-resistance can be attributed to multitude of different sources but expression of rhTRAIL-binding receptors DR4 and DR5 is a major regulatory point for rhTRAIL-sensitivity. Located at the most apical part of the rhTRAIL-induced apoptotic pathway, multiple reports claim that rhTRAIL-resistance is associated with the decreased expression of DRs on the cancer cell membrane (13,14). Consequently, the membrane expression of DR4 and DR5 may be potential markers for predicting a patient's sensitivity to rhTRAIL and therefore discriminate which patients would benefit from rhTRAIL-therapy from those who will not. We propose the development of an *in vitro* test to analyze isolated tumor cells for their membrane expression of DR4 and DR5 in order to determine a patient's suitability for rhTRAIL-treatment. Here we test the value of measuring the membrane expression DR4 and DR5 by flow cytometry on a panel of advanced metastatic malignant melanoma cell lines as a prospective marker for rhTRAIL-sensitivity.

**3.3 Methods.**

A. **Drugs and Chemicals.** Recombinant human Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhTRAIL) was produced according to well defined and previously detailed protocols (15–17).

B. **Cell Culture.** Cells were incubated in a 90% humidified atmosphere with 5% CO2 at $37^0$C. Human adult primary epidermal melanocytes (ATCC PCS-200-013) were maintained in Dermal Cell Basal Medium supplemented with an Adult Melanocyte Growth kit (ATCC PCS-200-042) and 1% Antibiotic/Antimycotic Solution. The malignant melanoma cell lines, A375 (ATCC CRL-1619), WM9 and WM164 were

maintained in DMEM and MeWo (ATCC HTB-65) was maintained in RPMI, both supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic/Antimycotic Solution. WM9 and WM164 were a kind gift from Dr. Daniel J. Lindner from the Cleveland Clinic Foundation, Cleveland, OH. Malignant melanoma lines are derived from patient tumors. A375 was derived from an epithelial skin biopsy. MeWo, WM9 and WM164 are metastatic malignant melanomas isolated from the lymph nodes.

C. **Death Receptor Membrane Expression.** Untreated and rhTRAIL-treated cells were collected with enzyme-free phosphate-buffered saline (PBS)-based cell dissociation buffer (Gibco Life Technologies) and stained with mouse anti-human DR4 or DR5 conjugated to phycoerythrin (PE) (eBioscience). Briefly, 0.25x106 cells were incubated in 100 µl of staining buffer (2% FBS and 0.02% sodium azide in PBS) and 5 µl anti-DR4 or anti-DR5 for one hour on ice in the dark. As a negative control to account for non-specific antibody binding, all four melanoma lines were stained with mouse IgG1κ, the same antibody isotype as DR4 and DR5, under the same conditions. After incubation, cells were washed twice with staining buffer and resuspended in 500 µl of staining buffer and analyzed on BD FACS Canto II using Diva software (BD Bioscience, San Jose, CA).

D. **Apoptosis Assay.** Cells were trypsinized, harvested, washed twice with cold PBS and resuspended in 100 µl of Annexin-V binding buffer at a concentration of 1x103 cells/µl. According to manufacturer's protocol, cells were incubated with 5 µl of FITC-Annexin-V and propidium iodide (PI) for 15 minutes at room temperature in the dark (FITC-Annexin-V Kit Apoptosis Detection Kit I, BD Pharminogen).

Stained cells were analyzed on BD FACS Canto II using Diva software. Single color controls (Annexin-V or PI only) were used to set up compensation and quadrants for FACS.

**E. Western Blot Analysis.** Total cell lysates were prepared using RIPA lysis buffer (Sigma) containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris, pH 8.0 plus a 1x cocktail of protease inhibitors (Protease Inhibitor Cocktail Set I, Calbiochem). Cells were lysed for 30 minutes at 4°C followed by centrifugation for 10 minutes at 10,000 rpm at 4°C. Protein concentrations were determined using BCA protein assay (Pierce). A 35 μg protein aliquot was mixed with 4x Laemmli's SDS sample buffer (0.02% Bromophenol Blue (BPB), 8% Beta-mercaptoethanol (BME), 8% SDS, 40% glycerol and 250 mM Tris-HCl, pH 6.8). Cell lysates were heated for five minutes at 100°C, resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk or 5% BSA for ≥1 hour and incubated with primary antibodies for PARP and caspase 8 (Cell Signaling). After incubation, the membrane was incubated with secondary anti-rabbit or mouse horseradish peroxidase (HRP)-conjugated antibodies (Biorad). Proteins were visualized through development by enhanced chemiluminescence (ECL 2 Western Blotting Substrate, Pierce) and exposure on X-ray film. The blots were reprobed for β-actin to confirm equal protein loading.

**F. Statistical Analysis.** Student t-test was used to determined significance. P values less than 0.05 were deemed significant.

### 3.4 Results.

***Melanoma Death Receptor Expression.*** To test rhTRAIL-sensitivity, malignant melanoma lines were treated with and without 100 ng/ml of rhTRAIL for 72 hours. Prior to treatment, control experiments were done to determine the optimal conditions for rhTRAIL-treatment (data not shown). The treatment of 100 ng/ml for 72 hours was selected to best distinguish between rhTRAIL-sensitive and rhTRAIL-resistant melanomas. Post-treatment cells were analyzed for induction of apoptosis through FITC-Annexin-V and PI staining followed by FACS analysis (Fig. 2A&B). Two of the four lines, A375 and WM9, underwent apoptosis in response to rhTRAIL-treatment, whereas, the other two, MeWo and WM164, did not. In response to rhTRAIL-treatment, A375 formed 29.2±2.0% apoptotic cells ($P<0.001$) and WM9 formed 36.9±0.5% apoptotic cells ($P<0.001$), however, MeWo and WM164 did not form significant apoptotic cells as compared to the control ($P>0.05$). To confirm the event of rhTRAIL-induced apoptosis, western blotting was employed using antibodies to various components of the apoptotic cascade. rhTRAIL significantly induced apoptosis in A375 and WM9 noted by the fragmentation of the DNA repair enzyme Poly-(ADP) Ribose Polymerase (PARP) but this did not occur in rhTRAIL-resistant melanomas, MeWo and WM164 (Fig. 2C). Treatment with rhTRAIL was able to effectively initiate the extrinsic pathway of apoptosis in rhTRAIL-sensitive lines, A375 and WM9, as indicated by the cleavage of procaspase 8 to caspase 8 which was not present in rhTRAIL-resistant lines, MeWo and WM164, post-rhTRAIL-treatment (Fig. 2C).

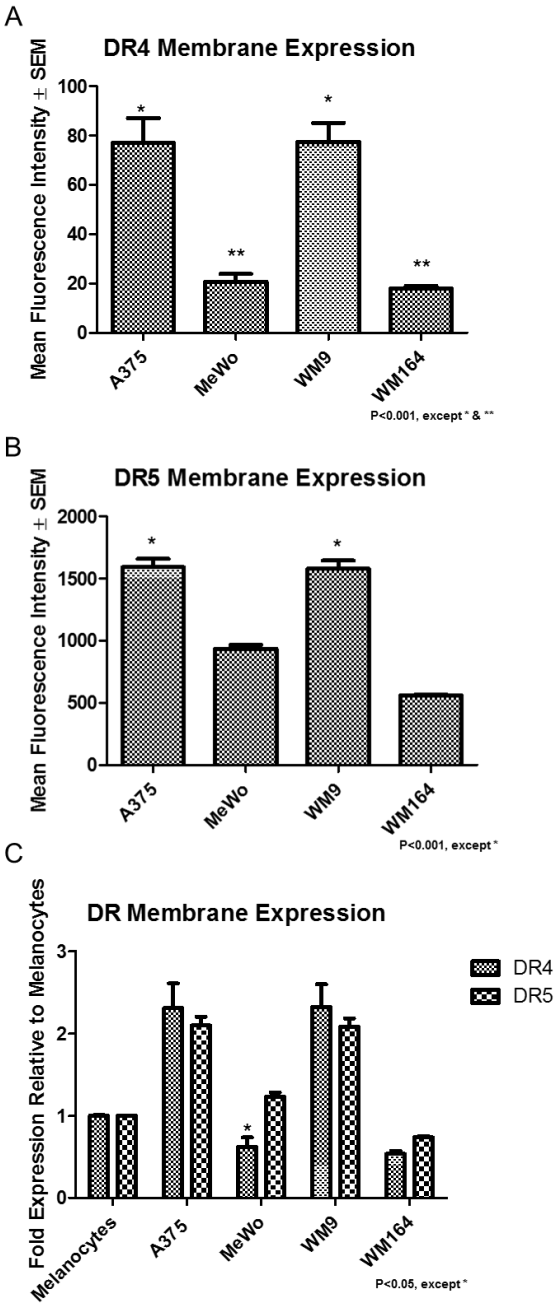


**Figure 3.1. Death Receptor Membrane Expression**. Membrane expression of rhTRAIL-binding receptors DR4 and DR5 on metastatic malignant melanomas. A) Membrane expression of DR4. Mean Fluorescent Intensity (MFI) ± SEM (n=9). B) Membrane expression of DR5. MFI ± SEM (n=9). D) Melanoma DR expression normalized to non-cancerous melanocytes.

***Melanoma rhTRAIL-Sensitivity.*** To test rhTRAIL-sensitivity, metastatic malignant melanoma lines were treated with and without 100 ng/ml of rhTRAIL for 72 hours. Post-treatment cells were analyzed for induction of apoptosis through FITC-Annexin-V and PI staining followed by FACS analysis (Fig. 2A&B). Two of the four lines, A375 and WM9, underwent apoptosis in response to rhTRAIL-treatment, whereas, the other two, MeWo and WM164, did not. In response to rhTRAIL-treatment, A375 formed 29.2±2.0% apoptotic cells ($P<0.001$) and WM9 formed 36.9±0.5% apoptotic cells ($P<0.001$), however, MeWo and WM164 did not form significant apoptotic cells as compared to the control ($P>0.05$). To confirm the event of rhTRAIL-induced apoptosis, western blotting was employed using antibodies to various components of the apoptotic cascade. rhTRAIL significantly induced apoptosis in A375 and WM9 noted by the fragmentation of the DNA repair enzyme Poly-(ADP) Ribose Polymerase (PARP) but did not occur in rhTRAIL-resistant melanomas, MeWo and WM164 (Fig. 2C). Treatment with rhTRAIL was able to effectively initiate the extrinsic pathway of apoptosis in rhTRAIL-sensitive lines, A375 and WM9, as indicated by the cleavage of procaspase 8 to caspase 8 which was not present in rhTRAIL-resistant lines, MeWo and WM164, post-rhTRAIL-treatment (Fig. 2C).

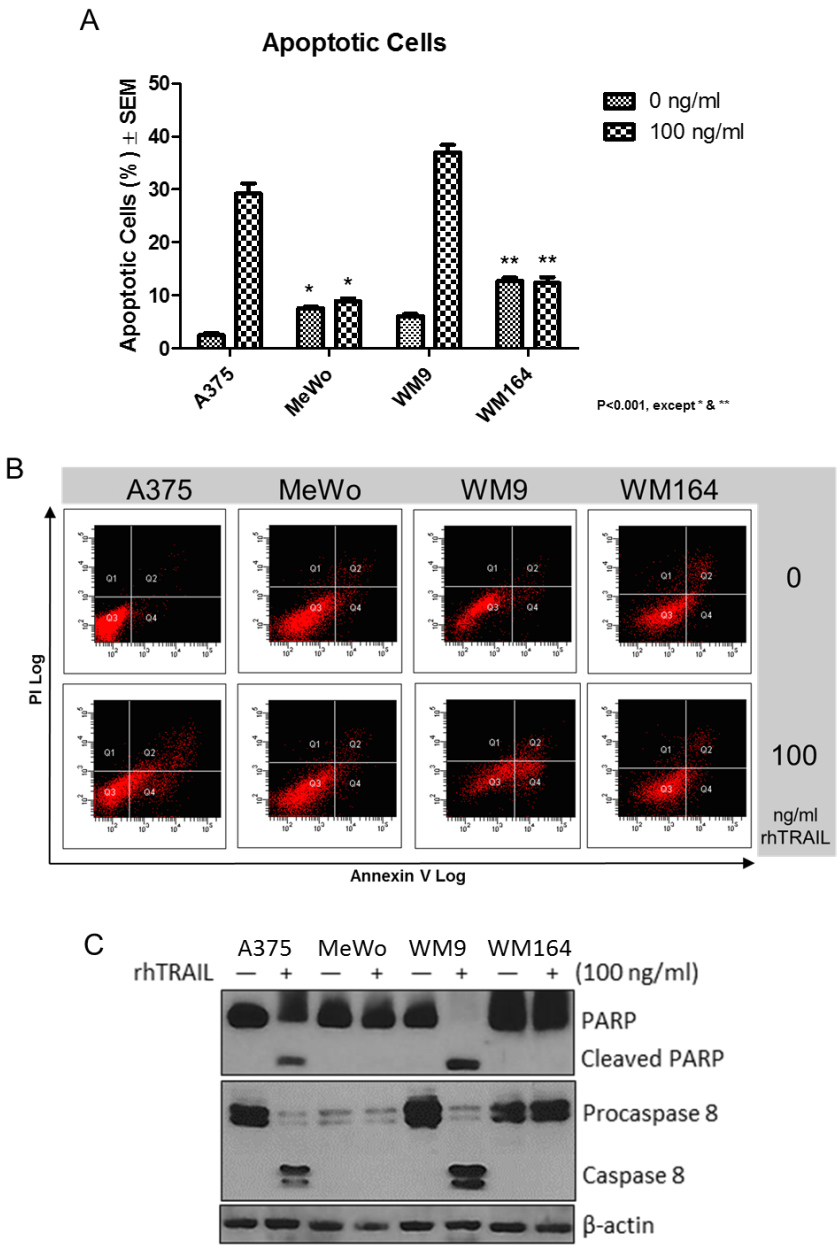


**Figure 3.2. rhTRAIL-sensitivity.** A) Percent apoptotic cells ± SEM. Average of three independent assays (n=9). B) Representative histogram. Lower left quadrant: Viable cells (Annexin$^-$/PI$^-$), Lower right quadrant: Early apoptotic cells (Annexin$^+$/PI$^-$), Upper right quadrant: Late apoptotic cells (Annexin$^+$/PI$^+$). C) Melanoma lines ± rhTRAIL subjected to western blot analysis and probed with anti-PARP or anti-caspase 8. β-actin was used as a loading control for each membrane. Representative β-actin is depicted.

### 3.5 Discussion.

rhTRAIL holds great promise as an anti-cancer therapeutic. However, there are still challenges for selecting patients that will benefit from rhTRAIL-based treatments as the use of rhTRAIL is only advantageous to patients with rhTRAIL-sensitive tumors. It is instrumental to develop a sensitive and rapid test to identify patients who are well-suited for rhTRAIL-based therapy. Here we propose a flow cytometry-based companion diagnostic assay to measure the membrane expression of DRs on tumors cells isolated from cancer patients. By first determining DR membrane expression, rhTRAIL can be more appropriately applied to patients with tumors pre-disposed to the effects of rhTRAIL. rhTRAIL clinical trials held with patients pre-screened for DR expression will allow for the more successful clinical application of rhTRAIL. Using an advanced metastatic malignant melanoma model, the potential utilization of DR4 and DR5 as markers for rhTRAIL-sensitivity was tested.

Heterogeneity in rhTRAIL-sensitivity was seen in the panel of four malignant melanoma lines analyzed. In response to rhTRAIL-treatment, two out of the four lines were sensitive to rhTRAIL-induced apoptosis and two were resistant. In rhTRAIL-sensitive lines, A375 and WM9, the hallmark events of apoptosis, generation of apoptotic cells (Annexin-V+ cells) (18) and the fragmentation of the DNA repair enzyme PARP (19), occurred. However, these events did not transpire in rhTRAIL-resistant lines, MeWo and WM164, in response to rhTRAIL-treatment. The mechanism of rhTRAIL-induced apoptosis is mediated through the activation of the extrinsic pathway of apoptosis. The extrinsic apoptotic pathway is initiated by the binding of rhTRAIL to DR4 and/or DR5

followed by the subsequent activation of initiator caspase, caspase 8, from its pro-form procaspase 8. Caspase 8 can then cleave and activate downstream executioner caspases which ultimately carry out the events of apoptosis (7,20). Here we show that rhTRAIL is able to efficiently activate the extrinsic pathway of apoptosis in rhTRAIL-sensitive melanoma lines but not in rhTRAIL-resistant lines. This is evidenced by the formation of caspase 8 in rhTRAIL-sensitive lines, A375 and WM9, but not in rhTRAIL-resistant lines, MeWo and WM164, in response to rhTRAIL-treatment. The use of metastatic malignant melanomas for the analysis of potential markers for rhTRAIL-based treatments is pertinent due to the varying sensitivity among malignant melanomas to rhTRAIL (21,22). This variability represents the broad range of rhTRAIL-sensitivities seen in the patient population (23). Through tumor profiling, patients can be matched with treatments that are more likely to be effective with fewer side effects. Highlighting the need to develop an *in vitro* clinical test to select patients that will benefit from rhTRAIL-treatment.

Decades of research have uncovered numerous mechanisms cancer cells use to evade rhTRAIL-induced apoptosis. One mechanism of rhTRAIL-resistance is mediated by the upregulation of anti-apoptotic proteins termed inhibitors of apoptosis proteins (IAPs), specifically cFLIP, XIAP, cIAP and survivin, which inhibit caspase activity and prevent cell death (24–26). Likewise, the equilibrium between pro- and anti-apoptotic members of the Bcl-2 family play an important role in regulating rhTRAIL-sensitivity. Overexpression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, correlate highly with rhTRAIL-resistance. While, downregulation of pro-apoptotic Bcl-2 proteins, Bax and Bak, render cells resistant to rhTRAIL (27–29). Lastly, the optimum expression of pro-

apoptotic rhTRAIL-receptors is indispensable in promoting apoptosis. rhTRAIL binds to five receptors: two pro-apoptotic and three anti-apoptotic. Pro-apoptotic receptors, DR4 and DR5, contain cytoplasmic death domains through which rhTRAIL can initiate apoptosis. Anti-apoptotic receptors, decoy receptor 1 (DcR1), decoy receptor 2 (DcR2) and osteoprotegerin (OPG), act as antagonistic receptors. These receptors lack an intracellular death domain and cannot transmit an apoptotic signal upon rhTRAIL-binding (30).

Finding a predictive marker for rhTRAIL-sensitivity is challenging due to the extreme complexity of rhTRAIL-resistance. However, the membrane expression of DRs holds the most promise. Since DRs are at the most apical part of the rhTRAIL-induced apoptotic pathway without adequate levels of membrane-bound DRs there can be no induction of apoptosis regardless of other anti-apoptotic factors. Originally, the differential expression of pro- verses anti-apoptotic receptors was thought to be the cause for the difference in rhTRAIL-sensitivity. However, studies have shown that the distribution between pro- and anti-apoptotic receptors does not correlate with sensitivity (9). Several studies have demonstrated that downregulation of pro-apoptotic receptors DR4 and DR5 on the membrane of cancer cells is instrumental in conveying rhTRAIL-resistance (21,31,32). Data shows that lack of DR membrane expression correlates with rhTRAIL-resistance regardless of alterations in the levels of IAPs or anti-apoptotic Bcl-2 proteins. Additionally, although mRNA for DR4 and DR5 is present in the vast majority of cancer cells, due to post-translational modifications and intracellular trafficking, total receptor expression does not reflect the functional membrane expression of DR4 and DR5 (13,33).

Therefore, the best reflection of rhTRAIL-sensitivity is the amount of DRs on the cancer cell membrane. Additionally, the marker for rhTRAIL-sensitivity needs to be applicable to a rapid and high-throughput clinical test. For measuring DR4 and DR5, there are already sensitive and specific fluorescent antibodies commercially available. Finally, the utilization of a flow cytometry-based technique allows for the rapid interrogation of isolated tumor cells with little sample preparation that can be easily integrated into clinical testing.

Preliminary results show a direct correlation between DR expression and sensitivity to rhTRAIL-induced apoptosis. rhTRAIL-resistant lines, MeWo and WM164, had significantly less DR4 and DR5 expressed on their membrane expression compared to the rhTRAIL-sensitive lines, A375 and WM9. On average the rhTRAIL-sensitive melanoma lines had nearly four-fold more DR4 membrane expression. For DR5, on average the rhTRAIL-sensitive melanoma lines had over two-fold more membrane expression than rhTRAIL-resistant lines.

Here we provide preliminary data for the association between DR membrane expression and rhTRAIL-sensitivity. rhTRAIL-sensitive cancers have significantly higher membrane expression of both DR4 and DR5 than rhTRAIL-resistant cancers. Yet, even resistant cancers still express some levels of DR4 and DR5; although, they do not undergo apoptosis in response to rhTRAIL-treatment. We have shown that the non-transformed counterpart of melanomas, melanocytes, are resistant to rhTRAIL-induced apoptosis even

though they do expressed DR4 and DR5 on their cell membrane. However, normal melanocytes have a much lower membrane expression of both DR4 and DR5 as compared to rhTRAIL-sensitive melanoma cells (6). For a cell to undergo rhTRAIL-induced apoptosis a threshold for apoptosis activation must be surpassed. In order for a cell to activate the process of apoptosis a certain amount of caspase 8 must be activated through the binding of DR4 and/or DR5 by rhTRAIL (34). Conclusively, cancer cells must express an optimum amount of DRs on the cell membrane to overcome the apoptotic threshold and render them sensitive to rhTRAIL. Our data suggests that a two-fold increase in DR expression compared to normal cells is enough to render cancer cells sensitive to rhTRAIL-induced apoptosis. More research is needed to confirm the threshold between DR membrane expression and rhTRAIL-sensitivity. Utilizing both established cancer cell lines and patient samples, a clinical reference range of DR expression must be set to determine a patient's suitability for rhTRAIL-treatment and for the successful execution of this companion diagnostic test.

Utilizing a flow cytometry-based technique, we show the value of measuring DR membrane expression to predict rhTRAIL-sensitivity on established malignant melanoma cell lines. This technique can be easily translated into a clinical test to characterize tumor cells isolated from patients, applicable to both solid and hematological cancers. The use of flow cytometry to analyze solid tumors is relatively novel and includes samples from surgical specimens, fine needle aspirations or frozen or paraffin-embedded tissues. Analysis of solid tissue involves the disaggregation of the sample into a single cell suspension. This can be accomplished either mechanically or with enzymes such as

collengase or a combination of both (35–37). Flow cytometry in the analysis and diagnosis of hematological cancers such as leukemia and lymphoma is well established and is currently clinically utilized (38,39). The application of multiparameter flow cytometry allows for the rapid and sensitive detection of abnormal cells in a tumor sample or whole blood. Abnormal cells are identified by the presence of antigens such as clusters of differentiation (CD) that differs significantly from their normal counterpart (40). Therefore, DR4 and DR5 can be easily added to the panel of markers used to diagnosis these cancers. However, the clinical development of measuring DRs to predict rhTRAIL-sensitivity requires the optimization of blood volume and tissue collected to ensure an adequate number of cells is obtained.

The pre-clinical and early clinical trials of rhTRAIL demonstrated promising results. rhTRAIL acted as a potent pro-apoptotic molecule and could be systemically applied without any major adverse effects. However, once rhTRAIL proceeded further into clinical trials, alone and in combination with traditional therapies, the results were disappointing. Clinical trials were performed without prescreening patients' tumors for DR membrane expression and the unselected treatment of patients with rhTRAIL resulted in a lack of clinical benefit. These data provide the rationale to implement a flow cytometry-based technique to profile tumor cells isolated from patients for DR membrane expression in order to determine their suitability for rhTRAIL-treatment. If patients can be screened for DR expression, they can be more appropriately selected for rhTRAIL clinical trials and give rhTRAIL a fighting chance to act as an effective anti-cancer therapy by giving it to patients that are well-suited for it.

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# CHAPTER IV

# SENSITIZATION OF RHTRAIL-RESISTANT MALIGNANT MELANOMAS BY QUERCETIN

## 4.1 Abstract.

Malignant melanoma is the most commonly-diagnosed skin cancer associated with a high rate of metastasis. Early stage melanoma is easily treated, but metastatic malignant melanoma is one of the most treatment-refractory malignancies with low survival rates. The application of recombinant human Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhTRAIL) for the treatment of metastatic malignant melanoma holds considerable promise due to its selective pro-apoptotic activity towards cancer cells and not non-transformed cells. Unfortunately, the clinical utilization of rhTRAIL has been terminated due to the resistance of many cancer cells to undergo apoptosis in response to rhTRAIL. However, rhTRAIL-resistance can be abrogated through the co-treatment with compounds derived from "Mother Nature" such as quercetin that can modulate cellular components responsible for rhTRAIL-resistance. Here we show that rhTRAIL-resistant malignant melanomas can be sensitized by quercetin mediated by the upregulation of

rhTRAIL-binding receptors DR4 and DR5 and the proteasome-mediated downregulation of the anti-apoptotic protein FLIP.

**4.2 Introduction.**

The frequency of malignant melanoma has been on the rise over the last 30 years. Although it is the least diagnosed of the skin cancers, it is associated with the highest rate of mortality. When the melanoma is localized to the epidermis, the survival rate is 98%. However, once the cancer metastasizes the 5-year survival rate decreases to 17% (1). The decrease in survival rate correlates with the lack of effective treatment options for metastatic malignant melanoma. Current therapies (chemotherapy, radiation therapy, targeted therapy and immunotherapy) are characterized by slow effectiveness and only transient anti-tumor properties due to acquired resistance. In addition, a high degree of negative side effects are associated with these therapies which deeply impacts the patient's quality of life and limits the optimum drug dose. As a result, there is no standard therapeutic regimen for metastatic malignant melanoma (2,3). It is vital to invest in the development of novel therapeutics for metastatic malignant melanoma and increase the survival outcome for these patients.

The application of recombinant human Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhTRAIL) as a treatment for human cancer holds great promise due to its ability to selectively induce apoptosis in cancer cells while not harming normal non-transformed cells (4,5). rhTRAIL, the optimized form of the cytokine TRAIL, consists of

only the biologically active C-domain of endogenous molecule. rhTRAIL selectively induces apoptosis by interacting with membrane-bound extracellular receptors, death receptor (DR) 4 and DR5, which are more abundantly expressed on cancer cells compared to normal cells (6). Upon rhTRAIL-binding, the extrinsic pathway of apoptosis is initiated characterized by the activation of caspase 8 followed by the activation of executioner caspases 3, 6 and 7. Additionally, mediated by the caspase 8 cleavage of BID to tBID, rhTRAIL can indirectly activate the intrinsic pathway of apoptosis independently of p53. Once activated, tBID will translocate to the mitochondria and stimulate the release of cytochrome c into the cytosol and ultimately resulting in the activation of caspase 9. Caspase 9 can then cleave and activate executioner caspases which carry out the hallmark events of apoptosis including DNA fragmentation and cytoplasmic condensation (7).

Although the application of rhTRAIL as an anti-cancer therapeutic holds great promise, the clinical use has been limited due to the heterogeneity seen in rhTRAIL-sensitivity among cancers. This is especially prevalent in cases of advanced metastatic malignant melanoma. Nearly two-thirds of melanoma cells are resistant to rhTRAIL-induced apoptosis, including melanomas directly isolated from cancer patients (8,9). Melanoma rhTRAIL-resistance can be attributed to a number of different causations. Specifically, the decreased membrane expression of pro-apoptotic rhTRAIL-binding receptors DR4 and DR5 (10,11)(site DR paper). The lack of receptor expression may be associated with gene mutations or decreased transcription (12,13). Additionally, failure to transport the receptors to the membrane or constitutive endocytosis can lead to the decreased expression of DRs on cancer cells (14–16). Moreover, the upregulation of

intracellular anti-apoptotic proteins plays an indispensable role in rhTRAIL-resistance. The equilibrium between pro- and anti-apoptotic members of the Bcl-2 family takes part in regulating rhTRAIL-sensitivity. Overexpression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, correlates highly with rhTRAIL-resistance. While, inactivation of the pro-apoptotic Bcl-2 proteins, Bax and Bak, along with the Bcl-xL-mediated sequestering of tBID, renders cells resistant to rhTRAIL (17–19). Another mechanism of rhTRAIL-resistance is mediated through the increased expression of inhibitors of apoptosis proteins (IAPs) such as FLIP, XIAP, cIAP and survivin that inhibit caspase activity (12,20). It is essential to understand the molecular mechanism of rhTRAIL-resistance and develop ways to overcome this resistance through combination therapy.

Epidemiological evidence shows that a plant-based diet is associated with a decrease incidence of various types of cancers. The benefit of which is most accredited to the natural phytochemicals such as flavonoids present. One of the most prevalent flavonoids with potent physiological activity is quercetin. Quercetin is found in a wide variety of sources ranging from onions and apples to red wine (9). As a pleiotropic molecule, quercetin exhibits its anti-cancer effects on a number of different pathways such as cell survival pathways, cell cycle arrest, upregulation of tumor suppressor genes, downregulation of anti-apoptotic proteins and pro-apoptotic pathways (21–26). Most noteworthy is the impact quercetin has on the expression of DR5. Several studies show that quercetin can upregulate DR5 on the membrane of cancer cells through enhanced transcription (27–31). Moreover, it has been demonstrated that quercetin is able to downregulate a number of anti-apoptotic proteins that promote rhTRAIL-resistance,

specifically FLIP, Mcl-1 and survivin (26,32). Overall, this evidence shows the potential of quercetin as a co-treatment for rhTRAIL. The ability of quercetin to sensitize rhTRAIL-resistant malignant melanomas has yet to be evaluated. Here we test the combination rhTRAIL plus quercetin to overcome the intrinsic resistance of metastatic malignant melanomas to rhTRAIL.

**4.3 Methods.**

A. **Drugs and Chemicals.** Recombinant human Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (rhTRAIL) was produced according to well defined and previously detailed protocols (6). Quercetin dihydrate (Calbiochem) was dissolved in Polyethylene Glycol (PEG)-400 (Fisher Scientific). MG132 (Calbiochem) was dissolved in dimethyl sulfoxide (DMSO).

B. **Cell Culture.** WM164 cells were maintained in DMEM and MeWo cells were maintained in RPMI, both supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic/Antimycotic Solution. Cells were incubated in a 90% humidified atmosphere with 5% CO2 at $37^0$C.

C. **Apoptosis Assay.** Cells were trypsinized, harvested, washed twice with cold PBS and resuspended in 100 µl of Annexin-V binding buffer at a concentration of 1x103 cells/µl. According to manufacturer's protocol, cells were incubated with 5 µl of FITC-Annexin-V and Propidium Iodide (PI) for 15 minutes at room temperature in the dark (FITC-Annexin-V Kit Apoptosis Detection Kit I, BD Pharminogen). Stained cells were analyzed on BD FACS Canto II using Diva software. Single

color controls (Annexin-V or PI only) were used to set up compensation and quadrants for FACS.

**D. Western Blot Analysis.** Total cell lysates were prepared using RIPA lysis buffer (Sigma) containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris, pH 8.0 plus a 1x cocktail of protease inhibitors (Protease Inhibitor Cocktail Set I, Calbiochem). Cells were lysed for 30 minutes at 4°C followed by centrifugation for 10 minutes at 10,000 rpm at 4°C. Protein concentrations were determined using BCA protein assay (Pierce). A 35 μg protein aliquot was mixed with 4x Laemmli's SDS sample buffer (0.02% Bromophenol Blue (BPB), 8% Beta-mercaptoethanol (BME), 8% SDS, 40% glycerol and 250 mM Tris-HCl, pH 6.8). Cell lysates were heated for five minutes at 100°C, resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk or 5% BSA for ≥1 hour and incubated with primary antibodies for PARP, caspase 8, caspase 3, Bid, caspase 9, caspase 6, caspase 7, FLIP, DR4 and DR5 (Cell Signaling). After incubation, the membrane was incubated with secondary anti-rabbit or mouse horseradish peroxidase (HRP)-conjugated antibodies (Biorad). Proteins were visualized through development by enhanced chemiluminescence (ECL 2 Western Blotting Substrate, Pierce) and exposure on X-ray film. The blots were reprobed for β-actin to confirm equal protein loading.

**E. Cytochrome c Release.** Cells were resuspended in permeabilization buffer (400 μg/mL digitonin, 75 mM KCl, 1mM NaH2PO4, 8 mM Na2HPO4 and 250 mM

sucrose) plus protease inhibitors. Samples were incubated for 10 minutes at 4°C, centrifuged at 16,000 g for five minutes at 4°C and the supernatants were kept as the cytosolic fraction. Samples were quantified as described above and 60 µg was resolved on a 15% gel. As described above, the gel was transferred to PVDF, blocked and incubated with anti-cytochrome c (Cell Signaling) and visualized by chemiluminescence.

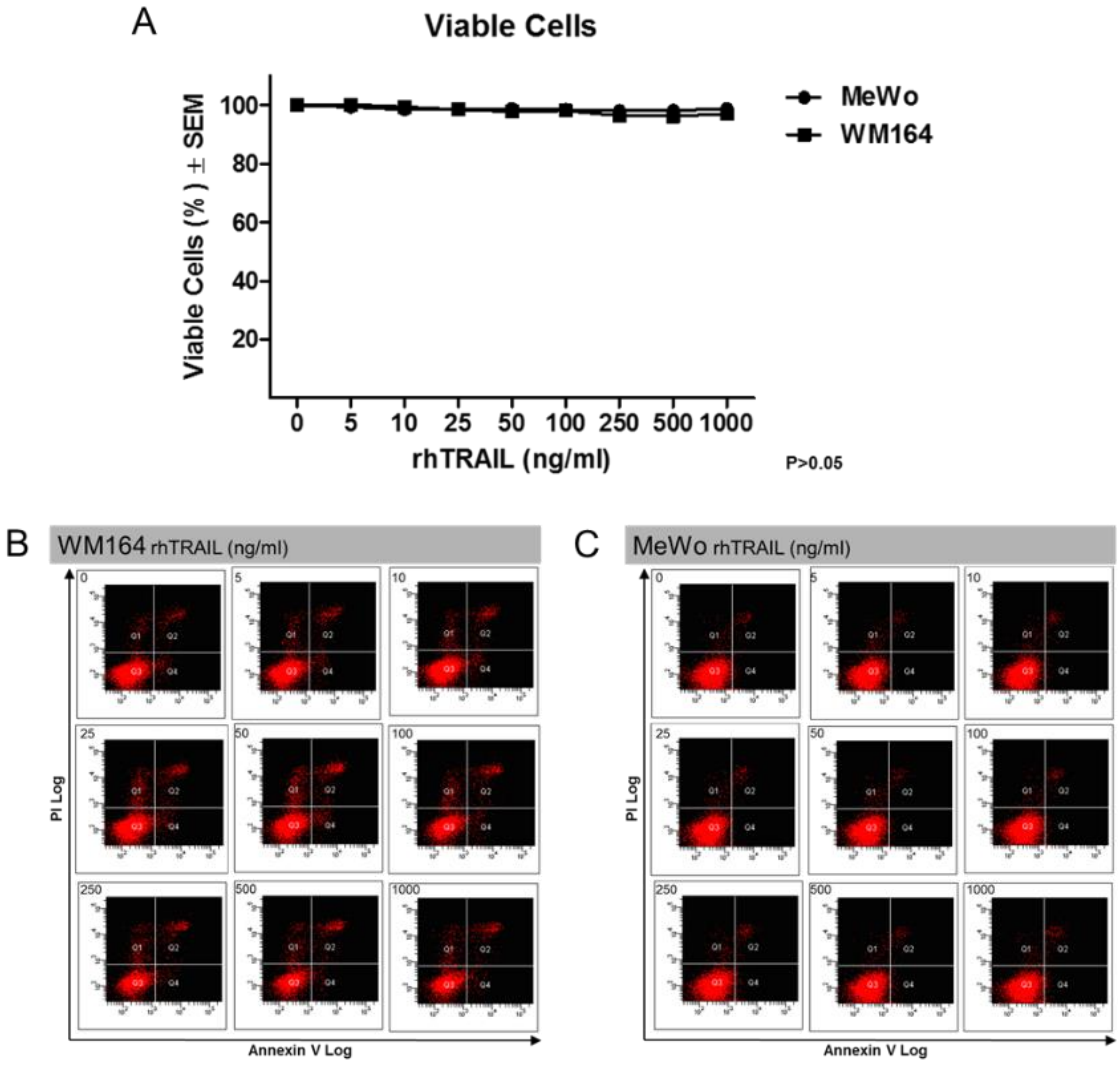
**F. Death Receptor Membrane Expression.** Cells were collected with enzyme-free PBS-based cell dissociation buffer (Gibco Life Technologies) and stained with mouse anti-human DR4 and DR5 conjugated to phycoerythrin (PE) (eBioscience). Briefly, 0.25x106 cells were incubated in 100 µl of staining buffer (2% FBS, 0.02% sodium azide in PBS) and 5 µl anti-DR4 or anti-DR5 for one hour on ice in the dark. As a negative control, cells were stained with mouse IgG1κ isotype antibody under the same conditions. After incubation, cells were washed twice with staining buffer and resuspended in 500 µl of staining buffer and analyzed on BD FACS Canto II using FACS Diva software. For the permeabilization experiments, after the first antibody incubation cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with 0.1% saponin for 5 minutes at room temperature. Cells were then incubated with anti-IgG1κ, DR4 or DR5 for 30 minutes in the saponin buffer and analyzed by FACS. To calculate the cytoplasmic DR expression, the permeablized cells representing the total DR expression was subtracted from the unpermeablized cells representing membrane-bound DRs.

**G. Reverse Transcription-PCR.** Total RNA was extracted using TRIzol reagent (Ambion) and treated with DNase according to manufacturer's protocol (Invitrogen Deoxyribonuclease I, Amplification Grade). RT-PCR was performed following the manufacturer's protocol (Invitrogen SuperScript III One-Step RT-PCR System with Platinum® Taq DNA Polymerase). Human DR5 mRNA was amplified using the forward primer 5'-GGGAGCCGCT-CATGAGGAAGTTGG-3' and the reverse primer 5'-GGCAAGTCTCTCTCCCAGCGTCTC-3'. For DR4, forward primer 5'-GAGCAACGCAGACTCGCT-3'and the reverse primer 5'-TCACTCCAAGGACACGGC-3' were used. For FLIP, forward primer 5'-CTTGGCCAATTTGCCTGTAT-3'and the reverse primer 5'-CCCATGAACATCCTCCTGAT-3' were used. For β-actin, the forward primer 5'-TGACGGGGTCACCCACACTGTGCC-3'and the reverse primer 5'-CTGCATCCTGTCGGCAATGCCAG-3' were used. cDNA synthesis was performed at 60ºC for 30 minutes using the Applied Biosystems GeneAmp PCR System 9700. The PCR cycling conditions (30 cycles) were as follows: denature for 2 minutes at 94ºC, anneal for 30 seconds at 55ºC for FLIP and DR4 and 65ºC for DR5 and β-actin, extend for 1 minute and 30 seconds at 68ºC, and execute a final extension for 10 minutes at 68ºC. Reaction products were analyzed on 1.2% agarose gels. The bands were visualized by ethidium bromide and an UV.

**H. Statistical Analysis.** Student t-test was used to determined significance. P values less than 0.05 were deemed significant.

## 4.4 Results.

***rhTRAIL sensitivity.*** Metastatic malignant melanoma cell lines MeWo and WM164 were tested for their sensitivity to rhTRAIL-induced apoptosis *in vitro*. Cells were treated with increasing concentrations of rhTRAIL ranging from 5 ng/ml to 1 µg/ml for 72 hours. Levels of rhTRAIL-induced apoptosis were determined by FITC-Annexin-V and PI staining followed by FACS analysis (Fig. 1 A-C). Both melanoma lines were completely resistant to rhTRAIL-induced apoptosis. Treatment with rhTRAIL, even at the highest tested treatment concentration of 1 µg/ml, did not result in the formation of apoptotic cells as indicated by the lack of Annexin-V+ and/or Annexin-V+ and PI+ cells as compared to the control ($P>0.05$). The rhTRAIL-resistant status of the melanoma lines were confirmed by western blotting utilizing antibodies to various components of the apoptotic cascade (Fig. 1 D&E). Both lines were completely resistant to rhTRAIL-induced apoptosis and did not show any Poly (ADP-ribose) polymerase (PARP) cleavage or activation of any proteins in the apoptotic pathway.


A
Viable Cells
Viable Cells (% ) ± SEM
100
80
60
40
20
0 5 10 25 50 100 250 500 1000
rhTRAIL (ng/ml)
MeWo
WM164
P>0.05
B
WM164 rhTRAIL (ng/ml)
0
5
10
25
50
100
250
500
1000
PI Log
Annexin V Log
C
MeWo rhTRAIL (ng/ml)
PI Log
Annexin V Log

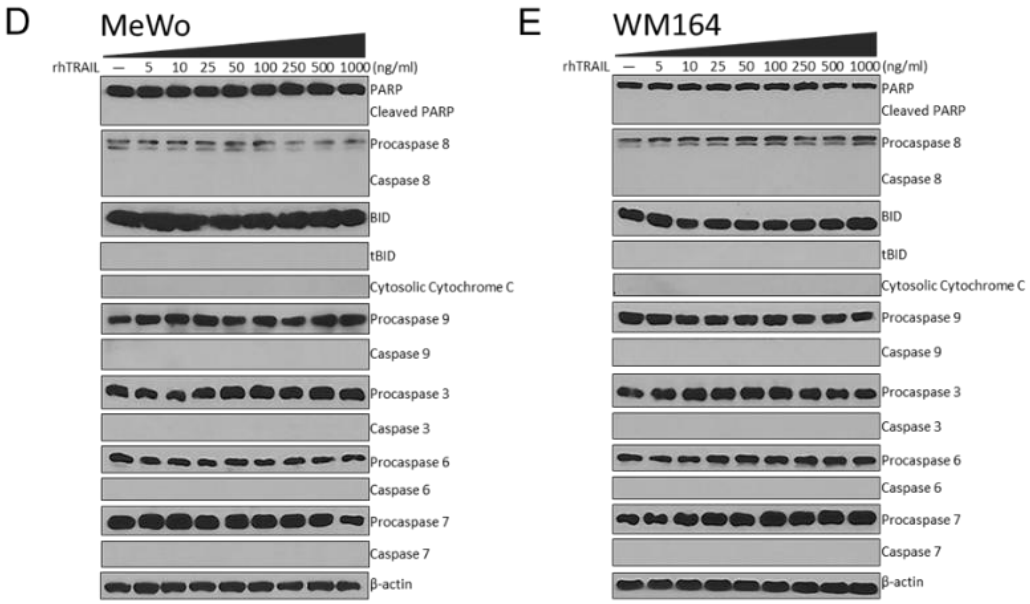
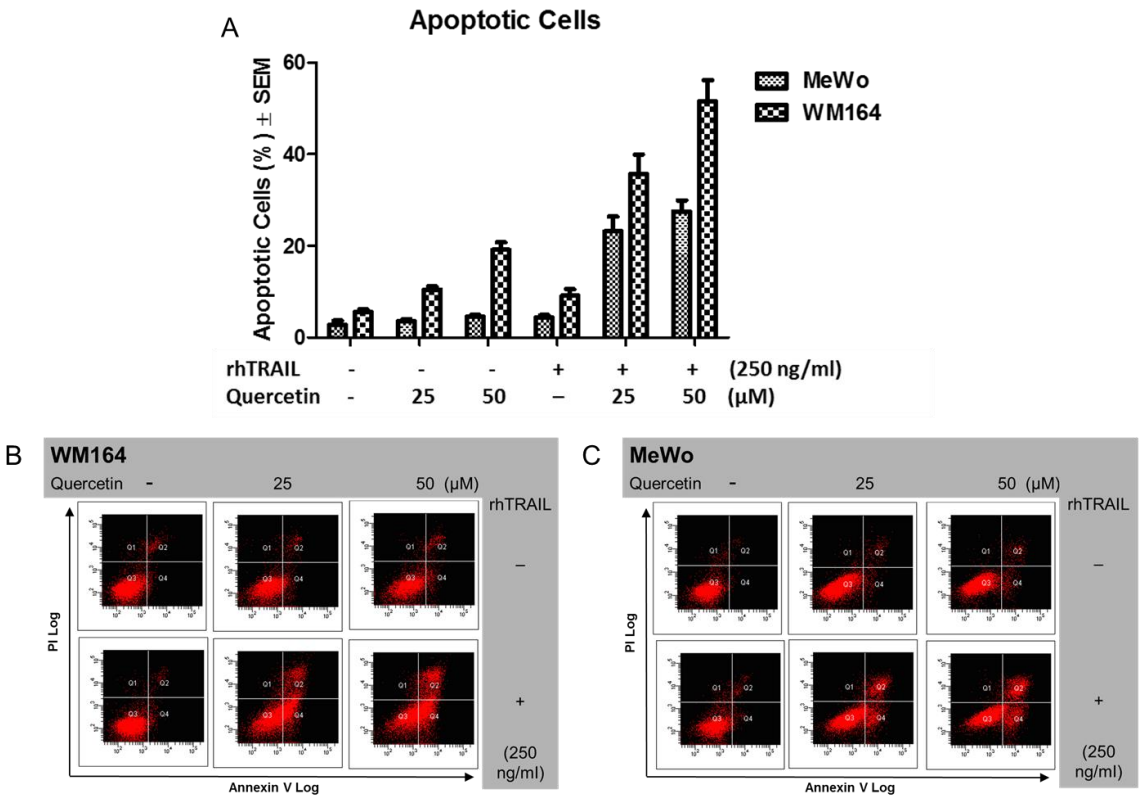



**Figure 4.1. rhTRAIL sensitivity.** Sensitivity of malignant melanomas MeWo and WM164 to rhTRAIL *in vitro*. A) Average of three independent assays ± SEM. B) Representative histogram of MeWo. C) Representative histogram of WM164. Lower left quadrant: Viable cells ($Annexin^-/PI^-$), Lower right quadrant: Early apoptotic cells ($Annexin^+/PI^-$), Upper right quadrant: Late apoptotic cells ($Annexin^+/PI^+$). D) MeWo and B) WM164 ± rhTRAIL subjected to western blot analysis and probed with anti-PARP, caspase 8, Bid, cytochrome c, caspase 9, caspase 3, caspase 6 and caspase 7. β-actin was used as a loading control for each membrane. Representative β-actin is depicted.

***rhTRAIL plus quercetin apoptosis.*** To overcome the intrinsic rhTRAIL-resistance of MeWo and WM164, the combination treatment of rhTRAIL plus quercetin was employed. Melanoma lines were treated with single agent rhTRAIL at 250 ng/ml, quercetin at 25 and 50 μM and with the combination of the single agents. Post treatment cells were collected and analyzed for induction of apoptosis through Annexin-V and PI staining and western blotting probing for key proteins in the apoptotic pathways (Fig. 2). Alone, quercetin induced minimal levels of apoptosis in the rhTRAIL-resistant melanoma cells. For MeWo there was no significant induction of apoptosis when treating with both concentrations of quercetin marked by the lack of Annexin-V+ and/or Annexin-V+ and PI+ cells as compared to the control ($P>0.05$) (Fig. 2 A&B). The lack of quercetin-induced apoptosis was confirmed by western blot where there was no PARP cleavage or activation of any pro-apoptotic proteins (Fig. 2D). However, in WM164, quercetin was able to induce minimal levels of apoptosis with the formation of 10.5±0.7% apoptotic cells at 25 μM and 19.1±1.6% apoptotic cells at 50 μM ($P<0.05$) (Fig. 2 A&C). Probing for apoptotic proteins did not reveal any PARP cleavage or protein activation the in quercetin-treated WM164 cells as the low levels of apoptosis was not sufficient to be detected by western blotting (Fig. 2E). Furthermore, by combining rhTRAIL plus quercetin the minimal levels of apoptosis induced in the single agent treatments were dramatically increased in both melanoma lines. This was evidenced by the significantly higher formation of Annexin-V+ and/or Annexin-V+ and PI+ cells as compared to any single agent treatments ($P<0.05$) (Fig. 2 A-C). The augmentation of apoptosis occurred dose-dependently in respect to quercetin. The ability of quercetin to sensitize rhTRAIL-resistant melanomas to undergo apoptosis was confirmed by western blotting (Fig. 2 D&E). In both melanoma lines, PARP

was cleaved in only the co-treatment group dose-dependently. By adding quercetin to the rhTRAIL-treatment, the once resistant cells were sensitized to activate the rhTRAIL-mediated extrinsic pathway of apoptosis as marked by the cleavage of pro-caspase 8 to caspase 8. Additionally, through the co-treatment the intrinsic pathway of apoptosis was activated in WM164 but not in MeWo as noted by the cleavage of BID to tBID followed by the release of cytochrome c into the cytosol and the activation of caspase 9 from pro-caspase 9. Finally, through the co-treatment executioner caspases were activated. In both melanoma lines caspase 3 was activated through the co-treatment, caspase 6 in only the highest co-treatment in WM164 and caspase 7 in the highest co-treatment in both melanoma lines.


A
Apoptotic Cells
Apoptotic Cells (% ) ± SEM
60
40
20
0
MeWo
WM164
rhTRAIL - - - + + + (250 ng/ml)
Quercetin - 25 50 – 25 50 (µM)
B
WM164
Quercetin - 25 50 (µM)
rhTRAIL
–
+
(250 ng/ml)
PI Log
Annexin V Log
C
MeWo
Quercetin - 25 50 (µM)
rhTRAIL
–
+
(250 ng/ml)
PI Log
Annexin V Log

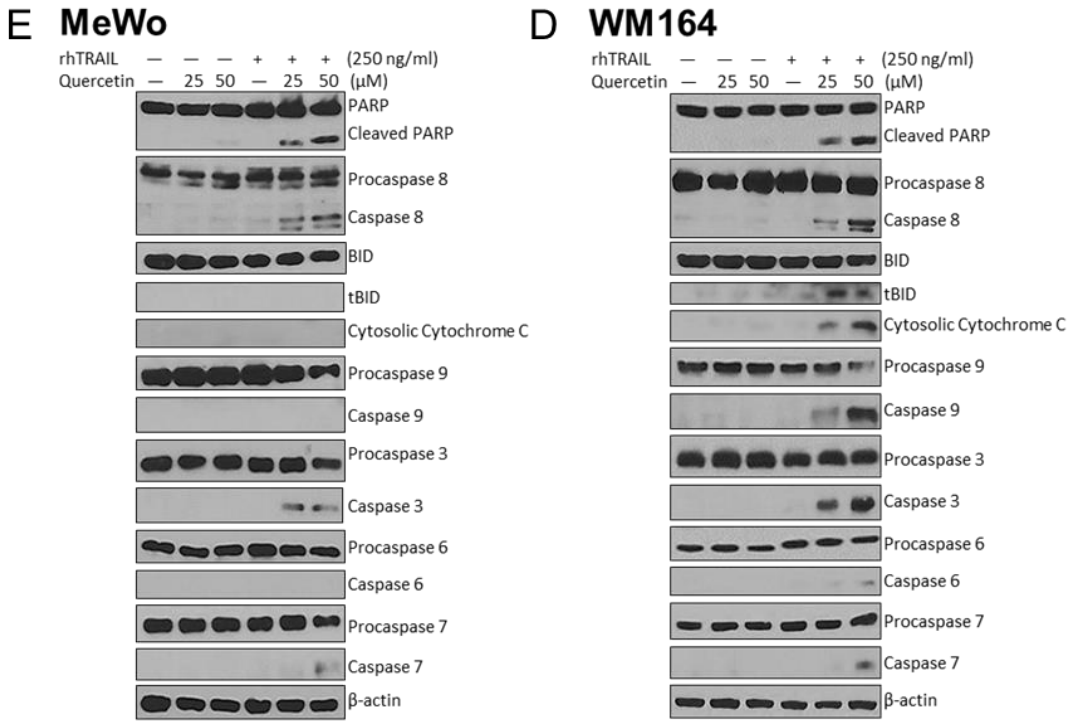



**Figure 4.2. rhTRAIL plus quercetin apoptosis.** Combination treatment-induced apoptosis. A) Average of three independent assays ± SEM. B) Representative histogram of MeWo. C) Representative histogram of WM164. Lower left quadrant: Viable cells ($Annexin^-/PI^-$), Lower right quadrant: Early apoptotic cells ($Annexin^+/PI^-$), Upper right quadrant: Late apoptotic cells ($Annexin^+/PI^+$). D) MeWo and B) WM164 ± rhTRAIL subjected to western blot analysis and probed with anti-PARP, caspase 8, Bid, cytochrome c, caspase 9, caspase 3, caspase 6 and caspase 7. β-actin was used as a loading control for each membrane. Representative β-actin is depicted.

***Quercetin regulation of death receptors.*** The addition of quercetin to rhTRAIL was able to promote the activation of the rhTRAIL-mediated extrinsic apoptotic pathway evidenced by the activation of caspase 8 in only the co-treatment groups. To elucidate the mechanism of quercetin sensitization, the most apical part of the extrinsic apoptotic pathway, expression of rhTRAIL-binding receptors DR4 and DR5 were examined in response to quercetin-treatment (Fig. 3). Quercetin was able to upregulate the membrane expression of DR4 in both melanoma lines dose-dependently (Fig. 3A). In response to quercetin-treatment, MeWo upregulated DR4 on the cell membrane over four-fold at 25 µM and over 11-fold at 50 µM ($P<0.001$). For WM164, DR4 was upregulated nearly five-fold and seven-fold at 25 µM and 50 µM, respectively ($P<0.001$). For DR5, the membrane expression was only upregulated in melanoma line WM164 dose-dependently with a quarter-fold increase at 25 µM and a half-fold increase at 50 µM ($P<0.001$) (Fig. 3B). However, this did not occur in MeWo cells ($P>0.05$). To further examine the effects of quercetin on the regulation of DR4 and DR5 the total protein expression and mRNA levels were analyzed post-treatment with quercetin (Fig. 3 C-E). For DR4 there was no change in the total protein expression or the mRNA message in both melanoma lines. For DR5, there was no change in the total protein expression or the mRNA in MeWo. However, there was a dose-dependent upregulation of the total DR5 protein and DR5 mRNA in WM164. To explain the increase in the membrane expression of DR4 but the lack of increase in total protein and mRNA in response to quercetin-treatment, the non-membrane levels of DR4 was measured (Fig. 3 F&G). In both melanoma lines there was significant levels of DR4 within the cell. However, with the application of quercetin the non-membrane levels of DR4 decreased while the membrane levels increased ($P<0.001$).

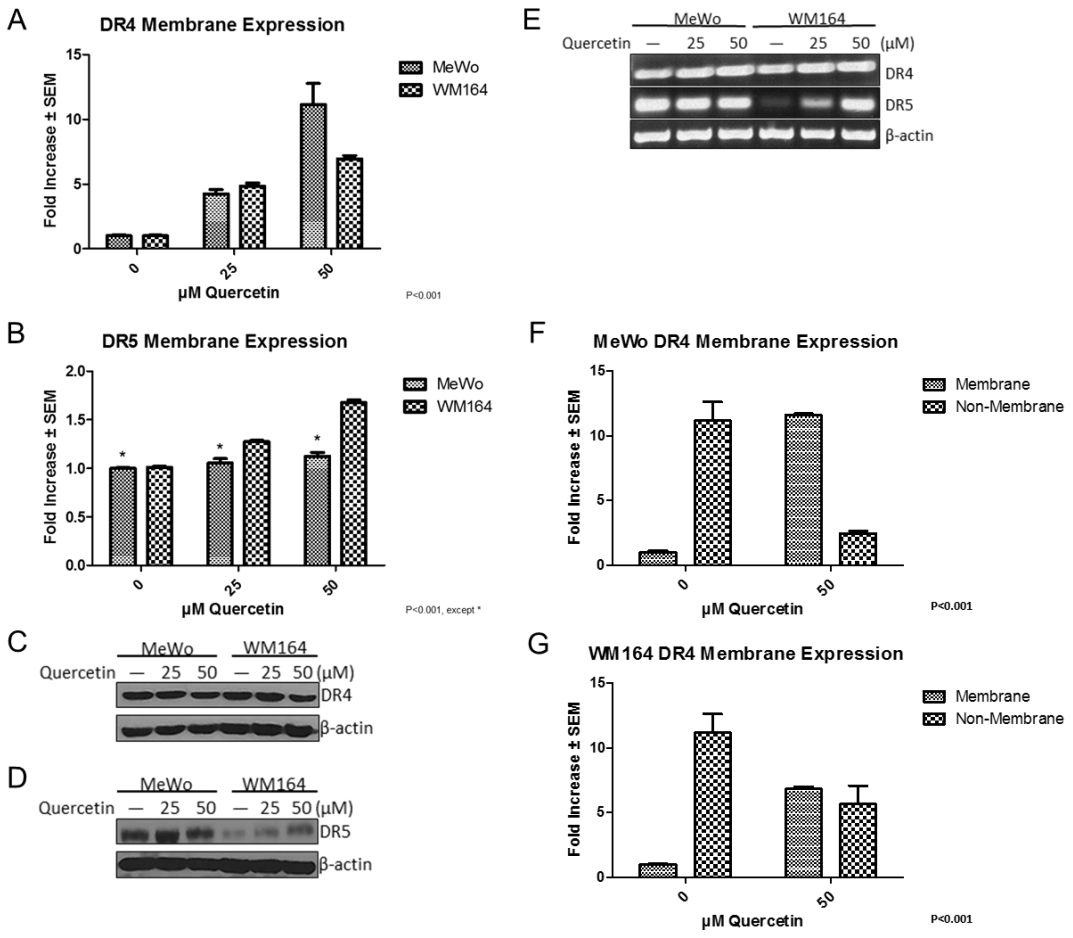



**Figure 4.3. Quercetin regulation of death receptors.** Effects of quercetin on DR4 and DR5 expression. A) Membrane expression of DR4. Mean Fluorescent Intensity (MFI) ± SEM. B) Membrane expression of DR5. MFI ± SEM. C) Total DR4 protein. D) Total DR5 protein. E) DR4 and DR5 mRNA signal. F) Membrane versus non-membrane DR4 expression in MeWo. G) Membrane versus non-membrane DR4 expression in WM164.

***Quercetin regulation of FLIP.*** Also a player in the most apical part of the extrinsic apoptotic pathway, FLIP expression is a major regulatory point for rhTRAIL-sensitivity. Here we wished to see the potential regulation of FLIP by quercetin. Quercetin was able to dose-dependently downregulate the protein expression of FLIP in both melanoma lines (Fig. 4A). To see if quercetin transcriptional downregulates FLIP, the mRNA signal of FLIP in response to quercetin-treatment was measured (Fig. 4B). Here we found no change in the transcript signal in both lines. Another mechanism of protein downregulation is mediated through proteasomal degradation. To test if quercetin promotes the downregulation of FLIP mediated through the proteasome, the proteasome inhibitor MG132 was employed (Fig. 4C). By co-treating quercetin and MG123 we were able to prevent the downregulation of FLIP and show that quercetin downregulates FLIP by promoting proteasome degradation.

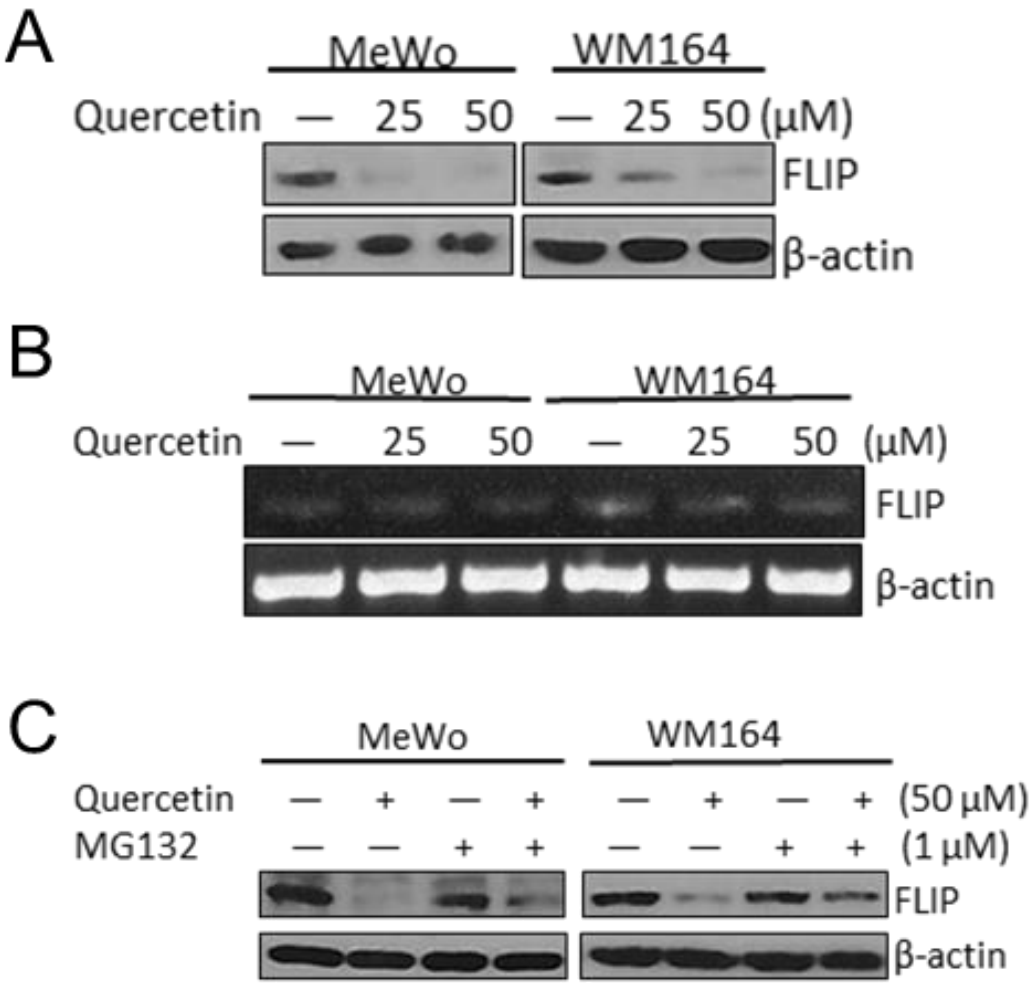



**Figure 4.4. Quercetin regulation of FLIP.** Effects of quercetin on FLIP expression. A) Total FLIP protein. B) FLIP mRNA signal. C) Quercetin plus proteasome inhibitor MG132 on total FLIP protein.

**4.5 Discussion.**

The selectively of rhTRAIL to induce robust levels of apoptosis in cancer cells makes it a prime candidate for the treatment of metastatic malignant melanoma. Unfortunately, the once promised molecule has been suspended due to the resistance exhibited by various cancers to undergo apoptosis in response to rhTRAIL. Understanding the molecular mechanisms of rhTRAIL-resistance is essential to formulating a combination therapy to sensitize resistant malignant melanomas. Here we evaluate the plant-derived flavonoid quercetin as a potential co-treatment for rhTRAIL to overcome melanoma resistance.

Apoptosis induced by pro-apoptotic receptor agonists such as rhTRAIL is mediated through the binding of extracellular receptors, DR4 and/or DR5, and the direct activation of the extrinsic apoptotic pathway along with the indirect activation of intrinsic pathway (33). Melanoma lines MeWo and WM164, both derived from advanced cases of metastatic malignant melanoma are among the melanomas resistant to rhTRAIL-induced apoptosis. Even treating with extremely high concentrations of l μg/ml no event of apoptosis was marked either lines. The lack of apoptotic Annexin-V+ cells, cleaved PARP or activation of any key proteins in either the extrinsic or intrinsic pathways evidenced this. However, the addition of quercetin was able to negate the rhTRAIL-resistance of both MeWo and WM164. Treating at subcytotoxic concentrations of both single agents, the combination of rhTRAIL plus quercetin was able to induce apoptosis in both lines apparent by the significant formation of Annexin-V+ cells, PARP cleavage and activation of executioner caspases 3, 6 and 7, dose-dependently in respect to quercetin. The combination treatment

allowed for the activation of the extrinsic apoptotic pathway as noted by the formation of caspase 8. It is interesting note that in WM164 and not MeWo the intrinsic pathway was activated through the co-treatment mediated by formation of tBID followed by the release of cytochrome c from the mitochondria and the activation of caspase 9.

Of the most significance is that the addition of quercetin to rhTRAIL allowed for the activation of rhTRAIL-mediated extrinsic apoptotic pathway. This is demonstrated by the cleavage of pro-caspase 8 to caspase 8, a marker for rhTRAIL-induced apoptosis (34). The activation of caspase 8 in only the co-treatment group implies that quercetin plays a role in regulating cellular components responsible for controlling rhTRAIL-sensitivity. To understand the mechanism of the sensitization of rhTRAIL-resistant malignant melanomas by quercetin, the effects of quercetin on the most apical parts of the extrinsic pathway were evaluated.

Multiple reports claim that low levels of DRs on the membrane of cancer cells confers rhTRAIL-resistance (14–16,35). We have previously shown that rhTRAIL-resistant melanomas MeWo and WM164 have two-fold less membrane expression of both DR4 and DR5 compared to rhTRAIL-sensitive melanomas (cite paper). Nonetheless, the membrane expression of both DR4 and DR5 can be upregulated on melanoma cells via quercetin. The mechanism in which quercetin executes the upregulation in melanoma cells differs between DR4 and DR5. DR expression can be transcriptionally regulated or through post-translation modifications including protein glycosylation, trafficking and

endocytosis (36). Here we show that quercetin promotes DR5 upregulation on the membrane of melanoma WM164 cells as a consequence of quercetin-stimulated gene transcription. This is illustrated by the correlation between the increased DR5 membrane expression and an increase in the total DR5 protein and mRNA levels, dose-dependently in response to quercetin. Several studies have shown that quercetin is capable of upregulating DR5 on the membrane of cancer cells mediated through the increased activity of transcription factors including p53, CHOP and SP1 (28,30,31,36). However, the transcriptional regulation of DR5 by quercetin has never been evaluated in melanoma cells. The specific cellular targets responsible for the upregulation of DR5 by quercetin is unknown and warrants additional study. However, a p53-mediated mechanism can be excluded because WM164 has an inactivating Y220C mutation in the p53 gene yet experienced DR5 upregulation (37,38).

Conversely, the robust upregulation of DR4 on the membrane of the melanoma cells was not by means of enhanced transcription. This is evident by the absence of an increase in the total protein and mRNA of DR4 in response to quercetin-treatment. Previous studies have shown that total protein and mRNA levels do not correlate with the functional membrane expression of DRs. Studies show that some melanoma cells, despite the presence of mRNA, lack DR4 membrane expression and have high levels of the receptor within the cytoplasm (13). Additionally, immunohistochemistry staining of DR4 reveals that the receptor can be localized to the trans-Golgi network in melanoma cells (39). The translocation of DRs from the trans-Golgi network to the plasma membrane is a complex system regulated by cargo transport proteins such as Arf and ARAP1.

Malfunctions in this pathway can result in DRs surface deficiency and increased localization to the cytoplasm (36). Here we show that both melanoma lines have substantial levels of DR4 within the non-membrane portion of the cell which substantially decreases upon quercetin-treatment. We hypothesize that quercetin promotes the vesicular movement of DR4 from the cytoplasm or the trans-Golgi network to the cellular membrane. The ability of quercetin to the promote DR4 upregulation on the cancer cell membrane is a novel finding. The quercetin-mediated upregulation of DR4 has never been described and warrants thorough examination of the exact mechanism in which quercetin promotes this movement.

Moreover, the binding of rhTRAIL to DR4 and/or DR5 results in the trimerization of the receptors leading to the assembly of the intracellular death-inducing signaling complex (DISC). At the DISC, the adaptor protein, Fas-associated death domain (FADD), acts as a bridge between the death-receptor complex and the death effector domain (DED) of the initiator caspase, procaspase 8. Induced proximity results in the autoproteolytic cleavage of procaspase 8 into its active form, caspase 8. However, the anti-apoptotic protein FLIP will compete for FADD binding, decreasing the formation of caspase 8 and impeding the pro-apoptotic signal generated by rhTRAIL-binding. This is a result of the homology between FLIP and procaspase 8, both possessing a DED yet FLIP lacks caspase activity (40). The ratio between caspase 8 and FLIP is a major regulator of rhTRAIL-sensitivity (20,41). Although quercetin has no effect on procaspase 8 levels, we are able to downregulate FLIP and push the ratio in favor of the pro-apoptotic procaspase 8 and therefore sensitize the rhTRAIL-resistant melanomas. FLIP levels are maintained by a

balance between transcription and degradation mediated by the ubiquitin-proteasome degradation system (42). Here we show that quercetin promotes the downregulation of FLIP mediated through degradation by the proteasome. This is apparent by the inhibition of the quercetin-mediated FLIP downregulation when a proteasome inhibitor is applied. Additional studies have also shown that quercetin is able to downregulate FLIP mediated by the proteasome however this has never been shown in melanoma (31,43).

Here we provide insight into the molecular mechanisms in which rhTRAIL-induced apoptosis can be modulated by quercetin in advanced metastatic malignant melanomas. The application of rhTRAIL is far superior to other therapeutics due the selectivity of rhTRAIL for only cancer cells while showing no harm to normal healthy cells. This selectively will result in potent anti-tumor activity with minimal side effects for the patient. However, the presentation of resistance limits the application of rhTRAIL. Nonetheless, resistance can be overcome through the co-treatment of rhTRAIL with quercetin and lead to the sensitization of rhTRAIL-resistant malignant melanomas.

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# CHAPTER V

# OVERALL CONCLUSION

## 5.1 Conclusion.

Currently, the incidence of skin cancer is on the rise with the malignant melanoma subtype being associated with the highest rate of chemo-resistance and death. Upon metastasis, the five-year survival rate dramatically decreases to 17% from 98% for early stage malignant melanoma. The lack of a standard therapeutic regimen for advanced cases malignant melanoma is responsible for the decline in survival rate (1,2). However, one promising treatment option for malignant melanoma is rhTRAIL.

Herein we show that rhTRAIL possesses potent pro-apoptotic activity *in vitro* and *in vivo* towards malignant melanoma cells. Traditional therapies such as chemotherapy also induce apoptosis in cancer cells; however, these types of therapeutics are non-specific. They only induce apoptosis as a result of causing DNA damage or other cellular stresses and cannot differentiate between cancerous and non-cancerous cells. The lack of specificity results in severe negative side effects for the patient which limits their quality of life and prevents the administration of the optimum drug dose (3,4). Conversely, rhTRAIL is specific for cancer cells only and does not show harm towards normal

healthy cells. This is apparent by the absence of rhTRAIL-induced apoptosis in the non-transformed counterpart of melanomas, melanocytes. The safety of rhTRAIL is further confirmed *in vivo*. The systemic application of rhTRAIL showed no harm to the mice model with no toxicity to any major organs. The exact mechanism for cancer cell-specific rhTRAIL-induced apoptosis is yet to be elucidated. However, we show that melanoma cells have a two-fold higher expression of both DR4 and DR5 compared to melanocytes. The higher expression of DRs may explain why cancer cells are more sensitive to rhTRAIL compared to normal cells.

Unfortunately, the clinical use of rhTRAIL is limited by the heterogeneity in sensitivity among cancers. This is especially observed in cases of malignant melanoma. Many melanomas do not undergo apoptosis in response to rhTRAIL-treatment, even at extremely high concentrations. Resistance can be due to a multitude of reasons. However, we find that the optimum expression of DRs on the cancer cell membrane is essential to overcome the apoptotic threshold and render melanomas sensitive to rhTRAIL.

Nonetheless, the clinical use of rhTRAIL can get back on the "trail" through specific measures. Firstly, clinical trials were performed without prescreening patients' tumors for DR membrane expression. The unselected treatment of patients with rhTRAIL resulted in a lack of clinical benefit, and the clinical trials failed. However, we show a correlation between DR membrane expression and rhTRAIL-sensitivity.

Therefore, the development of an *in vitro* clinical assay to screen patients' tumors for DR expression is essential. If patients are first screened for DR expression, they can be more appropriately placed in rhTRAIL clinical trials. Moreover, finding combination treatments for rhTRAIL in order to sensitize resistance melanomas is another method to overcome the limitations of rhTRAIL. Of special interest are natural compounds derived from "Mother Nature" that are able modulate some of the cellular components responsible for rhTRAIL-resistance (5). Here we show the successful co-treatment of rhTRAIL plus the plant-derived flavonoid quercetin. The resistance of malignant melanomas to rhTRAIL was negated by the addition of quercetin mediated by upregulation of DRs and downregulation the anti-apoptotic protein FLIP.

In the end, these data show the potential of rhTRAIL for the treatment of rhTRAIL-sensitive malignant melanomas. A treatment that would be far superior to traditional therapeutics in terms of tolerability. However, for malignant melanomas resistant to rhTRAIL, the addition of quercetin in a combination treatment can sensitive the resistance cells to the effects of rhTRAIL.

## 5.2 References.

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