RHTRAIL WITH SENSITIZING AGENTS FOR BREAST CARCINOMA TREATMENT

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May 2013

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY IN CLINICAL-BIOANALYTICAL CHEMISTRY

at the
CLEVELAND STATE UNIVERSITY

December 2018
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ACKNOWLEDGEMENTS

I would first like to thank Dr. Kalafatis. I would like to thank you for giving me the opportunity to work in your lab as an undergraduate and graduate student. Without you I would not have realized my desire and capability to obtain a PhD. I appreciate that you allowed me to learn how to become an independent scientist and being able to accomplish this success. I would also like to express my thanks to all of my committee members: Dr. Berdis, Dr. Lindner, Dr. Su, Dr. Weyman, and Dr. Zhou and for all being supportive throughout this challenging process and providing input on my research.

I would like to thank everyone from the Kalafatis lab, especially Katie. I appreciate your support and teaching throughout this process, and I am glad that we worked so well together to both achieve the success of obtaining our PhDs. Joe, Seema, and Jamila for being good role models and teachers. Thank you to all the people who have helped me along the way.

I would like to dedicate my dissertation to my loving family. I would like to express my thanks to my mother and father in that you continue to believe in me and have given me the opportunities in life to accomplish all my dreams and make you proud. You sacrificed everything to give me a good life and obtain a valuable education.
Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) shows therapeutic potential for cancer with its ability to induce apoptosis in cancer cells independent of p53, while exhibiting minimal toxicity to normal, non-transformed cells. Despite this, a majority of breast cancers display resistance to rhTRAIL treatment due to down-regulation of pro-apoptotic proteins, overexpression of anti-apoptotic proteins, and/or down-regulation of death receptors (DRs) 4 and 5. To overcome rhTRAIL resistance, combinatorial approaches with “mother nature”-derived compounds such as Quercetin (Q), silibinin, and Ursolic acid (UA) have been investigated as possible sensitizing agents because of their abilities to down-regulate anti-apoptotic proteins and up-regulate pro-apoptotic proteins in cancer cells. Therefore, the intention of this dissertation was to ascertain the capacity of these compounds to sensitize rhTRAIL-resistant breast cancers to rhTRAIL-induced apoptosis. Q’s augmentation in breast cancer was determined the proteasome-mediated degradation of c-FLIPL along with the up-regulation of DR5 in rhTRAIL-resistant breast cancer cells. Silibinin enhanced rhTRAIL-induced apoptosis in rhTRAIL-resistant triple negative breast cancers (TNBCs) through the down-regulation of the anti-apoptotic protein survivin and through the up-regulation of DR4 and DR5. UA sensitized rhTRAIL-resistant triple negative breast cancers via the down-regulation of c-FLIPL and through the up-regulation of DR4 and DR5. Thus, the data
strongly suggests that these “mother nature”-derived compounds are efficacious sensitizing agents for rhTRAIL-resistant breast cancers.
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CHAPTER I

INTRODUCTION

1.1 Breast carcinoma

Breast carcinoma is the most common cancer diagnosis for women in the United States with on average 230,000 women being diagnosed each year, but breast cancer mortality rates have declined over time as a result of earlier detection and access to more anti-cancer therapeutics (1). Moreover, breast cancer is prevalent among women with a family history of breast cancer and those who carry the BRCA1 and BRCA2 genes (1). The current treatment options for breast cancer are breast conserving surgery which involves the removal of a tumor and the surrounding tissue, mastectomy which is the removal of the breasts surgically, radiation therapy, chemotherapy before or after surgery, and hormone-targeted therapy. It should be noted that hormone-targeted therapy can just be applied to those diagnosed with estrogen receptor (ER) and progesterone receptor (PR) positive breast cancers and human epidermal receptor 2 (HER2) overexpression breast cancers but not for the more fatal triple negative breast cancers (TNBCs) that lack the expression of those receptors (1). However, in order to observe if the tumor has spread to other parts of the body, then the underarm lymph nodes need to be examined during the surgery (1). If the cancer has not spread, then the five year survival rate is 99%; whereas,
if the cancer has spread to other tissues and the lymph nodes, then the survival rate decreases to 84% (1). The survival rate further decreases to 24% when the cancer has spread to distant lymph nodes or organs (1). Therefore, there is a demand for effective and safe therapeutic strategies that will improve patients’ overall quality of life.

1.2 Dysregulation of apoptosis and cancer

Cancer is a disease characterized by improper proliferation, irregular cell survival, decreased apoptosis, cell immortalization, invasion of surrounding tissue and metastasis. Apoptosis or programmed cell death is a fundamental process to maintain homeostasis. Dysregulation in the apoptotic pathway or resistance to apoptotic stimuli is a key factor in the inception and advancement of cancer. Mechanisms of resistance consist of overexpression of oncogenes, inactivation of tumor suppressor genes, imbalance of pro- and anti-apoptotic proteins and inactivation of the intrinsic and extrinsic apoptotic pathways. Traditional cancer therapies such as chemotherapy and radiotherapy induce apoptosis in order to eliminate cancer cells. These anti-cancer therapeutic approaches induce apoptosis in a p53-dependent manner causing severe cell damage. The tumor suppressor gene, p53, is a crucial factor for the execution of the intrinsic pathway of apoptosis. It should be pointed out that more than 50% of all cancers possess a mutated or non-functional p53 gene, so these therapies are less efficacious. In addition, these traditional anti-cancer therapies target all cells not just cancer cells for cell death. Without specificity, toxic side effects could be observed in patients deterring patients’ overall quality of life and hindering the administration of optimal drug-dosing (2). Consequently, there is a need to develop more selective, safe, and specific anti-cancer therapies that can induce apoptosis through the p53-independent extrinsic pathway.
1.3 Pathways of apoptosis

The induction of apoptosis has been a target for different anti-cancer therapeutics (3, 4). Apoptosis does occur naturally in organisms to maintain homeostasis; it is also a defense mechanism against a foreign body (3, 4). It should be pointed out that there is a distinction between apoptosis and necrosis. Necrosis is an uncontrolled and passive process leading to cell death marked with inflammation; whereas, apoptosis is a controlled process that is dependent on energy to progress with no inflammation observed averting any potential negative side effects (4–6). The extrinsic and intrinsic are the two initiating pathways of apoptosis that both converge to the execution pathway to activate the executioner caspases 3, 6, and/or 7 depending on the cancer cell line (Figure 1.1) (3–6). An executioner caspase can then cleave poly (ADP-ribose) polymerase or PARP (a hallmark of apoptosis) which leads to cell death which is displayed by nuclear disruption including DNA fragmentation and chromatin condensation along with cellular blebbing and shrinkage (3–6). The extrinsic pathway can be triggered when pro-apoptotic death ligands binding to receptors with cytoplasmic death domains (DD) that are located on the extracellular membrane; the ligand of focus is TRAIL which can bind to DR4 and DR5. The binding of TRAIL leads to the trimerization of the receptors followed by the assembly of the death inducing signaling complex or DISC. DISC encompasses Fas-associated death domain or FADD which recruits and binds to pro-caspase 8 (7–9). Then pro-caspase 8 is autoproteolytically cleaved to the active form caspase 8 and thereby expediting the execution of the executioner pathway and eventual PARP (7, 8, 10). The proper execution of the extrinsic pathway can be inhibited when cellular FLICE-like inhibitory protein or c-FLIP binds to DISC to displace caspase 8 because c-FLIP is
structurally similar to caspase 8 (7). For the intrinsic pathway’s initiation, DNA or microtubule damage can initiate the mitochondrial-dependent or intrinsic pathway caused by chemotherapy, radiotherapy, hypoxia, starvation, or other forms of cell stress (7, 11). Normally, the p53 inhibitor Mdm2 (Hdm2 in humans) binds to p53 and keeps p53 stabilized by preventing p53 from being transcribed and from being degraded. However, the DNA damage causes the activation of p53 by breaking up the interaction of Mdm2 and p53 by the phosphorylation by the kinases ATM and ATR facilitating the heightening of p53’s transcriptional activity, DNA binding and posttranslational modifications involving protein stabilization. The subsequent translocation of Bax and Bad (pro-apoptotic members of the Bcl-2 family) is observed; the translocation causes a decrease in mitochondrial membrane potential resulting in the release of cytochrome c from the mitochondria in the presence of ATP (7, 11). Cytochrome c binds to apoptotic peptidase-activating factor 1 (Apaf-1) and pro-caspase 9 to form the apoptosome signaling complex; pro-caspase 9 is then activated to caspase 9 (7). The execution pathway can then be initiated directly by caspase 9 (7). There is some cross-talk between the extrinsic and intrinsic pathways in which caspase 8 can cleave Bid (pro-apoptotic Bcl-2 family member) to truncated bid (tBid) (7). tBid can travel to the mitochondria facilitating the release of cytochrome c release via the increased expression of Bax and Bad to execute the intrinsic pathway of apoptosis (7). There are several anti-apoptotic proteins that are involved including Bcl-2, Bcl-xL, and XIAPs (X-linked inhibitors of apoptosis) (7). Bcl-2 and Bcl-xL are members of the Bcl-2 family that prevent the release of cytochrome c from the mitochondria when overexpressed (7). In addition, IAPs such
as XIAP, cIAP, c-FLIP, and survivin are also anti-apoptotic proteins that can inhibit the activation of executioner caspases and caspase 9 (7).

**Figure 1.1: Schematic of apoptosis.** (Modified from Duiker et al. 2006)

1.4 TRAIL

TRAIL is a type II transmembrane protein and a member of TNF-gene superfamily; it possesses sequence homology to FasL and TNF (9, 12, 13). The extracellular region of the protein can form soluble TRAIL or sTRAIL when cleaved by metalloproteases (9). TRAIL is a potential anti-cancer agent because it can selectively kill cancer cells such as colon, lung, breast, kidney, brain, pancreas, prostate, skin,
leukemia, multiple myeloma, lymphoma, and non-Hodgkin’s lymphoma through the induction of apoptosis of cells without causing harm to normal, non-transformed cells including breast epithelial cells and melanocytes *in vitro* and *in vivo*; sTRAIL can also induce apoptosis those cancer cells (14). Moreover, the induction of apoptosis is independent of the p53 status of cells; whereas, chemotherapy and radiation therapy induce apoptosis utilizing a functional p53-tumor suppressor gene. Several cancer cells have a mutational p53 gene or a non-functional p53 gene, so a more effective therapeutic option for a variety of cancers would be bypassing the activation of p53 (9, 13, 15).

There are other members of the TNF-family that can cause the induction of apoptosis such as FasL, TNFα, and CD40L *via* the activation of the extrinsic pathway (14). However, FasL has been demonstrated to cause severe liver toxicity, and TNF infusion has been shown to cause a deadly inflammatory response similar to septic shock (9). Despite this, TRAIL has been revealed to specifically target cancer cells without affecting normal cells with little to no indications of toxicity (9, 15). TRAIL can be found on chromosome 3 at position 3q26, and TRAIL mRNA is expressed in a variety of different cells and tissues including the lymphoid system, spleen, prostate, and lungs. (12). In addition, TRAIL is naturally expressed in killer cells and assists in the immune system; TRAIL expression is increased in cells that mediate innate immunity after the activation of different pro-inflammatory cytokines (12, 16, 17). Recombinant human TRAIL (rhTRAIL) is the extracellular C-terminus of TRAIL which is the active portion of TRAIL and composed of amino acids 114-281; it has also been shown to have little effect on normal, non-transformed cells such as melanocytes and still target cancer cells.
for the induction of apoptosis (12, 13). rhTRAIL was administered to treat breast cancer cells.

1.5 TRAIL structure

The crystallography of TRAIL was determined (Figure 1.2); studies found that TRAIL has a homotrimeric structure with three TRAIL monomers and two antiparallel β-pleated sheets (12). TRAIL contains a cysteine residue, Cys230, which allows the three molecules of TRAIL to interact through the three cysteinyl sulfur atoms that are coordinated by a zinc and a chloride ion (12, 18). The zinc ion is necessary for the trimerization of TRAIL after binding to the DRs; the other TNF receptors do not utilize zinc-mediated trimerization (12, 13, 17). A study observed that if there was a change to the cysteine at 230 to alanine or serine, then there was a large decrease in the apoptotic activity of TRAIL (12). Without zinc, TRAIL would destabilize, the cysteines could be oxidized, and disulfide-linked dimers of TRAIL could form (19).

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

TRAIL induces apoptosis via the ligation of DR4 and DR5 along with the trimerization of TRAIL around a centrally located zinc atom linked through a cysteine (20). The trimerized receptors form aggregates that accumulate in the lipid raft microdomains located in the plasma membrane. Next, there is the assembly of the adaptor components including FADD and activation of caspase 8 into DISC which leads to the activation of the executioner caspases (17, 20). DR4 and DR5 are key receptors for the induction of apoptosis because they both contain an intracellular death domains (DDs) (21, 22). Decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2) are two membrane receptors that TRAIL can bind to, but both receptors lack a DD (Figure 1.3) (12, 22). This means that apoptosis cannot occur, so they both can act as antagonistic receptors (12). DcR1 does not have any transmembrane or cytoplasmic residues and is glycosylphosphatidylinositol linked to the cell surface (12). DcR2 has a partial DD, but it cannot mediate apoptosis (12). TRAIL can also bind to osteoprotegerin (OPG) which a soluble receptor that lacks any transmembrane and cytoplasmic residues (12, 17). The crystal structure of the ligand-receptor complex between TRAIL and DR4 and/or DR5 illustrates that the trimeric ligand interacts with three monomeric receptors, engaging at the interfaces between the monomers of TRAIL (Figure 1.2). This aspect prompted the development of the “ligand trimerization model” in which the incoming trimeric TRAIL recruits three receptor molecules, generating an intracellular signaling cascade (23).
1.7 TRAIL preparations

rhTRAIL is applied in this study and does not induce apoptosis on normal, non-transformed cells such as epithelial, endothelial, fibroblastic, smooth muscle, astrocytic and hematopoietic stem cells but does induce apoptosis in cancer cells by interacting with DR4 and DR5 (13, 20). However, the mechanism by which rhTRAIL promotes a pro-apoptotic effect in cancer cells and not normal, non-transformed cells has not been established (24). Even with this effect, a study has illustrated that rhTRAIL could cause some toxicity in hepatocytes using a polyhistidine-tagged version of rhTRAIL or
rhTRAIL.His that did induce apoptosis, but the normal version of rhTRAIL did not induce apoptosis in the hepatocytes (19). Later, it was determined that the amount of zinc was a factor in this difference between rhTRAIL.His and rhTRAIL having much higher levels of zinc compared to rhTRAIL.His (18). rhTRAIL was deemed to be safe when injected into severe combined immunodeficient (SCID) mice with hepatocytes intravenously (24). For this investigation, the rhTRAIL applied is an optimized preparation with the amino acids 114-281 and no exogenous sequence tags.

1.8 Preclinical and clinical trials of rhTRAIL

Optimized rhTRAIL without exogenous sequences tags has been utilized in clinical studies done by Genetech, Inc. Pharmacokinetic studies have revealed that the half-life of rhTRAIL is three to five minutes in rodents and 23-32 minutes in nonhuman primates (9). One phase I study involved doses of rhTRAIL ranging from 0.5 to 30 mg/kg that were applied intravenously over one hour on days one and five of a 21-day cycle for up to 24 weeks; there was a five day dosing period followed by 16 days of no treatment. They found that there was no correlation between any toxicity seen and the dose level of the treatment; even at 30 mg/kg there was no antibody formation or liver toxicity. The half-life of rhTRAIL in serum was about half an hour to one hour after infusion. At the end of second cycle, 46% of the 71 patients had a stable disease or better; no hepatotoxicity was observed. After six months, there were two patients with chondrosarcoma who had partial responses after that time period. There was antitumor activity in two of the five patients with chondrosarcoma (15).

There have been several other clinical trials done with rhTRAIL alone and in combination with a different anti-cancer therapeutic. Clinical trials with rhTRAIL alone
include treating Non-Hodgkin’s lymphoma, lymphoma, and non-small cell lung carcinoma (12). rhTRAIL has been combined with rituximab to treat Non-Hodgkin’s lymphoma where no toxicity was observed in the seven patients treated intravenously with 4 or 8 mg/kg of rhTRAIL for five days of a 21-day cycle with rituximab at 375 mg/m² weekly (25). The trial showed that two of the patients had a complete response, one patient had a partial response, and two patients had a stable disease response (12). rhTRAIL has also been attempted to be combined with gemcitabine for treatment of pancreatic cancer and also with bortezomib for treatment of myeloma. Both single agent rhTRAIL and combinatorial therapeutic approaches did not produce significant clinical benefit and trials were terminated due to this lack of clinical efficacy (12). Clinical trials have been carried out with monoclonal antibodies against DR4 or DR5 (26).

1.9 rhTRAIL resistance

Upon repeated exposure to rhTRAIL, some cancer cells can acquire resistance while others exhibiting resistance to rhTRAIL’s pro-apoptotic effects (27). In addition, rhTRAIL sensitivity was examined in one study involving breast cancer cell line panel; researchers treated the cell lines with increasing concentrations of TRAIL from 0 to 1000 ng/mL (28). Utilizing MTS colorimetric assays to determine cell proliferation, they found that breast cancer cell lines displayed a variety of rhTRAIL sensitivity (28). Since some cancer cells are resistant to rhTRAIL, combinatorial therapeutic approaches are a potential option to sensitize those resistant cancer cell lines to rhTRAIL-induced apoptosis (29). Some cancer cells that are resistant to rhTRAIL exhibit a decreased DR4 and/or DR5 surface expression and/or protein expression, so the expression of DRs may be a factor in the sensitization of cancer cells to rhTRAIL (29, 30). rhTRAIL resistance
could also be observed due to the overexpression of different IAPs including FLIP, XIAP, cIAP, and survivin along with the up-regulation of anti-apoptotic proteins of the Bcl-2 family and/or the down-regulation of pro-apoptotic proteins of the Bcl-2 family. Therefore, there is a need to discover ways to overcome rhTRAIL resistance in order to clinically apply rhTRAIL effectively.

1.10 rhTRAIL synergism

Since rhTRAIL resistance has been observed in a variety of cancer cell lines, researchers have proposed to combine rhTRAIL with other anti-cancer drugs to overcome this resistance. Studies have shown that combinatorial approach of rhTRAIL with established anti-cancer therapies such as chemotherapy, radiotherapy, irradiation, proteasome inhibitors, histone deacetylase inhibitors, and tyrosine kinase inhibitors have enhanced rhTRAIL’s pro-apoptotic effects on different cancer cell lines including breast, prostate, pancreatic, and colon in vitro (17, 22). Some studies have also observed an increase in the expression of DR5 in those cells treated with rhTRAIL and another anti-cancer therapy (9). In addition, “mother nature”-derived compounds are of interest to be applied as a co-treatment with rhTRAIL. These inexpensive natural compounds are often safe to ingest and often found in supplemental form because they have been used to treat various diseases; whereas, traditional chemotherapies are more expensive and can produce in negative side effects. Some of these natural compounds have shown to possess the capability to enhance rhTRAIL’s pro-apoptotic effects through the upregulation of DR4 and DR5 along with down-regulation of anti-apoptotic proteins promoting rhTRAIL-induced apoptosis. As single agents, natural compounds have the ability to activate apoptosis and potentiate rhTRAIL-induced apoptosis in cancer cells. Moreover,
these compounds have multiple mechanisms of action furthering a heightened sensitivity to rhTRAIL and thereby, are more effective. In this study, we compare three potential sensitizing agents: Q, silibinin, and UA and scrutinize their capacities to augment rhTRAIL-induced apoptosis in breast carcinoma.

1.11 Quercetin

One potential sensitizing agent is Quercetin (Q); it is a naturally occurring flavonol found in different vegetables, fruits, tea, red wine, and coffee (31–33). Q has been shown to produce anti-proliferative and pro-apoptotic effects in cancer cells such as prostate, cervical, lung, breast, and colon (31, 32, 34–38). Q can induce apoptosis in some cancer cell lines through the down-regulation of anti-apoptotic proteins survivin, Bcl-xL, and Bcl-2 and through the up-regulation of pro-apoptotic proteins Bad and Bax (31, 32, 34–38). Additionally, no major cytotoxic effects have been observed in different in vivo studies, and clinical trials have administered Q with no major cytotoxic effects cited (31–33). Accordingly, these findings suggest that Q has the potential to be an effective sensitizing agent.
Figure 1.4: Structure of Quercetin. Figure prepared on ChemDraw.
Figure 1.5: Content of Quercetin in selected food. (Modified from Miles et al. 2014; data obtained from the USDA Database for the flavonoid content of selected foods)

1.12 Silibinin

Silibinin is a flavonolignan derived from the seeds of *Silybum marianum* (milk thistle) (39, 40). Silibinin has been deemed safe for human use and been sold as a dietary supplement (40, 41). Silibinin has exhibited anti-cancer characteristics in various cancer cells including colon, lung, prostate, skin, hepatocellular, and breast cancer (42). *In vitro*, silibinin has been shown to promote apoptosis in hepatocellular, prostate, and breast cancers through the activation of caspase 8, subsequent activation of caspase 3, and the cleavage of PARP; in addition, silibinin-induced apoptosis occurred through the release of cytochrome c from the mitochondria facilitating caspase 9 activation, caspase 3 activation, and eventual PARP cleavage (39). Silibinin up-regulated DR4 and DR5 in
colon cancer cells (39). Silibinin decreased survivin expression in laryngeal squamous cell cancer cells and glioma cancer cells; silibinin down-regulated c-FLIP expression in human glioma cells (43, 44). Silibinin decreased hepatocellular and lung carcinoma xenografts in vivo (45, 46). All of these are encouraging results that make silibinin a safe and promising candidate for co-treatment with rhTRAIL.

![Structure of Silibinin](image)

**Figure 1.6: Structure of Silibinin.** Figure prepared on ChemDraw.

### 1.13 Ursolic Acid

Ursolic Acid (UA) is a pentacyclic triterpenoid derived from the leaves and berries of various plants and found in the coatings of fruits (47–49). UA has been determined to be safe for human use and has been sold as a dietary supplement (47, 50). UA has exhibited anti-cancer characteristics in various cancer cells including colon, lung, prostate, ovarian, hepatocellular, and breast (49–52). *In vitro*, UA has been demonstrated to promote apoptosis in hepatocellular, prostate, and breast cancers through the proper execution of both the extrinsic pathway and intrinsic pathway ending to PARP cleavage (48, 50, 53, 54). UA’s mechanism of apoptosis induction has been elucidated in prostate, gastric, and colorectal cancers through the up-regulation of DR5, down-regulation of c-
FLIP, Bcl-2, and survivin (50, 51, 54, 55). *In vivo*, UA decreased the growth of prostate and colorectal xenografts (49, 51). UA has shown to not impact non-tumorigenic breast epithelial MCF-10A cell growth (56).

![Figure 1.7: Structure of Ursolic Acid](image). Figure prepared on ChemDraw.

### 1.14 References


CHAPTER II

TRAIL-INDUCED APOPTOSIS IN TRAIL-RESISTANT BREAST CARCINOMA THROUGH QUERCETIN CO-TREATMENT

2.1 Abstract

Breast cancer is the most commonly diagnosed cancer in women. There is a continued interest for the development of more efficacious treatment regimens for breast carcinoma. Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) shows potential as a potent anti-cancer therapeutic for the treatment of breast cancer while displaying minimal toxicity to normal cells. However, the promise of rhTRAIL for the treatment of breast cancer is dismissed by the resistance to rhTRAIL-induced apoptosis exhibited by many breast cancers. Thus, a co-treatment strategy was examined by applying the natural compound Quercetin (Q) as a sensitizing agent for rhTRAIL-resistant breast cancer BT-20 and MCF-7 cells. Q was able to sensitize rhTRAIL-resistant breast cancers to rhTRAIL-induced apoptosis as detected by western blotting through the proteasome-mediated degradation of c-FLIP\(L\) and through the up-regulation of DR5 expression. Overall, these \textit{in vitro} findings establish that Q is an effective sensitizing agent for rhTRAIL-resistant breast cancers.

2.2 Introduction
Breast cancer is the most commonly diagnosed cancer in women (1–4). The development of more effective treatment regimens against the different forms of breast carcinoma are being explored (2, 4, 5). Patients with hormone-dependent and human epidermal growth factor receptor-2 (HER2) overexpression breast cancers often have a better prognosis because of the availability of hormone-targeted therapies (2, 5, 6). Whereas, triple negative breast cancers (TNBCs) are more challenging to treat because there is no specific hormone to target; hence, TNBC is the deadliest form of breast cancer (2, 4).

One promising anti-cancer therapeutic of interest is recombinant human tumor necrosis factor (TNF)-related apoptosis-inducing ligand (rhTRAIL), the optimized form of the endogenous death ligand TRAIL. rhTRAIL consists of the extracellular C-terminus of TRAIL amino acids 114-281 lacking exogenous sequence tags (7–11). rhTRAIL has shown great potential as an effective anti-cancer therapeutic due to its ability to induce apoptosis in cancer cells while exhibiting minimal toxicity to normal, non-transformed cells (7–11). rhTRAIL initiates the extrinsic pathway of apoptosis by binding to the extracellular death receptors DR4 and DR5 leading to trimerization of the receptors followed by the activation of caspase 8, the subsequent activation of the executioner caspases 3, 6, and 7, and the eventual cleavage of poly (ADP-ribose) polymerase or PARP (hallmark of apoptosis) (12–16). In addition, rhTRAIL can activate the intrinsic pathway of apoptosis independently of p53 through the caspase 8-mediated cleavage of Bid to truncated Bid (tBid) facilitating the release of cytochrome c from the mitochondria followed by the activation of caspase 9 and the subsequent activation of the executioner caspases. (13–15, 17–19). Despite this, a majority of breast cancer cells exhibit resistance
to TRAIL treatment due to the up-regulation of anti-apoptotic proteins such as cellular FLICE-like inhibitory protein (c-FLIP) and the down-regulation of DR5. Clinical trials have been completed with TRAIL, but further trials have since been terminated due to a limited therapeutic efficacy (7, 20–22). Consequently, many studies have focused on determining sensitizing agents that have the capability to overcome rhTRAIL-resistance.

One potential sensitizing agent is Quercetin (Q); it is a naturally occurring flavonol found in different vegetables, fruits, tea, red wine, and coffee (23–25). Q has been shown to produce antiproliferative and pro-apoptotic effects in cancer cells such as prostate, cervical, lung, breast, and colon (23, 24, 26–30). Q can induce apoptosis in some cancer cell lines through the down-regulation of anti-apoptotic proteins survivin, Bcl-xL, and Bcl-2 and through the up-regulation of pro-apoptotic proteins Bad and Bax (29, 31–33). Investigations involving human hepatoma and prostate cancer cells have demonstrated that Q can enhance TRAIL-induced apoptosis through the up-regulation of DR5 (28, 34, 35). Additionally, non-tumorigenic breast epithelial MCF-10A cells were not affected by Q treatment after 24, 48, and 72 hours supporting Q’s safety for systemic application (29, 36). Furthermore, no major cytotoxic effects have been observed in different in vivo studies, and clinical trials have administered Q with no major cytotoxic effects cited (24, 31, 37–39). Therefore, these findings suggest that Q has the potential to be an effective sensitizing agent.

The intention of this study was to investigate the capability of Q to sensitize rhTRAIL-resistant TNBC BT-20 cells (mutated p53) and hormone-dependent breast cancer MCF-7 cells (wild-type p53) and to elucidate the underlying mechanism for Q’s sensitization. Our study demonstrates that Q has the ability to induce the proteasome-
mediated degradation of c-FLIP_L and to induce the up-regulation of DR5 facilitating the execution of the extrinsic pathway and thereby, sensitizing breast cancers to rhTRAIL-induced apoptosis. Thus, the presented evidence reveals that Q is a good sensitizing agent for rhTRAIL-resistant breast cancers.

2.3 Methods

A. Drugs and chemicals

rhTRAIL was produced according to well defined and previously detailed protocols. (8–10) rhTRAIL was aliquoted and stored at -80°C. Quercetin dihydrate (lot# D00166146, molecular weight of 338.3 g/mol, Calbiochem) was dissolved in Polyethylene Glycol (PEG) molecular weight 400 (Fisher Scientific) at 7.5 mg/mL, filtered, aliquoted, and stored at -20°C (39). MG132 proteasome inhibitor (molecular weight of 457.6 g/mol, Calbiochem) was dissolved in dimethyl sulfoxide (DMSO) to produce a 10 mM stock that was filtered, aliquoted, and stored at -20°C.

B. Cell culture

Human breast cancer MCF-7 (ATCC HTB-22) and BT-20 (ATCC HTB-19) cells were cultured in DMEM (Cleveland Clinic Cell Services Media Core) supplemented with 10% FBS (Gibco), 1% antibiotics-antimycotics (Gibco), 1% L-glutamine (Gibco), 1% non-essential amino acids (Gibco), and 1% sodium pyruvate (Gibco). Human non-tumorigenic breast epithelial MCF-10A (ATCC CRL-10317) cells were cultured in HuMEC ready medium (Gibco). Cells were maintained in a humidified atmosphere with 5% CO2 at 37°C. Cells were treated with drugs 24 hours after plating, incubated with drugs for an additional 72 hours, and collected for the different assays described below (36,40).
C. Annexin V/PI assays-flow cytometry

Cells were trypsinized, spun at 1000 rpm for three minutes, and washed with PBS. Cells were incubated with Annexin V-FITC and propidium iodide (PI) solution ( Annexin V: FITC Apoptosis Detection Kit I, BD) for 15 minutes at room temperature in the dark. Apoptosis was detected utilizing the BD FACSCanto II and applying FACSDiva software and Flowing Software 2. Each experiment was performed in triplicate, and three independent experiments were conducted for each cell line to obtain the mean percent of apoptotic cells +/-SEM.

D. Determination of apoptotic associated protein levels by western blotting

Cells were collected and washed with PBS. Total cell lysates were prepared by lysing with RIPA buffer (Amresco) and a protease inhibitor cocktail (Calbiochem). The lysates were placed on ice for 30 minutes, were spun at 10,000 rpm for 10 minutes, and were quantified by applying the Pierce BCA Protein Assay (Thermo Scientific). Aliquots of 35 μg of protein were prepared, denatured with 4X Laemmlı sample buffer (250 mM Tris-HCl, 8% SDS, 40% glycerol, 8% BME, and 0.02% Bromophenol Blue), and separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were transferred to PVDF membranes (Millipore) employing the semi-dry transfer method (Bio-Rad). Each membrane was blocked with 5% non-fat milk at room temperature for one hour and incubated overnight at 4°C with a diluted primary antibody: anti-PARP, anti-caspase 3, anti-cleaved caspase 3, anti-caspase 7, anti-caspase 8, anti-cleaved caspase 8, anti-Bid (Cell Signaling Technology), or anti-FLIP (Enzo Life Sciences). The membranes were washed in TBS-Tween (0.15 M NaCl, 0.02 M Tris, and 0.3% Tween-20 with a pH of 7.4), incubated with a horse radish peroxidase (HRP)-conjugated secondary
antibody either goat anti-mouse IgG or goat anti-rabbit IgG (Bio-Rad) diluted in 5% non-fat milk for one hour at room temperature, and washed in TBS-Tween. The membranes were then exposed on HyBlot CL Autoradiography film (Denville Scientific); the protein bands were detected using chemiluminescence via Pierce ECL2 Western Blotting Substrate (Thermo Scientific). The membranes were then probed for β-actin (Promega) as the internal loading control.

E. Determination of cytochrome c release

Cells were collected, washed, and resuspended in permeabilization buffer (400 µg/mL digitonin, 75 mM KCl, 1mM NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose) with a protease inhibitor cocktail. All of the samples were kept on ice for 10 minute and spun at 16,000 g for five minutes at 4°C; the supernatants were kept as the cytosolic fractions. Protein quantification was executed as described above, and aliquots of 60 µg of protein were prepared, denatured, and separated on 15% SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes and blocked as above. The membranes was incubated overnight at 4°C with anti-cytochrome c (Cell Signaling Technology) diluted in 5% non-fat milk. The membranes were washed and developed as above.

F. Western blot analysis of DR4 and DR5 expression

Cells were collected with cell dissociation buffer (Gibco) and lysed with RIPA buffer as above. After the membranes were blocked, the membranes were incubated overnight at 4°C with anti-DR4 (Imgenex) in 5% non-fat milk or anti-DR5 (Cell Signaling Technology) in 5% BSA (Fisher Scientific). The membranes were washed and developed as above. Densitometry was calculated from Image J software.

G. Flow cytometry analysis of DR4 and DR5 expression
Cells were collected with cell dissociation buffer and spun at 1000 rpm for three minutes. Cells were resuspended in staining buffer (2% FBS, 0.02% sodium azide, and PBS) and incubated with anti-DR4-PE or anti-DR5-PE (eBioscience) for one hour in the dark at 4°C; a mouse IgG1 kappa isotype control (eBioscience) was used to compensate for any non-specific binding. Cells were washed twice with staining buffer and resuspended in staining buffer for analysis. DR4 and DR5 membrane expressions were analyzed on a BD FACSCanto II flow cytometer utilizing FACSDiva software. Histograms were prepared employing Flowing Software 2. Each experiment was performed in triplicate, and three independent experiments were conducted for each cell line to obtain the fold increase in DR4 or DR5 cell surface expression relative to the vehicle-treated control +/-SEM.

H. Reverse Transcription-PCR analysis for DR5 and c-FLIP\(_L\)

Total RNA was extracted from cells using TRIzol reagent (Ambion). Reverse transcription polymerase chain reaction (RT-PCR) was performed following the manufacturer’s protocol (Invitrogen SuperScript III One-Step RT-PCR System with Platinum\(^\circledR\) Taq DNA Polymerase). Human DR5 mRNA was amplified using the forward primer 5’-GGGAGCCGCT-CATGAGGAAGTTGG-3’ and the reverse primer 5’-GGCAAGTCTCTCTCCAGCGTCTC-3’. For c-FLIP\(_L\), forward primer 5’-CTTGGCCAATTTGCCTGTAT-3’ and the reverse primer 5’-CCCATGAACATCCCTCCTGAT-3’ were used. For β-actin, the forward primer 5’-TGACGG-GGTCAACCCACACTGTGCC-3’ and the reverse primer 5’-CTGCATCCTGTCGGCAATGCCAG-3’ were used. cDNA synthesis was performed at 60°C for 30 minutes using the Applied Biosystems GeneAmp PCR System 9700. The
PCR cycling conditions (40 cycles) were chosen as follows: denature for 2 minutes at 94°C, anneal for 30 seconds at 55°C for c-FLIP and 65°C for DR5 and β-actin, extend for 1 minute and 30 seconds at 68°C, and execute a final extension for 10 minutes at 68°C. Reaction products were analyzed on 1.2% agarose gels. The bands were visualized by ethidium bromide (Invitrogen) and an UV illuminator (UVP).

I. Examining post-translational effects of Q

Cells were treated with 0.25 µM MG-132 alone and in combination with 50 µM Q along with a vehicle-treated control. Cells were collected, washed, lysed, and quantified as above. Western blot analysis was performed as above probing for c-FLIP.

J. Co-Immunoprecipitation (Co-IP)

Columns were prepared according to the manufacturer’s instructions (Pierce Co-IP kit) with 5 µg of anti-c-FLIP. Cells were collected, washed, lysed, and quantified. Co-IP was preformed overnight at 4°C with 500 µg of pre-cleared lysate. Proteins were eluted according to the manufacturer’s instructions and analyzed by western blotting probing for ubiquitin (Cell Signaling Technology) and c-FLIP on 15% SDS-PAGE gels.

K. Statistical analysis

Data were analyzed using Student’s t-test and Anova, and the differences between experimental and control groups were considered statistically significant at p values less than 0.05.

2.4 Results

FACS analysis of rhTRAIL-induced apoptosis

FACS analysis was completed on breast cancer cells treated with increasing concentrations of Q (12.5, 25, and 50 µM) in the presence or absence of 100 ng/mL
rhTRAIL to ascertain Q’s sensitizing effects on rhTRAIL-induced apoptosis (Figure 2.1). Q enhanced rhTRAIL-induced apoptosis in both breast cancer cell lines. However, for breast cancer MCF-7 cells, Q did not have as a considerable impact on promoting rhTRAIL-induced apoptosis when compared to Q-mediated rhTRAIL-induced apoptosis in breast cancer BT-20 cells. For example, breast cancer MCF-7 and BT-20 cells treated with 50 µM Q produced on average about 15% and 20% apoptotic cells, respectively ($p<0.05$); whereas, the co-treatment of 50 µM Q and 100 ng/mL rhTRAIL on MCF-7 and BT-20 cells produced on average about 25% and 45% apoptotic cells, respectively ($p<0.05$). It should be noted that 100 ng/mL rhTRAIL alone did not produce a significant amount of apoptotic breast cancer cells when compared to the vehicle-treated control.
Figure 2.1: Q enhances rhTRAIL-induced apoptosis in breast cancer cells. Cells were treated with increasing concentrations of Q in the presence and absence of 100 ng/mL rhTRAIL for 72 hours. (A) For the representative FACS plots, the left top quadrants represent Annexin V+/PI⁻-dead cells, the right top quadrants represent Annexin V+/PI⁺-late apoptotic, dead cells, the left bottom quadrants represent Annexin V⁻/PI⁻-viable cells, and the right bottom quadrants represent Annexin V⁺/PI⁻-early apoptotic cells. Bar graphs (B) BT-20 and (C) MCF-7 display the average total percent of apoptotic cells (Annexin V⁺ cells) +/- SEM, and the average was calculated from three independent
experiments performed in triplicate (n=9). * represents $p<0.05$ relative to the vehicle-treated control. ** represents $p<0.01$ relative to the vehicle-treated control.

*Detection of the pathway of apoptosis*

Breast cancer cells were treated the same as the FACS analysis. The protein levels of caspase 8, cytosolic cytochrome c, caspase 3 (only for BT-20 cells because MCF-7 cells lack pro-caspase 3 expression), caspase 7, and cleaved PARP were all up-regulated with the co-treatment of Q and rhTRAIL while Bid expression was down-regulated when compared to the vehicle-treated controls and single agent treatments in both breast cancer cell lines (Figure 2.2). Densitometry results are illustrated in Figure 2.3. These results indicate that the extrinsic pathway was induced as marked by caspase 8 activation, executioner caspase 3 and/or caspase 7 activation, and PARP cleavage. In addition, Q alone did exhibit minimal pro-apoptotic effects on breast cancer cells as illustrated by PARP cleavage. Furthermore, cytochrome c was released from the mitochondria in both breast cancer cells treated with 50 μM Q demonstrating the initiation of the intrinsic pathway of apoptosis. Also, it should be noted that PARP fragmentation was not observed with single agent rhTRAIL treatment at 100 ng/mL for both breast cancer cell lines supporting data gathered from other researchers that breast cancer BT-20 and MCF-7 cells are TRAIL-resistant (41). In addition, TRAIL did not induce apoptosis in MCF-10A cells (42). These data together with the data from FACS shown in figure 2.1 strongly suggest that Q sensitized breast cancer cells to rhTRAIL-induced apoptosis through the induction of the extrinsic pathway of apoptosis.
Figure 2.2: Q and rhTRAIL induce the extrinsic pathway of apoptosis. Both breast cancer (A) BT-20 and (B) MCF-7 cells were sensitized to rhTRAIL-induced apoptosis via Q co-treatment as marked by the activation of caspase 8, the activation of executioner caspases 3 and 7, and the cleavage of PARP. β-actin was used as loading control and probed for each blot; the β-actins shown are representative results for each cell line.
BT-20

A

Ratio of Protein to β-actin

Apoptotic Protein

PARP
Cleaved PARP
Caspase 8
Cleaved Caspase 8
Bid
Cyt c
Caspase 3
Cleaved Caspase 3
Caspase 7
Cleaved Caspase 7

0 μM
12.5 μM
25 μM
50 μM
100 ng/mL TRAIL
12.5 μM and 100 ng/mL TRAIL
25 μM and 100 ng/mL
50 μM and 100 ng/mL
Figure 2.3: Densitometry results for apoptotic associated proteins. Densitometry results for immunoblots were calculated for breast cancer (A) BT-20 (B) MCF-7 cells using Image J software and graphs were generated applying GraphPad Prism.

Synergism of Q and rhTRAIL

The Chou-Talalay method was applied to calculate the combination index (CI) for the co-treatment of Q and rhTRAIL in both breast cancer cell lines (Figure 2.4). The CIs for both breast cancer cell lines were less than one indicating a synergic effect was observed and not an additive effect (CI=1) or an antagonistic effect (CI >1).
Combination indexes (CIs) were calculated and the co-treatment of Q and rhTRAIL displayed synergic effect (all CIs <1.0) in both breast cancer cell lines (A) BT-20 (B) MCF-7.

**Q induces the proteasome-mediated degradation of c-FLIP**

Considering that Q was shown to sensitize breast cancer to rhTRAIL-induced apoptosis through the extrinsic pathway by enhanced activation of caspase 8, Q’s impact on the expression of the caspase 8 inhibitor c-FLIP was assessed. Results gathered from western blot analysis demonstrate that Q down-regulated the expression of the long form of c-FLIP (c-FLIP\_L) in breast cancer in a dose-dependent manner after 72 hours (Figure 2.5A). Preliminary assays were performed with 24, 48, and 72 hour Q-treated breast cancer cells, and Q’s impact on c-FLIP\_L was not displayed until after 72 hours. At the highest concentration of Q used in our study (50 \mu M), c-FLIP\_L’s expression was down-regulated by about nine and three fold in comparison to the vehicle-treated control for breast cancer BT-20 and MCF-7 cells, respectively. In order to observe if Q-induced
down-regulation occurred through the proteasome-mediated degradation of c-FLIP<sub>L</sub>, a proteasome inhibitor MG132 was used. MG132 was used at a low concentration of 0.25 μM in presence and absence of 50 μM Q along with a vehicle-treated control. Inhibition of the proteasome resulted in the prevention of c-FLIP<sub>L</sub> down-regulation through Q treatment. In addition, c-FLIP<sub>L</sub> expression did not differ between the vehicle-treated control and breast cancer cells treated with only the proteasome inhibitor MG132 (Figure 2.5B). Furthermore, Co-IP was carried out probing for ubiquitin demonstrating that Q promoted the ubiquitination of c-FLIP<sub>L</sub> in breast cancer (Figures 2.5C and 2.5D). Hence, for breast cancer, Q enhances rhTRAIL-induced apoptosis through the proteasome-mediated degradation of c-FLIP<sub>L</sub> via increased ubiquitination.
Figure 2.5: Q promotes the proteasome-mediated degradation of c-FLIP<sub>L</sub>. Western blotting revealed that Q decreases c-FLIPL expression in (A) BT-20 and MCF-7 cells in a dose-dependent manner. (B) BT-20 and MCF-7 cells were treated with a proteasome inhibitor MG132 alone and in combination with Q for 72 hours, and the co-treatment of MG132 and Q recovered c-FLIP<sub>L</sub> protein expression in breast cancer. Co-IP was performed on (C) BT-20 and (D) MCF-7 cells treated in the presence and absence of 50 µM Q for 72 hours. Q enhanced the ubiquitination of c-FLIP<sub>L</sub> in breast cancer. Blots were also probed for c-FLIP<sub>L</sub> to confirm that the Co-IP was properly executed.

**Q’s impact on DR4 and DR5 expression**

Western blot and flow cytometry analyses were performed to discover Q’s effects on DR4 and DR5 protein and cell surface expression in breast cancer. DR5 protein expression was up-regulated in BT-20 cells with Q treatment dose-dependently only after 72 hours, but Q did not influence DR4 protein expression in BT-20 cells (Figure 2.6A).
DR5 and DR4 protein expressions did not change significantly in Q-treated MCF-7 cells (Figure 2.7A). Moreover, FACS analysis revealed that BT-20 cells express both receptors on the cell surface (Figures 2.6B-6D), and DR4 expression was not up-regulated significantly with Q ($p>0.05$) for all treatments when compared to control) while Q did induce the up-regulation of DR5 membrane expression. FACS analysis also showed that MCF-7 cells express both receptors on the cell surface (Figures 2.7B-2.7D). DR4 cell surface expression was not up-regulated significantly with single agent Q treatment ($p>0.05$ for all treatments when compared to control), but 50 μM Q slightly up-regulated DR5 membrane expression when compared to the vehicle-treated control. The results obtained from FACS analysis agree with the data derived from western blot analysis for both cell lines. Therefore, Q-induced DR5 up-regulation is an additional factor for the heightened rhTRAIL sensitivity observed in TNBC cells.
Figure 2.6: DR4 and DR5 expression in Q-treated breast cancer BT-20 cells. (A) DR4 and DR5 (mature form) protein levels were assessed after 72 hours of treatment; Q up-regulated DR5 expression in a dose-dependent manner. (B) DR5 and (C) DR4 cell surface expression levels for Q-treated BT-20 cells were analyzed by flow cytometry. For the representative histograms (B) and (C), vehicle-treated control=green, 12.5 µM Q=yellow, 25 µM Q=black, and 50 µM Q=blue. (D) The bar graphs represent the average fold increase in DR4 or DR5 cell surface expressions relative to the vehicle-treated control +/-SEM from three independent experiments performed in triplicate (n=9). * represents $p<0.05$ relative to the vehicle-treated control. ** represents $p<0.01$ relative to the vehicle-treated control.
Figure 2.7: DR4 and DR5 expression for Q-treated breast cancer MCF-7 cells. (A) DR4 and DR5 (mature form) protein levels were analyzed after 72 hours of treatment; Q slightly up-regulated DR5 protein expression. Cell surface expression of (B) DR5 and (C) DR4 for Q-treated breast cancer MCF-7 cells were evaluated. For the representative histograms (B) and (C) vehicle-treated control=green, 12.5 µM Q=yellow, 25 µM Q=black, and 50 µM Q=blue. (D) The bar graphs represent the fold increase in DR4 or DR5 cell surface expression relative to the vehicle-treated control+/−SEM from three independent experiments performed in triplicate (n=9). * represents $p<0.05$ relative to the vehicle-treated control. ** represents $p<0.01$ relative to vehicle-treated control.

Comparing DR4 and DR5 expression in breast cells
Western blot and flow cytometry analyses were performed to compare the expression of DR4 and DR5 in breast cancer BT-20 and MCF-7 cell lines to DR4 and DR5 expression in non-tumorigenic breast epithelial MCF-10A cells. MCF-10A had the highest DR4 and DR5 membrane and protein expression, and BT-20 cells had the lowest DR4 and DR5 protein and membrane expression levels (Figure 2.8). In addition, Q did not affect the expression of DR4 and DR5 in MCF-10A cells. Overall, the data demonstrates that Q specifically upregulates death receptors in malignant breast cancer cell lines only.
Figure 2.8: Comparing DR4 and DR5 expression among breast cancer cells and normal breast cells. (A) DR4 and DR5 (mature form) protein levels were compared among breast cancer cells (BT-20 and MCF-7) and non-tumorigenic breast epithelial cells (MCF-10A). (B) DR5 and (C) DR4 cell surface expression for cells were also evaluated. For the representative histograms (B) and (C), BT-20=blue, MCF-7=red, MCF-10A=green. (D) The bar graphs represent the DR4 or DR5 cell surface expression +/-SEM from three independent experiments performed in triplicate (n=9). (E) MCF-10A cells were treated with Q for 72 hours, and there was no change in DR4 and DR5 expression.
**Figure 2.9:** Densitometry results for DRs and c-FLIP<sub>L</sub>. Densitometry results for western blots for DR4, DR5, and c-FLIP<sub>L</sub> were calculated using Image J software and graphs were generated applying GraphPad Prism. (A) BT-20 (B) MCF-7 (C) BT-20, MCF-7, and MCF-10A (D) MCF-10A.
Q’s effects on DR5 and c-FLIP \textsubscript{L} expression at the transcriptional level

Q was shown to increase the expression of DR5 and decrease the expression of c-FLIP\textsubscript{L} in breast cancer, but it was unknown if Q acts at the transcriptional level to impact DR5 and c-FLIP\textsubscript{L}. Therefore, RT-PCR was executed applying β-actin as a positive control (Figure 2.10). Through RT-PCR analysis, we show that Q did not induce any change in c-FLIP\textsubscript{L} mRNA expression in breast cancer cells. The combined data suggest that Q-induced c-FLIP\textsubscript{L} down-regulation must occur at the post-translational level. Finally, Q did increase DR5 mRNA levels dose-dependently in TNBC BT-20 cells correlating with the increase in the protein expression observed through western blot and FACS analyses. Therefore, Q-induced DR5 up-regulation in TNBC occurs transcriptionally.

![Figure 2.10: Q increases DR5 mRNA expression. (A) BT-20 and (B) MCF-7 cells were treated with increasing concentrations of Q for 72 hours, and RT-PCR was executed with β-actin as a positive control. Q up-regulated DR5 mRNA expression in TNBC BT-20 dose-dependently, but Q did not impact c-FLIP\textsubscript{L} mRNA expression in breast cancer.](image-url)
2.5 Discussion

Breast cancer impacts women worldwide. Traditional chemotherapy and radiation treatments for breast cancer rely on p53 to induce the intrinsic pathway of apoptosis, but many cancers possess a non-functional p53 gene resulting in necrosis rather than apoptosis after chemotherapy and radiation producing adverse side effects in patients. rhTRAIL possesses the ability to induce apoptosis through the induction of the extrinsic pathway of apoptosis in cancer cells and induce apoptosis through the induction of the intrinsic pathway of apoptosis independent of p53 (7–10, 43, 44). rhTRAIL has been proposed to be applied as an anti-cancer therapeutic. However, clinical trials applying rhTRAIL as a potential anti-cancer therapeutic were terminated due to a lack of clinical efficacy, and in vitro studies had found that rhTRAIL treatment had limitations due to many cancer cell lines being resistant. This study focused on evaluating the potential of Q as a potent sensitizing agent for rhTRAIL-induced apoptosis in rhTRAIL-resistant TNBC BT-20 and hormone-dependent breast cancer MCF-7 cells; it should be noted that the combinatorial treatment of Q and rhTRAIL has not been examined before in the breast cancer cell lines of interest (28, 34, 35, 45). Through in vitro analysis, we show that Q possesses the capability to act as a sensitizing agent for rhTRAIL-resistant breast carcinoma.

In order to assess the interaction of Q and rhTRAIL, western blot analysis and Annexin V/PI assays were performed after 72 hours of treatment. The time course of 72 hours was chosen after preliminary experiments showed that Q’s impact on c-FLIP<sub>L</sub> and DR5 did not occur until after 72 hours. Other investigations have treated cells with Q at greater concentrations (100, 150, 175, and 200 µM) for 24 and 48 hours whereas, our
investigation applies at most 50 µM of Q (23, 34, 40, 46). Both assays confirmed that Q augments rhTRAIL-induced apoptosis in breast cancer BT-20 and MCF-7 cells via the execution of the extrinsic pathway enhanced the activation of caspase 8, the activation of executioner caspas 3 and 7, and the cleavage of PARP; furthermore, the results demonstrated that Q had the ability to promote apoptosis as a single agent, but rhTRAIL did not. In addition, the co-treatment of Q and rhTRAIL exhibited a synergic effect in breast BT-20 and MCF-7 cells. Therefore, Q’s mechanism of sensitization needed to be elucidated.

Previous studies have proposed that one reason cancer cell lines are resistant to rhTRAIL-induced apoptosis is through the up-regulation of the c-FLIP, a significant inhibitor of the extrinsic pathway (47). The c-FLIP_L is structurally similar to caspase 8, and at high expression levels, it has the ability to prevent caspase 8 activation when bound to the death-inducing signaling complex (DISC) and thus, suppressing the death receptor signaling pathway (47–49). Consequently, c-FLIP_L expression was evaluated and found to be significantly down-regulated in Q-treated breast cancer cells.

Considering Q-induced down-regulation of c-FLIP_L in breast cancer, the underlying mechanism for Q’s sensitization needed to be elucidated. Through RT-PCR analysis, Q was found to not impact the mRNA expression of c-FLIP_L in either breast cancer cell line. Analysis was then performed at the post-translational level through the treatment of both breast cancer cell lines with the 50 µM Q alone and in combination with the proteasome inhibitor MG132, and c-FLIP_L expression was assessed. We have established that Q promoted the ubiquitination of c-FLIP_L in breast cancer which has not been determined before in cancer cell lines. This is most likely the only pathway for c-
FLIP<sub>L</sub> degradation because if proteasome inhibitor applied at a greater concentration then a more pronounced effect of c-FLIP<sub>L</sub> recovery would be seen but the dead cells could be due to the high concentration of the inhibitor rather than Q. Thus, from these findings we can conclude that Q sensitizes TNBC and hormone-dependent breast cancer cells to rhTRAIL-induced apoptosis through the proteasome-mediated degradation of c-FLIP<sub>L</sub> because of enhanced ubiquitination.

Previous investigations have proposed that some rhTRAIL-resistant cancer cell lines express low levels DR4 and DR5 and thereby, making them less sensitive to rhTRAIL’s pro-apoptotic effects (28, 34). Q has been shown to up-regulate DR5 expression level in prostate and hepatoma cancer but not in breast cancer (28, 34). Hence, DR4 and DR5 protein expressions were examined by western blotting and DR4 and DR5 cell surface expressions were examined by FACS. The data proved that Q had the ability to significantly up-regulate DR5 expression in TNBC BT-20 cells, but Q had less of an effect on DR5 expression in hormone-dependent breast cancer MCF-7 cells. When comparing the expression levels of DRs, non-tumorigenic breast epithelial MCF-10A cells had the highest expression of DRs while TNBC BT-20 cells had the lowest expression of DRs. These results match another investigation comparing DR4 and DR5 protein expression in breast cancer MCF-7 cells and non-tumorigenic breast epithelial MCF-10A cells where MCF-10A cells expressed more DR4 and DR5 in comparison to MCF-7 cells (50). In addition, Q did not impact DR4 and DR5 protein expression in non-tumorigenic breast epithelial MCF-10A cells.

Since Q-induced DR5 up-regulation breast cancer, the underlying mechanism for this augmentation of rhTRAIL sensitivity needed to be elucidated. RT-PCR analysis
demonstrated that Q enhanced DR5 mRNA expression in a dose-dependent manner most significantly in TNBC BT-20 cells.

Overall, the data presented here demonstrate that Q is an effective sensitizing agent for rhTRAIL-resistant breast carcinoma. Through in vitro analysis, the co-treatment of Q and rhTRAIL proved efficacious for hormone-dependent breast cancer and TNBC. Our results suggest that the enhanced ubiquitination of c-FLIP by Q could be a novel mechanism underlying the down-regulation of c-FLIP, facilitating the enhanced rhTRAIL-sensitivity. Therefore, this co-treatment should be explored further in vivo in order to determine its clinical efficacy as a potential breast carcinoma therapeutic regimen especially for the more fatal TNBCs.

2.6 References


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CHAPTER III
SENSITIZATION OF RHTRAIL-RESISTANT TRIPLE NEGATIVE BREAST CARCINOMA THROUGH SILIBININ CO-TREATMENT

3.1 Abstract

Triple negative breast cancer (TNBC) is the most fatal form of breast cancer due to the shortcomings of therapies. However, recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) is a promising anti-cancer therapeutic that possesses the capability to promote the induction of apoptosis in cancer cells, but some TNBCs are resistant to rhTRAIL’s pro-apoptotic effects. Therefore, a combinatorial treatment approach with silibinin and rhTRAIL was considered in order to sensitize rhTRAIL-resistant TNBCs. The co-treatment of rhTRAIL and silibinin’s impact on apoptosis induction in rhTRAIL-resistant TNBC BT-20 and HCC1937 cells was inspected via the application of Annexin V/PI assays and western blot analysis. Silibinin possessed the ability to sensitize the examined rhTRAIL-resistant TNBC cells to rhTRAIL-induced apoptosis through the up-regulation of death receptors 4 and 5 and the down-regulation of survivin. These findings suggest that silibinin is a good sensitizing agent for rhTRAIL-resistant TNBCs.

3.2 Introduction
Triple negative breast cancers (TNBCs) are the most severe form of breast cancer carcinoma (1). TNBCs do not express estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2), so traditional hormone-targeted therapies for breast cancer cannot be applied. Developing and examining therapeutics against TNBCs is more problematic due to the absence of targeted molecular-oriented therapies resulting in worse clinical outcomes; hence, there is scientific interest in discovering and applying non-hormone related targeted therapies (1).

A potentially significant anti-cancer therapeutic is recombinant human tumor necrosis factor (TNF)-related apoptosis-inducing ligand (rhTRAIL), the optimized form of the endogenous death ligand TRAIL. rhTRAIL consists of the extracellular C-terminus of TRAIL amino acids 114-281 lacking exogenous sequence tags (2–6). The appealing aspect of rhTRAIL is that it possesses the capacity to induce apoptosis in cancer cells without producing a toxic impact on normal, non-transformed cells (2–6). The extrinsic pathway of apoptosis is induced when rhTRAIL interacts with the extracellular death receptors (DRs) 4 and 5 stimulating the trimerization of the receptors, and consequently leading to the activation of caspase 8, activation of the executioner caspases 3, 6, and 7, and the ultimate cleavage of poly (ADP-ribose) polymerase or PARP (hallmark of apoptosis) (7). Nonetheless, the pitfall of rhTRAIL treatment is apoptotic resistance detected in a majority of breast cancer cells due to the up-regulation of anti-apoptotic proteins such as survivin and the down-regulation of DRs. Some phase I clinical trials were completed with rhTRAIL, but further trials were terminated due to a limited therapeutic efficacy (2). In response, many investigations have concentrated on alternative combinatorial approaches to combat resistance by assessing the abilities of
various “mother-nature”-derived therapeutic agents to augment rhTRAIL-induced apoptosis in rhTRAIL-resistant cancers.

Silibinin is a flavonolignan derived from the seeds of Silybum marianum (milk thistle) (8, 9). Silibinin has been deemed safe for human use and been sold as a dietary supplement (9, 10). Silibinin has exhibited anti-cancer characteristics in various cancer cells including colon, lung, prostate, skin, hepatocellular, and breast cancer (11). In vitro, silibinin has been shown to promote apoptosis in hepatocellular, prostate, and breast cancers through the activation of caspase 8, subsequent activation of caspase 3, and the cleavage of PARP; in addition, silibinin-induced apoptosis occurred through the release of cytochrome c from the mitochondria facilitating caspase 9 activation, caspase 3 activation, and eventual PARP cleavage (8). Silibinin up-regulated DR4 and DR5 in colon cancer cells (8, 12, 13). Silibinin decreased survivin expression in laryngeal squamous cell cancer cells and glioma cancer cells; silibinin down-regulated c-FLIP expression in human glioma cells (14, 15). Silibinin decreased hepatocellular and lung carcinoma xenografts in vivo (16, 17). All of these are encouraging results that make silibinin a safe and promising candidate for co-treatment with rhTRAIL.

This investigation focused on evaluating the effectiveness of silibinin as a sensitizing agent for rhTRAIL-resistant TNBC BT-20 and HCC1937 cells (both with mutated p53 genes) and elucidating the underlying mechanism for silibinin’s sensitization. Through experimentation, we determined that silibinin retained the capability to decrease survivin expression and increase the expression of DRs in TNBC cells and thus, boosting rhTRAIL sensitivity in TNBC cells. Therefore, the presented evidence proposes that silibinin is an effective sensitizer for rhTRAIL-resistant TNBCs.
3.3 Methods

A. Drugs and chemicals

rhTRAIL was produced according to well defined and previously detailed protocols (3–5). Silibinin (molecular weight of 482.44 g/mol, Sigma) was dissolved in Polyethylene Glycol (PEG) molecular weight 400 (Fisher Scientific) at 10 mg/mL.

B. Cell culture

Human breast cancer BT-20 (ATCC HTB-19) cells were cultured in DMEM (Cleveland Clinic Cell Services Media Core) supplemented with 10% FBS (Gibco), 1% antibiotics-antimycotics (Gibco), 1% L-glutamine (Gibco), 1% non-essential amino acids (Gibco), and 1% sodium pyruvate (Gibco). Human breast cancer HCC1937 (ATCC CRL-2336, Manassas, VA, USA) cells were cultured in RPMI (Cleveland Clinic Cell Services Media Core) supplemented with 10% FBS (Gibco), 1% antibiotics-antimycotics (Gibco), and 1% L-glutamine (Gibco). Human non-tumorigenic breast epithelial MCF-10A (ATCC CRL-10317) cells were cultured in HuMEC ready medium (Gibco). Cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. Cells were treated with drugs 24 hours after plating, incubated with drugs for a further 72 hours, and collected for the various analyses detailed below.

C. Annexin V/PI assays-flow cytometry

Apoptosis was detected on the FACSCanto II after incubation with Annexin V-FITC and propidium iodide (PI) solution by following the procedure described by the manufacturer (Annexin V- FITC Apoptosis Detection Kit I, BD).
Analyses were completed by applying FACSDiva software and Flowing Software 2.

D. Western blot analysis

Cells were collected and total cell lysates were prepared according to previously published protocols (6). Aliquots of 35 μg of protein were prepared and separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels after denaturation with 4X Laemmli sample buffer (250 mM Tris-HCl, 8% SDS, 40% glycerol, 8% BME, and 0.02% Bromophenol Blue). After proteins were transferred to PVDF membranes (Millipore) via the semi-dry transfer method (Bio-Rad), the membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with a diluted primary antibody: anti-PARP, anti-caspase 3, anti-cleaved caspase 3, anti-caspase 7, anti-caspase 8, anti-cleaved caspase 8, anti-DR4, anti-DR5 or anti-survivin (Cell Signaling Technology). The membranes were washed and developed as described previously (6). 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. After incubation, the membrane was incubated with secondary anti-rabbit or mouse horseradish peroxidase (HRP)-conjugated antibodies (Bio-rad). 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 (Promega) to confirm equal protein loading.

**F. Flow cytometry analysis of DR4 and DR5 expression**

Cells were collected, prepared, and incubated with anti-DR4-PE, anti-DR5-PE or a mouse IgG1 kappa isotype control (eBioscience) according to previously published protocols (6); the isotype control was used to compensate for any non-specific binding. DR4 and DR5 membrane expressions were analyzed on a BD FACSCanto II flow cytometer applying FACSDiva software.

**G. Reverse Transcription-PCR analysis for DR4, DR5, and survivin**

Total RNA was extracted from cells using TRIzol reagent (Ambion). Reverse transcription polymerase chain reaction (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’-GGGAGCCGCTCATGAGGAAGTT-GG-3’ and the reverse primer 5’-GGC-AAGTCTCTCTCCCAGGTCTC-3’. Human DR4 mRNA was amplified using the forward primer 5’-
GAGCAACGCAGACTCGCT -3’ and the reverse primer 5’-TCACT-CCAAGGACACGGC-3’. For survivin, forward primer 5’-CAAGGACCACCGCATCT-CTAC-3’ and the reverse primer 5’-AGTCTGGCTCGTTCTCAGTG -3’ were used. For β-actin, the forward primer 5’-TGACGGGGGTCAACCCACACTGTGCC-3’ and the reverse primer 5’-CTGCATCCTGTCGCAATGCCAG-3’ were used. cDNA synthesis was performed at 60ºC for 30 minutes using the Applied Biosystems GeneAmp PCR System 9700. The PCR cycling conditions (40 cycles) were chosen as follows: denature for 2 min at 94ºC, anneal for 30 sec at 55ºC for survivin and DR4 and 65ºC for DR5 and β-actin, extend for 1 min and 30 sec at 68ºC, and execute a final extension for 10 min at 68ºC. Reaction products were analyzed on 1.2% agarose gels. The bands were visualized by ethidium bromide (Invitrogen) and an UV illuminator (UVP).

H. Statistical analysis

Data were analyzed using the Student’s t-test, and the differences between experimental and control groups were deemed statistically significant at \( p \) values less than 0.05.

3.4 Results

_Evaluating rhTRAIL-induced apoptosis through FACS analysis_

FACS analysis was conducted on TNBC cells treated with 25 or 50 µM silibinin in the presence or absence of 100 ng/mL rhTRAIL for 72 hours to determine silibinin’s sensitizing effects on rhTRAIL-induced apoptosis (Figure 3.1). Silibinin augmented rhTRAIL-induced apoptosis in both TNBC cell lines. For example, TNBC BT-20 and
HCC1937 cells treated with 50 µM of silibinin generated on average about 15% and 10% apoptotic cells, respectively ($p<0.05$); yet, the co-treatment of 50 µM Q and 100 ng/mL rhTRAIL on BT-20 and HCC1937 cells generated on average about 25% apoptotic cells ($p<0.05$). In addition, it should be pointed out that the single agent treatment of 100 ng/mL rhTRAIL did not generate a significant amount of apoptotic TNBC cells in comparison to the vehicle-treated control ($p>0.05$).

Figure 3.1: Silibinin augments rhTRAIL-induced apoptosis in breast cancer cells. Cells were treated with silibinin in the presence and absence of 100 ng/mL rhTRAIL for 72 hours. (A) For the representative FACS plots, the left top quadrants represent Annexin V⁻/PI⁻-dead cells, the right top quadrants represent Annexin V⁺/PI⁺-late apoptotic, dead cells, the left bottom quadrants represent Annexin V⁺/PI⁻-viable cells, and the right
bottom quadrants represent Annexin V+/PI-early apoptotic cells. Bar graphs (B) BT-20 and (C) HCC1937 display the average total percent of apoptotic cells (Annexin V+ cells) +/-SEM calculated from three independent experiments carried out in triplicate (n=9). * represents p<0.05 relative to the vehicle-treated control. ** represents p<0.01 relative to vehicle-treated control.

Observing the induction of the pathway of apoptosis

TNBC cells were treated with the single agents of silibinin (25 and 50 µM) and rhTRAIL (100 ng/mL) along with the co-treatments for 72 hours. The expression of proteins caspase 8, caspase 3, caspase 7, and cleaved PARP were all assessed in the treated cells, and the co-treatment of silibinin and rhTRAIL enhanced the expression of the examined apoptotic proteins in comparison to the vehicle-treated controls and single agent treatments (Figure 3.2). The data confirms that apoptosis was induced by the co-treatment through the extrinsic pathway as indicated by the activation of caspase 8, the activation of executioner caspases 3 and 7, and the cleavage of PARP. Furthermore, it should be recognized that PARP cleavage was not detected in rhTRAIL-treated TNBC cells; this aligns with other researchers’ results that TNBC BT-20 and HCC1937 cells are rhTRAIL-resistant (18). Moreover, silibinin did not influence proliferation of non-tumorigenic breast epithelial MCF-10A cells (19). In addition, TRAIL did not induce apoptosis in MCF-10A cells (20). This evidence supports the notion that the co-treatment of rhTRAIL and silibinin will impact breast cancer cells not normal, non-tumorigenic breast cells. Overall, these findings (Figure 3.2) in conjunction with the FACS analysis (Figure 3.1) indicate that silibinin bolsters the sensitization of rhTRAIL-resistant TNBC
cells to rhTRAIL-induced apoptosis via the stimulation of the extrinsic pathway of apoptosis.

Figure 3.2: Silibinin and rhTRAIL trigger the induction of apoptosis. TNBC (A) BT-20 and (B) HCC1937 cells were sensitized to rhTRAIL-induced apoptosis through silibinin co-treatment as indicated by activation of caspase 8, activation of executioner caspases 3 and 7, and PARP cleavage. β-actin was used as loading control and probed for each blot; the β-actins shown are representative results for each cell line.
Figure 3.3: Densitometry results for apoptotic associated proteins. Densitometry results for immunoblots were calculated for breast cancer (A) BT-20 (B) HCC1937 cells using Image J software and graphs were generated applying GraphPad Prism.
Synergism of silibinin and rhTRAIL

The Chou-Talalay method was applied to calculate the combination index (CI) for the co-treatment of silibinin and rhTRAIL in both TNBC cell lines (Figure 3.4). The CIs for both breast cancer cell lines were less than one indicating a synergic effect was observed and not an additive effect (CI=1) or an antagonistic effect (CI >1).

Figure 3.4: Combination indexes for the co-treatment of silibinin and rhTRAIL.

Combination indexes (CIs) were calculated and the co-treatment of silibinin and rhTRAIL displayed synergic effect (all CIs <1.0) in both breast cancer cell lines (A) BT-20 (B) HCC1937.

Silibinin’s effects on DR4 and DR5 expression

Western blot and flow cytometry analyses were conducted to ascertain silibinin’s effect on DR4 and DR5 protein and cell surface expression in TNBCs. Silibinin promoted the up-regulation of the protein expression of DR5 in TNBC cells in a dose-dependent
manner after 72 hours of treatment (Figure 3.5); in addition, silibinin significantly enhanced the protein expression of DR4 in HCC1937 ($p<0.05$) but not in BT-20 cells. The FACS analysis of the cell surface expressions of DR4 and DR5 in TNBC cells treated with silibinin were in alignment with the increased protein expression of DR5 in TNBC cells and the increased protein expression of DR4 in only TNBC HCC1937 cells. Silibinin did not affect the expression of DRs in non-tumorigenic breast epithelial MCF-10A cells after 72 hours of treatment (Figure 3.5E). Thus, silibinin induces DR5 up-regulation in TNBC cells and DR4 up-regulation in TNBC HCC1937 cells; the up-regulation of DRs by silibinin improves rhTRAIL sensitivity in these TNBC cells.
Figure 3.5: Expression of DRs in silibinin-treated TNBC cells. (A) DR4 and DR5 (mature form) protein levels were assessed in BT-20 cells; silibinin increased the expression of DR5 in BT-20 cells in a dose-dependent manner after 72 hours of treatment. (B) DR4 and DR5 cell surface expression levels for silibinin-treated BT-20 cells were analyzed by flow cytometry; the bar graphs represent the average fold increase in DR4 or DR5 cell surface expressions relative to the vehicle-treated control +/-SEM from three independent experiments performed in triplicate (n=9). * represents $p<0.05$ relative to the vehicle-treated control. ** represents $p<0.01$ relative to vehicle-treated control. (C) DR4 and DR5 (mature form) protein levels were analyzed in HCC1937 cells; silibinin increased the expression of DR4 and DR5 in HCC1937 cells. Cell surface
expression of (D) DR4 and DR5 for silibinin-treated breast cancer HCC1937 were assessed by flow cytometry. (E) DR4 and DR5 (mature form) protein levels were assessed for MCF-10A cells treated with silibinin, and DR4 and DR5 expressions were not significantly altered.

Silibinin’s influence on survivin expression

Survivin is a member of the inhibitor of apoptosis (IAP) gene family; it is a short-lived protein that is overexpressed in cancer cells especially breast cancer (21, 22). Survivin acts as an inhibitor of cell death by preventing the proper execution of the extrinsic and intrinsic pathways of apoptosis, but the mechanism of this inhibition has not been fully elucidated (21, 22). Through western blot analysis, silibinin down-regulated survivin expression in a dose-dependent manner in TNBC cells and thereby, facilitating enhanced rhTRAIL sensitivity via the full execution of the extrinsic pathway of apoptosis (Figure 3.7A).
Figure 3.6: Densitometry results for DRs and survivin. Densitometry results for western blots for DR4, DR5, and survivin were calculated using Image J software and graphs were generated applying GraphPad Prism. (A) BT-20 (B) HCC1937.

Silibinin’s effect on DRs and survivin mRNA expressions

Through analyses, silibinin demonstrated the ability to enhance the expression of DR5 in TNBC cells, enhance the expression of DR4 in TNBC HCC1937 cells, and diminish the expression of survivin in TNBC, so the next step was to assess if silibinin effects the expression of those apoptotic proteins transcriptionally. Thus, RT-PCR was carried out and β-actin was applied as a positive control (Figure 3.7B). The RT-PCR analyses revealed that silibinin did decrease survivin mRNA expression in both TNBC cells. In addition, silibinin did increase the expression of DR4 mRNA in HCC1937 cells but not in BT-20 cells; also, silibinin did increase the expression of DR5 mRNA in both TNBC cells. These results provide the mechanism of action by which silibinin influences...
rhTRAIL sensitivity in rhTRAIL-resistant TNBCs by effecting the mRNA expressions of DRs and survivin.

**Figure 3.7: Silibinin’s impact on mRNA expression of DRs and survivin. (A)**

Western blotting detected that silibinin reduces the expression of the anti-apoptotic protein survivin in TNBC cells in a dose-dependent manner. (B) BT-20 and HCC1937 cells were treated with single agent silibinin for 72 hours and RT-PCR was completed with β-actin as a positive control. Silibinin down-regulated survivin mRNA expression in TNBC cells, up-regulated DR5 mRNA expression in TNBC cells, and up-regulated DR4 mRNA expression in HCC1937 cells.

### 3.5 Discussion

TNBC is a deadly form of breast carcinoma. rhTRAIL induces apoptosis through the extrinsic pathway and through the intrinsic pathway independent of p53 in cancer cells. On the other hand, traditional chemotherapy and radiation treatments are dependent on p53 for the full execution of the intrinsic pathway of apoptosis (2–5, 23, 24). rhTRAIL
is a more favorable therapeutic agent because many cancers have a non-functional TP53 gene that leads to necrosis over apoptosis, so if chemotherapy and radiation are applied, then negative side effects could be observed in patients. Despite this, there are factors that are implicated in resistance to rhTRAIL that lead to clinical trials being terminated due to the absence of clinical efficacy (25–28). Our study sought to combat rhTRAIL resistance in TNBC BT-20 and HCC1937 cells through the application of the “mother-nature”-derived silibinin as a sensitizing agent. *In vitro* examination, revealed that silibinin is an effective sensitizer for the rhTRAIL-resistant TNBC BT-20 and HCC1937 cells.

Western blot analysis and Annexin V/PI assays were executed in order to evaluate the consequences of the co-treatment of silibinin and rhTRAIL. Results gathered from both assays concluded that silibinin enhances rhTRAIL-induced apoptosis in TNBC BT-20 and HCC1937 cells through the completion of the extrinsic pathway as presented by the augmented caspase 8 activation, executioner caspases 3 and 7 activation, and PARP cleavage (a hallmark of apoptosis). Consequently, the mechanism for silibinin’s sensitization needed to be established.

Some rhTRAIL-resistant cancer cell lines have displayed low expression levels of DR4 and DR5, and some studies have postulated that this lack of expression of DRs hinders rhTRAIL from inducing apoptosis in those cancer cell lines (25, 28). Accordingly, this study inspected the protein and cell surface expressions of DR4 and DR5 in silibinin-treated TNBC cells *via* western blot and FACS analyses. From these analyses, we determined that silibinin induced the up-regulation of DR5 in both TNBC cells and the up-regulation of DR4 in TNBC HCC1937 cells. In order to confirm that silibinin only affects DR4 and DR5 expression in cancer cell lines, we checked the
expression of DRs in silibinin-treated non-tumorigenic breast epithelial MCF-10A cells and affirmed that silibinin did not impact the expression of either DR in those non-tumorigenic breast epithelial cells.

Additionally, silibinin’s influence on the expression of the IAP survivin was probed for because survivin is expressed at high levels in breast cancer cells and interferes in the proper execution of the extrinsic pathway of apoptosis (21, 22). We discovered that silibinin significantly down-regulated the expression of survivin in both TNBC cells and thus aiding in intensifying rhTRAIL sensitivity in the examined TNBC cell lines. The underlying mechanism for this decrease in expression was then assessed through RT-PCR analysis.

Elucidation of silibinin’s mechanism of sensitization was carried out through RT-PCR analysis of DR4, DR5, and survivin. Silibinin increased DR5 mRNA expression in both TNBC cell lines, silibinin increased DR4 mRNA expression in HCC1937, and silibinin decreased survivin mRNA expression in both TNBC cell lines.

The aim of this study was to optimize the therapeutic efficacy of rhTRAIL through the application of the “mother-natured”-derived compound silibinin. Our findings indicate that silibinin was effective in overcoming the challenge of rhTRAIL-resistance in TNBC BT-20 and HCC1937 cells through the up-regulation of DRs and down-regulation of surviving transcriptionally and thereby, boosting rhTRAIL sensitivity in TNBC cells. In conclusion, silibinin is a potent sensitizing agent for rhTRAIL-resistant.
3.6 References


CHAPTER IV

SENSITIZATION OF RHTRAIL-RESISTANT TRIPLE NEGATIVE BREAST CARCINOMA THROUGH URSOLIC ACID CO-TREATMENT

4.1 Abstract

Triple negative breast cancer (TNBC) is the most deadly form of breast cancer due to a lack of efficacious therapies available. There is an encouraging anti-cancer therapeutic recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) with the capacity to induce apoptosis in cancer cells, but there are TNBCs that are not susceptible to rhTRAIL’s effects on apoptosis induction. A co-treatment method to sensitize rhTRAIL-resistant TNBCs with the “mother nature”-derived Ursolic acid (UA) and rhTRAIL was assessed. Annexin V/PI assays and western blot analysis were executed for rhTRAIL and UA treated rhTRAIL-resistant TNBC BT-20 and HCC1937 cells to evaluate apoptosis induction. Through the up-regulation of death receptors 4 and 5 and through the down-regulation of c-FLIP$L$, UA stimulates the sensitization of the rhTRAIL-resistant TNBC cells to rhTRAIL-induced apoptosis. The data presented here suggests that UA is a potent sensitizing agent for rhTRAIL-resistant TNBCs.

4.2 Introduction
Triple negative breast cancers (TNBCs) are the most severe type of breast carcinoma often resulting in poor prognosis (1). This highlights the priority for the development and application of multi-targeted and molecular-oriented therapies that are more effective and safe in these troublesome TNBCs in comparison to traditional chemotherapy and radiation therapy. TNBCs are regarded as hormone-independent because these breast cancers do not express estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2), so TNBCs are more aggressive and complicated to treat with common anti-breast cancer therapies such as hormone-targeted therapies. Therefore, there is scientific importance to produce and employ hormone-independent targeted therapies (1).

A compelling, well-studied anti-cancer therapeutic is recombinant human tumor necrosis factor (TNF)-related apoptosis-inducing ligand (rhTRAIL), the optimized form of the endogenous death ligand TRAIL. rhTRAIL is comprised of the extracellular C-terminus of TRAIL amino acids 114-281 without exogenous sequence tags (1–5). rhTRAIL maintains the capability to induce apoptosis (an area of interest in cancer research) in a variety of cancer cells and not toxically burdening normal, non-transformed cells (1–5). rhTRAIL prompts the extrinsic pathway of apoptosis by triggering the trimerization of death receptors (DRs) 4 and 5 facilitating the activation of caspase 8, the activation of the executioner caspases 3, 6, and 7, and the pivotal cleavage of poly (ADP-ribose) polymerase or PARP (hallmark of apoptosis) (6). However, several studies have provided evidence that the response to rhTRAIL treatment is variable in a majority of breast cancer cells as a result of the up-regulation of anti-apoptotic proteins such as c-FLIP<sub>L</sub> and the down-regulation of DRs. Some phase I clinical trials were carried out with
rhTRAIL, but further trials were discontinued due to inadequate therapeutic efficacy (1). Consequently, many researchers have focused on developing and implementing new therapeutic strategies with different combinatorial approaches to overcome apoptotic resistance by ascertaining the capacity of different “mother nature”-derived therapeutic agents to enhance rhTRAIL-induced apoptosis in rhTRAIL-resistant cancers (7, 8).

In our previous studies, we have demonstrated the ability of “mother nature”-derived therapeutic compounds Quercetin (Q) and silibinin to sensitize rhTRAIL-resistant cancers to rhTRAIL-induced apoptosis (7–9). Q enhanced DR4 and DR5 expression in rhTRAIL-resistant malignant melanoma, and Q increased DR5 expression in rhTRAIL-resistant TNBC. Q promoted the degradation of the long form of c-FLIP (c-FLIPL) in rhTRAIL-resistant malignant melanoma and breast carcinoma. Silibinin boosted DR4 and DR5 expression and simulated the down-regulation of survivin in rhTRAIL-resistant TNBCs. Thus, this evidence supports the notion to further examine other combinatorial approaches with “mother nature”-derived compounds and rhTRAIL to treat rhTRAIL-resistant cancers.

One potential therapeutic agent of interest is Ursolic acid (UA) which is a pentacyclic triterpenoid derived from the leaves and berries of various plants along with the coatings of fruits (10–12). UA has been determined to be safe for human use and has been sold as a dietary supplement (10, 13). UA has exhibited anti-cancer characteristics in a diverse array of cancer cells including colon, lung, prostate, ovarian, hepatocellular, and breast (12–15). In vitro, UA has shown the capacity to promote apoptosis in hepatocellular, prostate, and breast cancers through the proper execution of both the extrinsic pathway and intrinsic pathway ending in PARP cleavage (characteristic hallmark of apoptosis) (11, 13,
16, 17). UA’s mechanism of apoptosis induction has been elucidated in prostate, gastric, and colorectal cancers through the up-regulation of DR5 and the down-regulation of Bcl-2 and survivin (13, 14, 17, 18). In vivo, UA decreased the growth of prostate and colorectal xenografts (12, 14). Previous studies have shown that UA does not impact non-tumorigenic, breast epithelial MCF-10A cell growth (19). Thus, the “mother nature”-derived UA warrants in-depth investigation as a potential sensitizing agent for rhTRAIL-resistant TNBCs.

The sensitization of rhTRAIL-resistant TNBC BT-20 and HCC1937 cells (both with mutated p53 genes) was observed when co-treated with UA. UA promoted the up-regulation of DRs along with the down-regulation of c-FLIPL facilitating the induction of the extrinsic pathway of apoptosis. Therefore, this accumulating mechanistic evidence implicates UA as an efficacious sensitizing agent for rhTRAIL-resistant TNBCs.

4.3 Methods

A. Drugs and Chemicals

rhTRAIL was produced according to well defined and previously detailed protocols (3–5). UA (molecular weight of 456.7g/mol) was dissolved in Polyethylene Glycol (PEG) molecular weight 400 (Fisher Scientific) at 10 mg/mL.

B. Cell Culture

Human breast cancer BT-20 (ATCC HTB-19) cells were cultured in DMEM (Cleveland Clinic Cell Services Media Core) supplemented with 10% FBS (Gibco), 1% antibiotics-antimycotics (Gibco), 1% L-glutamine (Gibco), 1% non-essential amino acids (Gibco), and 1% sodium pyruvate (Gibco). Human breast cancer HCC1937 (ATCC CRL-2336) cells were cultured in RPMI
supplemented with 10% FBS (Gibco), 1% antibiotics-antimycotics (Gibco), and 1% L-glutamine (Gibco). Human non-tumorigenic breast epithelial MCF-10A (ATCC CRL-10317) cells were cultured in HuMEC ready medium (Gibco). Cells were cultivated in a humidified atmosphere with 5% CO₂ at 37°C. Cells were treated with drugs 24 hours after plating, incubated with drugs for additional 72 hours, and collected for the different assays described below.

C. **Annexin V/PI assays-flow cytometry**

Apoptosis was detected on the FACSCanto II after incubation with Annexin V-FITC and propidium iodide (PI) solution by abiding by the procedure detailed by the manufacturer (Annexin V- FITC Apoptosis Detection Kit I, BD). Assays were completed by employing FACSDiva software and Flowing Software 2.

D. **Assessing apoptotic associated protein levels**

Cells were harvested and total cell lysates were prepared according to previously published protocols (7, 8). Aliquots of 35 μg of protein were prepared and separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels after denaturation with 4X Laemmli sample buffer (250 mM Tris-HCl, 8% SDS, 40% glycerol, 8% BME, and 0.02% Bromophenol Blue). After proteins were transferred to PVDF membranes (Millipore) via the semi-dry transfer method (Bio-Rad), the membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with a diluted primary antibody: anti-PARP, anti-caspase 3, anti-cleaved caspase 3, anti-caspase 8, anti-cleaved caspase 8 (Cell Signaling Technology), or anti-FLIP (Enzo Life Sciences). The membranes were washed and developed as outlined previously (7, 8).
E. **Western blot analysis of DR4 and DR5 expression**

Cells were harvested and prepared according to previously published protocols (7, 8). PVDF membranes were blocked, incubated overnight at 4°C with anti-DR4 or anti-DR5 (Cell Signaling Technology), washed, and developed as above.

F. **Flow cytometry analysis of DR4 and DR5 expression**

Cells were collected, prepared, and incubated with anti-DR4-PE, anti-DR5-PE or a mouse IgG1 kappa isotype control (eBioscience) according to previously published protocols (7, 8); the isotype control was used to compensate for any non-specific binding. DR4 and DR5 membrane expressions were evaluated on a BD FACSCanto II flow cytometer implementing FACSDiva software.

G. **Reverse Transcription-PCR analysis for DR4, DR5, and c-FLIP\(_L\)**

Total RNA was extracted from cells using TRIzol reagent (Ambion, Pittsburgh, PA, USA). Reverse transcription polymerase chain reaction (RT-PCR) was executed according to 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’-GGGAGGCGGCT-CATGAGGAAGTTGG-3’ and the reverse primer 5’-GGCAAGGTCTCTCTCTC-CCAGGCTTC-3’. Human DR4 mRNA was amplified using the forward primer 5’- GAGCAAC-GCAGACTCGCT -3’ and the reverse primer 5’- TCACTCAAAGGACAGGGC -3’. For c-FLIP\(_L\) mRNA, forward primer 5’-CTTGGCCAATTTGCCTGTAT-3’ and the reverse primer 5’-CCCA-TGAACATCTCCTCCTGAT-3’ were used. For β-actin, the forward primer 5’-TGACGCGTCA-CCCACACTGTGCC-3’ and the reverse primer 5’-CTGCATCCTGTCGGAATGCCAG-3’ were used. cDNA synthesis was performed at
60°C for 30 minutes using the Applied Biosystems GeneAmp PCR System 9700. The PCR cycling conditions (40 cycles) were chosen as follows: denature for 2 min at 94°C, anneal for 30 sec at 55°C for c-FLIP_L and DR4 and 65°C for DR5 and β-actin, extend for 1 min and 30 sec at 68°C, and execute a final extension for 10 min at 68°C. Reaction products were assessed on 1.2% agarose gels. The bands were visualized by ethidium bromide (Invitrogen) and an UV illuminator (UVP).

H. Statistical analysis

Data were scrutinized utilizing the Student’s t-test, and the differences between experimental and control groups were deemed statistically significant at $p$ values less than 0.05.

4.4 Results

*Detecting rhTRAIL-induced apoptosis through FACS analysis*

In order to determine the rhTRAIL-inducing apoptosis abilities, FACS analysis was carried out on TNBC cells treated with 25 or 50 μM UA in the presence or absence of 100 ng/mL rhTRAIL for 72 hours to assess UA’s contribution to promoting rhTRAIL-induced apoptosis (Figure 4.1). UA potentiated rhTRAIL-induced apoptosis in both TNBC cell lines. For instance, TNBC BT-20 and HCC1937 cells were treated with 50 μM of UA to yield on average about 20% and 15% apoptotic cells, respectively ($p <0.05$); meanwhile, the combined treatment of 50 μM UA and 100 ng/mL rhTRAIL on BT-20 and HCC1937 cells produced on average about 30% apoptotic cells ($p <0.05$). Moreover, the single agent treatment of 100 ng/mL rhTRAIL did not yield a significant amount of apoptotic TNBC cells in comparison to the vehicle-treated control ($p >0.05$).
Figure 4.1: UA enhances rhTRAIL-induced apoptosis in TNBCs. TNBC cells were treated with UA with and without 100 ng/mL rhTRAIL for a time course of 72 hours. (A) For the displayed FACS plots, the left top quadrants represent Annexin V+/PI- dead cells, the right top quadrants represent Annexin V+/PI+ late apoptotic, dead cells, the left bottom quadrants represent Annexin V-/PI+-viable cells, and the right bottom quadrants represent Annexin V+/PI- early apoptotic cells. Bar graphs (B) BT-20 and (C) HCC1937 illustrate the average total percent of apoptotic cells (Annexin V+ cells) +/-SEM calculated from three independent experiments performed in triplicate (n=9). * represents $p<0.05$ relative to vehicle-treated control. ** represents $p<0.01$ relative to vehicle-treated control.

Examining the induction of the pathway of apoptosis
The execution of the caspase cascade is crucial for the adequate apoptotic signal transduction, so the pathway of apoptosis was investigated through western blot analysis by checking the expression levels of apoptotic associated proteins. The examined TNBC cells were treated with UA (25 and 50 µM) and rhTRAIL (100 ng/mL) alone and in combination for 72 hours. For these treated cells, the levels of expression for various apoptotic proteins caspase 8, caspase 3, and cleaved PARP were ascertained; the combination treatments of UA and rhTRAIL augmented the protein expression of the evaluated apoptotic proteins when compared the vehicle-treated controls and single agent treatments (Figure 4.2). These findings affirm that the co-treatment of UA and rhTRAIL induced apoptosis through the execution of the extrinsic pathway as illustrated by caspase 8 activation, caspase 3 activation, and PARP cleavage. Moreover, it is noteworthy that PARP cleavage was not observed in rhTRAIL-treated TNBC cells in agreement with other researchers’ evidence that TNBC BT-20 and HCC1937 cells are rhTRAIL-resistant (20). Also, both single agents UA and TRAIL did not impact non-tumorigenic breast epithelial MCF-10A cell proliferation which maintains the proposal that the co-treatment of UA and rhTRAIL influences breast cancer cells not normal, non-tumorigenic breast cells (21, 22). By taking the data complied from western blot analyses (Figure 4.2) in association with the data compiled from FACS analyses (Figure 4.1), UA demonstrates the capability to intensify rhTRAIL-induced apoptosis in rhTRAIL-resistant TNBC cells through the promotion of the completion of the extrinsic pathway of apoptosis.
Figure 4.2: UA and rhTRAIL facilitates the execution of apoptosis. TNBC (A) BT-20 and (B) HCC1937 cells were sensitized to rhTRAIL-induced apoptosis with UA co-treatment as demonstrated by activation of caspase 8, activation of executioner caspase 3, and PARP cleavage. β-actin was used as loading control and probed for each blot; the β-actins shown are representative results for each cell line.
A

BT-20

Ratio of Protein to β-actin

Apoptotic Protein

0 μM
25 μM
50 μM
100 ng/mL TRAIL
25 μM and 100 ng/mL
50 μM and 100 ng/mL

PARP
Cleaved PARP
Caspase 8
Cleaved Caspase 8
Caspase 3
Cleaved Caspase 3
Figure 4.3: Densitometry results for apoptotic associated proteins. Densitometry results for immunoblots were calculated for breast cancer (A) BT-20 (B) HCC1937 cells using Image J software and graphs were generated applying GraphPad Prism.

**Synergism of Q and rhTRAIL**

The Chou-Talalay method was applied to calculate the combination index (CI) for the co-treatment of Q and rhTRAIL in both breast cancer cell lines (Figure 4.4). The CIs for both breast cancer cell lines were less than one indicating a synergic effect was observed and not an additive effect (CI=1) or an antagonistic effect (CI >1).
Figure 4.4: Combination indexes for the co-treatment of UA and rhTRAIL.

Combination indexes (CIs) were calculated and the co-treatment of UA and rhTRAIL displayed synergic effect (all CIs <1.0) in both breast cancer cell lines (A) BT-20 (B) HCC1937.

UA’s influence on DR4 and DR5 expression

Western blot and flow cytometry analyses were carried out to identify UA’s effect on DR4 and DR5 protein and cell surface expression in TNBCs. The time period of 72 hours was selected following the completion of preliminary experiments that determined UA’s influence on c-FLIP_L and DRs was not displayed until after a 72 hour time period. UA significantly increased the protein expression of DR5 in TNBC cells in a dose-dependent manner following 72 hours of treatment \( (p<0.05) \); in addition, UA significantly enhanced the protein expression of DR4 in TNBC cells \( (p<0.05) \) (Figures 4.5A and 4.5B). Cell surface expressions of DR4 and DR5 in TNBC cells treated with
UA were assessed via FACS analyses (Figures 4.5D and 4.5E) and were in agreement with the heightened protein expression of DRs in TNBC cells. UA did not influence the expression of DRs in non-tumorigenic breast epithelial MCF-10A cells following 72 hours of treatment (Figure 4.5C). Thus, UA stimulates DR4 and DR5 up-regulation in TNBC cells; the enhanced expression of DRs further verifies the role of UA to boost rhTRAIL sensitivity in these TNBC cells.
Figure 4.5: **DR4 and DR5 expression in UA treated cells.** DR4 and DR5 (mature form) protein levels were assessed in (A) BT-20 cells and (B) HCC1937; UA boosted DR4 and DR5 expression in TNBCs in a dose-dependent manner following 72 hours of treatment. (C) DR4 and DR5 (mature form) protein levels were assessed for MCF-10A cells treated with UA, and DR4 and DR5 expressions were not significantly altered. DR4 and DR5 cell surface expression levels for UA-treated (D) BT-20 and (E) HCC1937 cells were analyzed by flow cytometry; the bar graphs represent the average fold increase in DR4 or DR5 cell surface expressions relative to the vehicle-treated control +/-SEM from three independent experiments performed in triplicate (n=9). * represents $p<0.05$ relative to vehicle-treated control. ** represents $p<0.01$ to vehicle-treated control.
Figure 4.6: Densitometry results for DRs and c-FLIP_L. Densitometry results for western blots for DR4, DR5, and c-FLIP_L were calculated using Image J software and graphs were generated applying GraphPad Prism. (A) BT-20 (B) HCC1937.
UA’s effects on c-FLIP<sub>L</sub> expression

On the basis of the results demonstrating UA’s capability to sensitize breast cancer to rhTRAIL-induced apoptosis through the extrinsic pathway by elevated expression of caspase 8, UA’s effect on the expression of the caspase 8 inhibitor c-FLIP was checked. Findings obtained from western blot analysis establish that UA diminished the expression of the long form of c-FLIP (c-FLIP<sub>L</sub>) in a dose-dependent manner after 72 hours in TNBC cells (Figure 4.7A).

UA’s impact on DRs and c-FLIP<sub>L</sub> mRNA expressions

RT-PCR analyses were performed to examine the potential transcriptional effects on DR4, DR5, c-FLIP<sub>L</sub> with β-actin was applied as a positive control. The results show that DR4 and DR5 mRNA expressions were increased in UA-treated TNBC cells (Figure 4.7B). In addition, c-FLIP<sub>L</sub> mRNA expression was decreased in TNBC cells.
Figure 4.7: UA’s impact on mRNA expression of DRs and c-FLIP\textsubscript{L}. (A) UA was detected to decrease the expression of c-FLIP\textsubscript{L} in TNBC cells in a dose-dependent manner via western blot analysis. (B) BT-20 and HCC1937 cells were treated with single agent UA for 72 hours and RT-PCR was executed with β-actin as a positive control. UA diminished c-FLIP\textsubscript{L} mRNA expression in TNBC cells and boosted DR4 and DR5 mRNA expression in TNBC cells.

4.5 Discussion

There is a high prevalence of breast cancer among commonly diagnosed cancers in women, so the development of multi-targeted and effective therapies are essential to deter poor prognosis especially in TNBCs. Apoptosis is widely accepted as an ideal mechanism for reduction of breast cancers. Chemotherapy and radiation are the conventional treatment methods that are administered to treat breast cancer through apoptosis induction, but the long-term application of these treatments usually worsen
patients’ conditions. Chemotherapy and radiation require a functional, non-mutated p53 gene for the absolute activation of the intrinsic pathway of apoptosis. On the contrary, rhTRAIL does not need a functional p53 gene to activate the extrinsic pathway of apoptosis. Therefore, rhTRAIL is of interest as an anti-cancer therapy over traditional chemotherapy and radiation because there are many cancers that possess a mutated or nonfunctional p53 gene hindering the effectiveness of that therapeutic strategy and can often result in patients in a worse condition due to negative side effects (1–4, 23, 24). Accordingly, our main objective is to apply the less toxic rhTRAIL in conjunction with “mother nature”-derived compounds such as UA as an alternative strategy to treat TNBCs.

In order to appraise the TNBC cells’ reactions to the co-treatment of UA and rhTRAIL, western blot analysis and Annexin V/PI assays were carried out. Noteworthy, TNBC BT-20 and HCC1937 cells have not been treated and examined with UA and rhTRAIL. Data was collected from both assessments, and it was deduced that UA augments rhTRAIL-induced apoptosis in TNBC BT-20 and HCC1937 cells by achieving the full execution of the extrinsic pathway as illustrated by the intensified activation of caspase 8, activation of executioner caspase 3, and cleavage of PARP. Based on these findings, the mechanism for UA’s sensitization had to be elucidated.

Low expression protein levels of DR4 and DR5 have been detected in rhTRAIL-resistant cancer cell lines, and some researchers have hypothesized that there is a deficiency in the expression of DR4 and DR5 preventing the full execution of the extrinsic pathway of apoptosis via rhTRAIL treatment (25, 26). Hence, protein and cell surface expressions of DR4 and DR5 were reviewed in UA-treated TNBC cells through
western blot and FACS analyses. The culminated data revealed that UA induced the up-regulation of DR4 and DR5 in both TNBC cells. The expression of DRs in UA-treated non-tumorigenic breast epithelial MCF-10A cells were examined, and the results verified that UA did not affect the expression of DRs in those non-tumorigenic breast epithelial cells. Thus, UA’s ability to impact on the up-regulation of expression of DRs is observed in breast cancer cells and not normal, non-transformed breast cells.

Following these results, we elucidated the underlying molecular mechanism for UA’s sensitizing effects through RT-PCR analysis. DR4 and DR5 mRNA expressions were up-regulated by UA, and c-FLIP_L mRNA expression was down-regulated facilitating the full execution of the extrinsic pathway of apoptosis.

In conclusion, molecular mechanisms of UA’s sensitizing effects of rhTRAIL were identified as evidenced by FACS, western blot, and RT-PCR analyses. Thus, UA is an effective sensitizing agent for rhTRAIL-resistant TNBCs and strongly justifies more investigation in vivo.

4.6 References


3. Shen YL, Zhang Y, Sun AY, Xia XX, Wei DZ and Yang SL. High-level production of soluble tumor necrosis factor-related apoptosis-inducing ligand


CHAPTER V
OVERALL CONCLUSION

5.1 Conclusion
Breast carcinoma is the most prevalent form of cancer for women with TNBCs being the deadliest form (1). Patients with hormone-dependent breast cancers do have access to hormone-targeted therapies, but those targeted treatments cannot be applied to patients with TNBCs. Therefore, there is a desire to develop and administer more effective and safe therapeutic approaches for any form of breast carcinoma. Hence, we pursued the combinatorial therapeutic approach of rhTRAIL with “mother nature”-derived compounds as a potential agents of interest for breast carcinoma.

Overall, this investigation shows that “mother nature”-derived sensitizing agents can enhance rhTRAIL’s pro-apoptotic effects in breast cancer cells in vitro (2-4). Traditional therapies such as chemotherapy induce apoptosis non-specifically in that the induction of apoptosis by this therapy cannot distinguish between cancerous and non-cancerous cells resulting in poor side effects for cancer patients deterring their quality of life. Whereas, the therapeutic agent rhTRAIL induces apoptosis in breast cancer cells and not in normal, non-tumorigenic breast epithelial cells; the precise mechanism for rhTRAIL-induced apoptosis in cancer cells specifically has not been established. Despite the potential clinical use of rhTRAIL, there are many breast carcinomas that are resistant to rhTRAIL treatment which could be due an array of reasons such as low DR
expressions, low pro-apoptotic protein levels, and high anti-apoptotic protein levels (5, 6). Thus, combinatorial methods with “mother-nature”-derived compounds have been proposed to overcome rhTRAIL’s limitations.

This study demonstrates that the combinatorial treatments of “mother nature”-derived sensitizers: Q, silibinin, and UA with rhTRAIL are an effective therapeutic approaches for rhTRAIL-resistant breast cancers (Figure 5.1). Q promoted the up-regulation of DR5 and the proteasome-mediated down-regulation of cFLIPL, silibinin mediated the up-regulation of DR4 and DR5 and the down-regulation of survivin, and UA induced the up-regulation of DR4 and DR5 and the down-regulation of cFLIPL in order to overcome rhTRAIL-resistance in breast cancer cell lines (2-4). Additionally, our lab also examined the co-treatment of Q and rhTRAIL in rhTRAIL-resistant malignant melanoma MeWo and WM164 cells; Q augmented rhTRAIL-induced apoptosis through DR4 and DR5 up-regulation and proteasome-mediated down-regulation of cFLIPL in the examined rhTRAIL-resistant malignant melanoma cell lines (4).
Figure 5.1: Chart summarizing sensitizers’ mechanisms of action. This chart summarizes the impact of the sensitizers Q, silibinin, and UA on apoptotic associated proteins to facilitate rhTRAIL-induced apoptosis in rhTRAIL-resistant breast cancers BT-20, MCF-7, and HCC1937 and rhTRAIL-resistant malignant melanoma MeWo and WM164. NA=not applicable

In conclusion, the findings from this investigation illustrate the promising co-therapeutic regimen of “mother nature”-derived sensitizing agents and rhTRAIL for rhTRAIL-resistant breast cancers. The future directions should be to combine all three sensitizing agents with rhTRAIL to observe through western blotting and flow cytometry.
analysis if the combination could further potentiate rhTRAIL’s pro-apoptotic effects in a variety of cancer cell lines. Throughout this study we observed that the different agents targeted different inhibitors of apoptosis and death receptors, so by targeting multiple inhibitors of apoptosis and death receptors there could be a greater enhancement of rhTRAIL-induced apoptosis in different cancer cell lines. Additionally, one should pre-treat different cancer cells with the sensitizing agents for 48 hours and then in the last 24 hours add in rhTRAIL to observe if that impacts the western blotting and flow cytometry results; this method could also further augment rhTRAIL’s pro-apoptotic effects in different cancer cell lines. Furthermore, the 72 hour co-treatment method of sensitizing agents and rhTRAIL and the co-treatment method of pre-treating with the sensitizing agents for 48 hours and rhTRAIL for the last 24 hours should be tested in vivo to determine if tumor growth decreases overtime.

5.2 References


