SR-B1 DIRECTED NANOPARTICLES AS A DRUG DELIVERY SYSTEM FOR THE TREATMENT OF TRIPLE NEGATIVE BREAST CANCER

DISSERTATION

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ABBREVIATIONS

- ABC---ATP-binding cassette transporter
- ACAT---acyl coenzyme A: cholesterol acyltransferase
- CE---cholesteryl ester
- CEH---cholesteryl ester hydrolase
- CETP---cholesteryl ester transfer protein
- DNA---deoxyribose nucleic acid
- ER---estrogen receptor
- FC---free cholesterol
- FDA---food and drug administration
- HDL---high-density lipoprotein
- HMG-CoA---3-hydroxy-3-methylglutaryl coenzyme A
- LCAT---lecithin: cholesterol acyltransferase
- LDL---low density lipoprotein
- LDLR---LDL-receptor
- MPS---mononuclear phagocyte system
- NIH---national institutes of health
- NP---nanopariticle

- NSF---national science foundation
- PC---phosphatidylcholine
- PD-1---Programmed cell death receptor 1
- PDZK1--- PSD95/Drosphilia dises-large/tight junction protein ZO1 containing 1
- PEG---poly ethylene glycol
- RCT---reverse cholesterol transport
- rHDL---reconstituted high-density lipoprotein
- RNA---ribose nucleic acid
- SR-BI---scavenger receptor class B, type I
- TG---triglyceride
- TNF---tumor necrosis factor
- VLDL--- very low density lipoprotein

ABSTRACT

The overall goal of this research was to determine the effectiveness of reconstituted high-density lipoprotein (rHDL) nanoparticles as a drug delivery system against metastatic triple negative breast cancer (TNBC). TNBC patients have a less favorable prognosis than those with hormone positive breast cancers. TNBC does not respond to current endocrine treatment. Consequently, the fiveyear survival rate for patients with metastatic TNBC is < 30%. The studies performed here were intended to fill a void in the treatment of metastatic TNBC with the use of targeted reconstituted high-density lipoprotein (rHDL) nanoparticles, an innovative approach. The rHDL nanoparticles are small, biocompatible, non-immunogenic complexes, targeted to the high-density lipoprotein receptor (scavenger receptor class B type 1 [SR-B1]). While most malignant cells and tumors overexpress the SR-B1 receptor, its expression levels are nearly undetectable in most normal tissues. These findings present the opportunity to exploit a key vulnerability of cancerous tumors as a "Trojan horse" therapeutic strategy and thus markedly limit the toxic impact of chemotherapy. Accordingly, we loaded rHDL nanoparticles with the anti-cancer drugs:

valrubicin and lapatinib and tested their effectiveness against TNBC cells and cardiomyocytes. The outcome of these studies show that: (1) The rHDL encapsulated drugs performed significantly better than their free (unencapsulated) counterparts, (2) The enhancement of the therapeutic effect of the drugs delivered via the rHDL nanoparticles was likely due to the overexpression of the SR-B1 receptor by the TNBC cells. This was confirmed by the enhanced uptake of valrubicin when delivered as a component of the rHDL complex. 3. We have also found that the combination of lapatinib and valrubicin may be ultimately more effective than the respective single drugs for the therapy of TNBC.

Keywords: nanoparticle, rHDL, TNBC, chemotherapy

CHAPTER I INTRODUCTION

Significance of Cancer

Cancer is an overwhelming disease in the United States and worldwide (WHO, 2014). It is a heterogeneous group of diseases with a high rate of recurrence. It leaves almost no one untouched, regardless of age, race, or socioeconomic status. From 2008 to 2012, global cancer incidence has increased 11% and is expected to grow another 70% to total 25 million cases within the next 20 years (IARC, 2014). Though in the United States a "war on cancer" was declared in 1971, cancer deaths still continue to rise, closing in on the number one cause of mortality. In 2011, 596,577 deaths occurred as a result of heart disease, while 576,691 deaths resulted from cancer causes (Siegel, 2015). Currently, cardiovascular disease remains the dominant cause of death in the United States. However, with the consistent rise of cancer-related mortality, predictions suggest that cancer may soon overcome heart disease as the leading cause of death (Siegel, 2015).

Significance of the Study

Metastatic breast cancer, in most women, remains an incurable disease. The overall goals of treatment are to elevate the quality of life, attenuate symptoms and

extend survival (Miles, 2009). Although 15–20% of all breast cancer cases are of the Triple negative breast cancer (TNBC) sub-type, TNBC accounts for a disproportional amount of cancer deaths (Cleator, 2007; Rakha, 2008). Accordingly, woman suffering from metastatic TNBC have a 5-year survival rate of < 30%(Liedtke, 2008). Due to a lack of targeted therapies and a high incidence of recurrence and metastasis within five years of diagnosis, TNBC patients have a less favorable prognosis than those with hormone positive breast cancers (Badve, 2011; Oakman, 2010). The inevitable development of multidrug resistance (MDR) within the cell has become a hallmark for unsuccessful cancer chemotherapy (Lee, 2005; Patel, 2011). MDR frequently develops as a result of permeability glycoprotein (Pgp) an ATP-binding cassette transporter glycoprotein (Patel, 2011). Because the drugs susceptible to P-gp activity include anthracyclines and taxanes, which are also used in TNBC treatment, a therapeutic level of the drug at the active site of the disease becomes a challenge (Oliveras-Ferraros, 2008). In addition the increase of dose concentration to overcome P-gp activity must be balanced with the potential increase toxicity to surrounding cells.

As an alternative to traditional therapy, nanoparticles provide a mechanism for drug delivery to malignant cells, significantly limiting exposure of these potentially harmful drugs to healthy tissue (Bos, 2009). Nanoparticles as cancer drug delivery vehicles provide a promising alternative to traditional therapy. Published data

suggest that nanoparticles can increase therapeutic index of cytotoxic drugs by increasing drug accumulation in the tumor, prolonging circulating half-life, and reducing the risk of off-target effects (Kamaly, 2012). Cancer cells proliferate much faster than healthy cells therefore; the requirement of cancer cells and tissues for energy and cell composing essentials is far more than healthy cells (Lacko, 2002). One way this is accomplished is by the mutation or overexpression of growth factor receptors thereby producing constitutive signaling. Besides essential nutrients, malignant cells have an exaggerated demand for cholesterol for membrane construction (Johnson, 2013), therefore cancer cells overexpress the high-density lipoprotein (HDL)/SR-B1 receptor (Fumoleau, 2012). rHDL nanoparticles exploit the over- expression of scavenger receptor class B type 1 (SR-B1) in cancer cells. By carrying anti-cancer drugs instead of cholesterol (see Figure 12), the drug loaded rHDL nanoparticles can serve as a "Trojan horse" and enhance the therapeutic efficacy of the encapsulated drugs against TNBC cancer cells (Johnson 2013; Lacko, 2006).

Cholesterol and Cancer

Cancer is a heterogeneous group of diseases, which has resulted from an overwriting of endogenous cell processes for the propagation and survival of abnormal cells. Though safeguards exist within the cell cycle to prevent replication

of mutated DNA, cancer has overcome such restrictions through mutations of such checkpoints or the regulators of those checkpoints. The result is a rapid uncontrolled proliferation of divergent cells. The cell cycle is not the only common metabolic pathway that is disrupted in cancer. Also altered in many cancers is cholesterol metabolism. Clinical evidence exists stating that the lipoprotein high-density lipoprotein (HDL) "the good cholesterol" is present at lower levels in the plasma of cancer patients while the degree of cholesterol in the tumor membrane is abundant (Murtola, 2012). Homeostasis levels of whole body cholesterol are maintained through a balance of synthesis, ingestion, and consequent absorption, and excretion. In the human body, cholesterol serves a precursor for steroid hormones, as well as bile acids, and vitamin D. The free form is also a key component in the production of a functioning plasma membrane (Likus, 2016). The majority of ingested cholesterol is free cholesterol, which is readily absorbed into the enterocytes of the small intestine (Tancharoenrat, 2014). Those ingested cholesterol molecules that are esterified interact with cholesterol ester hydrolase (CEH), which converts the esterified cholesterol into free cholesterol, which can then be incorporated into a bile acid micelle (Ikeda, 2002). Niemann-Pick C1-Like1 (NPC1L1) a transmembrane protein is now able to endocytose the cholesterol into the enterocyte of the small intestine. The free cholesterol will then be converted back into cholesterol esters by acetyl-CoA

acetyltransferase 2 (ACAT2) according to the cholesterol/cholesterol ester concentration gradient (Chang, 2009). Triglycerides, phospholipids and cholesterol esters will be encapsulated with Apo B-48 into a chylomicron by microsome triglyceride transfer protein (MTP). The chylomicron will exit the small intestines and enter the lymphatic system and finally reach the blood stream where it can deliver cholesterol to the cells before the liver removes it (Cooper, 1997). Though some cholesterol may be obtained from the diet or synthesized in the small intestine the majority of cholesterol in the body comes from synthesis in the liver (Morgan, 2016). Through a series of reactions, cholesterol is synthesized in the mevalonate pathway beginning with the conversion of acetyl-CoA to acetoacetyl-CoA or the direct conversion of acetyl-CoA to 3-Hydroxy-3- Methylglutaryl CoA (HMG-CoA; Likus, 2016). Cholesterol synthesis regulated by the subsequent step in the reaction, the conversion of HMG-CoA to mevalonate by the enzyme HMG-CoA reductase.

Cholesterol is fundamental to the fluidity of the cell membrane; as a result, it is vital to the continued progression of the cell cycle. When the cholesterol level is minimal within the cell the cell will not progress to the s-phase and will instead arrest at either of the g-phases until cholesterol or mevalonate become available again (Fernandez, 2004; Singh, 2013). Malignant cells ensure the supply of

cholesterol through the up-regulation of glycolysis, which provides two pyruvate molecules for each completed cycle. These pyruvate molecules are converted into acetyl-coA through a process called pyruvate decarboxylation. As a result of the up-regulation of glycolysis, there is an excess of acetyl-coA that can be used to initiate the mevalonate pathway ensuring cholesterol production (Likus, 2016).

Previous studies have revealed that the mevalonate pathway is deregulated in the basal/ mesenchymal subtype of breast cancer (Ginestier, 2012). Studies have also shown that the overexpression of the rate-limiting enzyme HMG-CoA reductase can transform the immortalized non-tumorigenic breast cell line MCF-10A. Increased HMG-CoA levels have also been described as a marker for a poor patient prognosis and were associated with a reduced survival outcome in a meta-analysis of 865 breast cancer patients (Clendening, 2010). These findings suggest that cholesterol may be useful to promote carcinogenesis and more specifically that an up-regulated level of HMG-CoA reductase could be an indicator of the aggressiveness of cancer.

Lipoproteins are endogenous cholesterol transporters. They help to maintain the homeostasis levels of cholesterol by delivering cholesterol to the peripheral tissues and removing and delivering excess free cholesterol back to the liver. It is known that malignant cells proliferate faster than normal cells. As a result, their

cholesterol requirement is higher than non-proliferating cells. One way cancerous cells acquire the needed quantity is through the over-expression of the HDL receptor scavenger receptor class B type 1 (SR-B1).

Scavenger Receptor Class B Type 1 (SR-B1)

Identified in 1996 as the receptor for the HDL molecule, the SR-B1 receptor is involved in the discriminating removal of cholesterol esters from the core of the HDL molecule and the delivery of excess free cholesterol to nascent or pre- β HDL molecule (Johnson, 1998). The SR-B1 receptor is encoded by the SR-B1 gene located on chromosome 12q24 (Johnson, 1998). It is an 82kDa protein consisting of 509 amino acids and a substantial carbohydrate moiety (Rhainds, 2004, Valacchi, 2011; see Figure 1). SR-B1 is thus a highly glycosylated receptor found in the caveolae of the cell membrane (Rhainds, 2004, Valacchi, 2011). The receptor contains two cytoplasmic domains: a transmembrane domain and a large extracellular domain (Rhainds, 2004). The SR-B1 protein is expressed in the liver, ovaries, testis, and the adrenal gland, and in other normal tissues that are actively proliferating (Shahzad, 2011). The SR-B1 receptor is part of the scavenger receptor class B protein receptor family. Within that group also exists another receptor that has a high affinity for HDL by the name of CD36 (Calza, 2006). The SR-B1 and CD36 share 30% homology; however, the CD36 does not facilitate

cellular lipid uptake (Oakman, 2010) and has no effect on the cholesterol aggregation within the plasma membrane (Calza, 2006, Valacchi, 2011).



Figure 1: *SR-B1 protein*. The image depicts the horseshoe shape of the receptor with its two transmembrane domains. The warning triangle () identifies essential domains necessary for CE selective uptake and N-glycosylation in the golgi apparatus. The smiley face identifies the PDZK1 binding site. PDZK1 is necessary for stability of the receptor in hepatocytes and cell signaling in epithelial cells (with permission, image taken from Rhainds,2004).

In addition to binding HDL SR-B1 also binds acetylated low-density lipoprotein (LDL), oxidized LDL, maleylated bovine serum albumin, and anionic phospholipids (Valacchi, 2011). However, it is to cholesterol ester-rich HDL molecules that SR-B1 has a greater affinity. SR-B1 is associated with an adapter by the name postsynaptic density protein PSD95/Drosphilia dises-large/tight junction protein ZO1 containing 1 (PDZK1). This protein is necessary for SR-B1 stability in hepatocytes and induction of cell signaling in other cells (Al-jarallah, 2010). SR-B1 binds to the HDL molecule via the helical portion of apolipoprotein A-I (apo AI), the major protein component of the HDL lipid-protein complex covering its outer surface (Steinberg, 2000). SR-B1 facilitates the selective removal of the cholesterol esters from the core of the HDL (Steinberg, 2000) and delivers it to the cell. The exact molecular mechanism of this process is yet to be delineated. However, it has been established that unlike the low-density lipoprotein receptor (LDL-R), the mechanism involving the SR-B1 receptor does not require endocytotic uptake of the whole HDL particle into the cell (Steinberg, 2000). In fact, the SR-B1 receptor accomplishes the transport of CE into the cell, leaving the rest of the HDL molecule intact (Lacko, 2006). The SR-B1 also facilitates the delivery of excess free cholesterol from the cell to the HDL molecule (Shah, 2011). The free cholesterol that HDL acquires is left on the surface of the particle until the lecithin-cholesterol acyltransferase (LCAT) molecule esterifies

the cholesterol. The cholesterol then moves into the core of the HDL making the particle spherical. At this point, the HDL can deliver its payload back to the liver for excretion or recycling; this process is referred to as reverse cholesterol transport (RCT; see Figure 2). Also, HDL can transfer its cholesterol esters; with the help of cholesteryl ester transfer protein (CETP), to Apo AE containing lipoproteins like LDL or VLDL (Shah, 2011), in that way LDL and VLDL also participate in RCT.

SR-B1 and Breast Cancer

Cancer cells are known to proliferate faster than normal cells (Lin, 2012). Consequently, malignant cells have adapted methods to acquire carbon, nitrogen sources as building blocks for new cells. Non-malignant cells do not take up nutrients from the surrounding environment unless stimulated by growth factors (Lin, 2012). Cancer cells have overcome this limitation either by altering their growth factor receptors so that they are constitutively signaling, or the receptor protein is over-expressed (Badve, 2011). The excessive requirements for nucleotides, amino acids, and lipids by proliferating cells are induced by their need to reproduce their entire cellular contents during mitosis (Lin, 2012).



Figure 2: Pathways involved in reverse cholesterol transport (RCT). The diagram depicts the role of the HDL, and LDL molecules and cholesterol ester transfer protein (CETP) in the process of RCT. B = (apo-B), A-1 = (apo-AI; with permission image taken from Shah, 2011).

Cancer cells overexpress the HDL (SR-B1) receptor on their cell surface to meet the excess requirement for cholesterol (Cruz, 2013; Calza, 2006). Prostate, breast, ovarian, pancreatic, hepatoma, colorectal, and nasopharyngeal cancer cell lines have been reported to overexpress the SR-B1 protein (Cao, 2004; Leon, 2010; Shahzad, 2011; Wadsack, 2003; Yuan, 2015; Zheng, 2013). Malignant cells manipulate this system and remove the cholesterol esters from the HDL particle for their survival.

SR-B1 is a key player in the aggressiveness and survival of breast cancer. Studies have shown that a high plasma cholesterol level increases the number of tumors present and induces metastasis in breast cancer (Danillo, 2013). Further in vitro studies have revealed that breast cancer cells show an increased proliferation and migration in the presence of HDL (Pan, 2012). Using the triple negative breast cancer (TNBC) cell model, MDA-MB-231 Danilo et al, published results indicating that migration can be induced by the addition of HDL₃. Danilo also revealed that attenuation of these effects could be accomplished with the knockdown of SR-B1 (Danilo, 2013). SR-B1 protects and supports the survival of breast cancer cells by binding HDL. The binding and uptake of CE from within the core lipoprotein result in the activation of the PI3K/Akt pathway leading to ERK1/2 and Akt induced activation of endothelial nitric oxide synthase (eNOS; Al Jarallah, 2010, Danilo, 2013). Also, SR-B1 activation of Akt permits SR-B1 to

regulate angiogenesis as well as inhibit apoptosis in breast cancer cells (Danillo, 2013). All of these effects were attenuated or inhibited with the knockdown of SR-B1. These data suggest that SR-B1 is complicit in promoting the survival and metastasis of breast cancer. Indeed, SR-B1 has been identified as a biomarker for poor prognosis in breast cancer patients (Li, 2016). Studies on the use of SR-B1 as a target for nanoparticles have been conducted in nasopharyngeal (NPC) carcinoma using HDL-mimetic nanoparticles. Pre-clinical results revealed an overexpression of SR-B1 in the NPC cell lines tested and in 75% of patient tissue samples. Results also showed that the HDL-mimetic nanoparticle could inhibit cell motility and colony formation without causing necrosis or inducing apoptosis (Zheng, 2013)

The following sections focus on the potential role of nanotechnology in therapeutic approaches for triple negative breast cancer, which were incorporated into this dissertation from our review article (Johnson et al., 2013).

The Potential Role of Nanotechnology in Therapeutic Approaches for Triple Negative Breast Cancer

ABSTRACT

Triple Negative Breast Cancer, TNBC, a highly aggressive and metastatic type of breast cancer, is characterized by loss of expression of the estrogen receptor (ER), progesterone receptor (PR), and a lack of overexpression of the human epidermal growth factor receptor 2 (HER2). It is a heterogeneous group of tumors with diverse histology, molecular uniqueness and response to treatment. Unfortunately, TNBC patients do not benefit from current anti-HER2 or hormone positive targeted breast cancer treatments; consequently, these patients rely primarily on chemotherapy. However, the 5-year survival rate for woman with metastatic TNBC is less than 30%. As a result of ineffective treatments, TNBC tumors often progress to metastatic lesions in the brain and lung. Brain metastases of invasive breast cancer are associated with 1 and 2 year survival rate of 20% and <2% respectively. Because the only current systemic treatment for TNBC is chemotherapy, alternative targeted therapies are urgently needed to improve the prognosis for these patients. This review is focused on opportunities for developing new approaches for filling the current void in an effective treatment for TNBC patients.

KEYWORDS: lipoprotein; nanoparticle; targeted therapy; triple negative breast cancer

Profiling and Current Therapeutic Approaches for Triple Negative Breast Cancer (TNBC)

Breast cancer is the second leading cause of cancer death among women in the US (CDC, 2012). TNBC, a highly aggressive and metastatic type of breast cancer, is characterized by loss of expression of the *estrogen receptor (ER)*, progesterone receptor (PR), and a lack of overexpression of the human epidermal growth factor receptor 2 (HER2) (Fornier, 2012). Histologically, 77%–90% of TNBC tumors are grade 3 at initial presentation (Rakha, 2008; Reis-Filho; 2008; Sorlie, 2001) and most patients are under the age of 50 at the onset (Banerjee, 2006; Carey, 2006; Rakha, 2007; Thike, 2010). TNBC is considered an interval cancer (appearing between mammograms), characterized by overexpression of the tumor suppressor p53 or a p53 loss-offunction mutation, as well as mutations in retinoblastoma (pRb) and p16, G1/S cell cycle regulators (Gauthier, 2007; Jumppanen, 2007; Langerod, 2007; Shakya, 2008; Subhawong, 2009). TNBC accounts for 15%–20% of all breast cancers (Carey, 2006; Cleator, 2007; Dent, 2007; Rakha, 2007) with a particularly high prevalence among African-American women. Accordingly 50% of all diagnosed cases of breast cancer among African-American women, in the under 40-age group, are of the TNBC type (Liu, 2011).

Breast cancer represents a varied group of diseases that can be divided into four groups (Table 1), according to their gene-expression profiles (GEP). These are:

luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)

amplification, and basal-like (Perou, 2000; Rakha, 2009). Triple negative breast cancer (TNBC) is a subtype of breast cancer that shares with the basal-like group many of its characteristics and GEP markers, including expression of basal *cytokeratins 5/6, 14,* and *17,* as well as the *epidermal growth factor receptor* (*EGFR*) and *vimentin* (Rakha, 2009). Though sixty- eighty percent of TNBC tumors are classified as basal-like, TNBC is a heterogeneous group with differences in histology, molecular profiles and response to treatment (Rakha, 2009). TNBC is further divided into six subtypes based on their GEP. They are: *basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like,* and *luminal androgen receptor (Rakha, 2009; see* Table 2).

Table 1. Breast cancer classification based on gene-expression profile (GEP)characteristics (Fornier, 2012; Perou, 2000)

Classes	ER	PR	HER2	GRADE	PROGNOSIS
Luminal A	Pos	Pos	Neg	Low	Good
Luminal B	Pos/Neg	Pos/Neg	Pos/Neg	Intermediate/High	Intermediate
HER2	Neg	Neg	Pos	High	Poor
Basal-like	Neg	Neg	Neg	High	Poor
Claudin-low	Neg	Neg	Neg	High	Poor

Pos = Positive; Neg = Negative

While TNBC and basal like malignancies have significant overlapping features, several differences have also been described (Calza, 2006; Jumppanen, 2007; Perou, 2000; Rakha, 2009; Rouzier, 2005; Sorlie, 2003; Tan, 2008). Bertucci et al. found that 23% of the tumors they investigated and classified as basal-like via GEP criteria (Bertucci, 2008; Schneider, 2008) could not be cross classified as TNBC also (Rakha, 2008). Another group representing 29% of those with the TNBC phenotype, were classified as non-basal-like according to GEP criteria. Despite this apparent conflict in classification, there is agreement that TNBC is characterized by loss of expression of the ER, PR, and a lack of overexpression of HER2 (Lin, 2012). Poorly differentiated ductal carcinomas make up 80%–93% of TNBC tumors (Carey, 2006; Oakman, 2010; Sorlie, 2003). TNBC rarely has a ductal carcinoma in situ (DCIS) component, due to the highly invasive nature of this tumor (Badve, 2011; Carey, 2006; Oakman, 2010).
Table 2. Triple negative breast cancer (TNBC) subtypes based on gene-expressionprofiles (GEP; Perou, 2003).

Subtype	GEP
Basal-like 1 (BLI)	Expresses cell cycle, DNA repair, and proliferating
	genes
Basal-like 2 (BL2)	Expresses growth factor signaling genes such as EGFR,
	MET, Wnt, IGF-IR
Immunomodulatory (IM)	Expresses genes involved in immune cell processes
Mesenchymal (M)	Expresses genes involved in cell motility,
	differentiation, and EMT processes
Mesenchymal stem-like (MSL)	Expresses growth factor signaling genes and low levels
	of proliferating genes
Luminal androgen receptor	Expresses androgen receptor and downstream genes
(LAR)	

The overall poor prognosis of TNBC is partly due to its high rate of recurrence and metastases within 5 years of the initial diagnosis (Oakman, 2010; Hudis, 2011) as well as lack of targeted therapies (Chitnis, 2008; Gril, 2008). TNBC tumors do not benefit from current anti-HER2 or hormone positive breast cancer treatments (Jones, 2007) as TNBC patients rely primarily on chemotherapy consisting of either anthracycline-based agents combined with cyclosphosphamide, followed by docetaxel or a combination of docetaxel, doxorubicin and cyclophosphamide (Chitnis, 2008). Despite the hypothesis that TNBC would respond well to chemotherapy due to the lack of *HER2* overexpression, these patients have a poorer overall survival than HER2 positive patients (Lehmann, 2011). The 5-year survival rate for women with metastatic TNBC is less than 30% (Liedtke, 2008). With 1118 patients enrolled, Liedtke *et al.* reported a higher proportion of complete responses with TNBC patients (22%) than with non-TNBC patients (11%). However, the 3-year progressive free survival (PFS) rates and overall survival (OS) were decreased among TNBC patients (Liedtke, 2008). These findings may be attributed to a group of TNBC patients with early onset drug resistance (Leidtke, 2008). As a result of ineffective treatments, TNBC tumors often progress to metastatic lesions in the brain and lung (Bos, 2009). Brain metastases of invasive breast cancer are associated with 1 and 2 year survival rate of 20% and <2% respectively (Bos, 2009). Effective treatment of significant

palliative benefit for patients with brain metastases is limited. The classical approach includes whole brain radiation or stereostatic radio surgery (Pestalozzi, 2009); however, these treatments do not increase the overall survival of the patient (El Guerrab, 2011), and in some cases has adverse effects on cognitive function (Fitzgerald, 2008). Because the only current systemic treatment for TNBC is chemotherapy, alternative targeted therapies are urgently needed to improve the prognosis for TNBC patients (Rakha, 2008). This review is focused on opportunities for developing new approaches for filling the current void in an effective treatment for TNBC patients.

Current Status of TNBC Therapeutics

Currently, the first-line treatment patterns for TNBC include a combination of surgery, radiation, and neoadjuvant/adjuvant chemotherapy, which can often lead to an improved prognosis for early stage TNBC. Kassam et al have demonstrated that, compared to other types of breast cancers, TNBC patients experience a higher proportion of metastatic recurrence (33.9% *vs.* 20.4%; p < 0.0001; Kassam, 2009). Furthermore, in advanced disease, a median overall survival is only 13.3 months, after initial diagnosis (Lin, 2008). This limited survival validates the urgent need for new approaches as a high priority, compared with other forms of metastatic breast cancers (Cancello, 2010; Reis-Filho, 2008).

As demonstrated by Liedtke *et al.* groups of patients with TNBC show marked differences with respect to response and prognosis subsequent to neoadjuvant chemotherapy (Liedtke, 2008). While some patients with TNBC may benefit from current chemotherapy regimens, there is a sizable group for whom there are only limited benefits. Consequently, four main issues need to be considered for the development of novel therapeutic approaches for TNBC patients: (1) Identification of patients with resistance to current chemotherapy regimens; (2) Development of novel biomarkers to improve the early diagnosis as well as the classification of patients with regards to their respective responses to therapy; (3) Development of alternative strategies for improved bioavailability and targeting of drugs; (4) Improvement of drug delivery vehicles to safely transport the anti-cancer agents to their tumor targets. TNBC patients represent a heterogeneous group with varying molecular profiles and response to treatment (Rakha, 2008). As a result several molecules and signaling pathways are likely targets for new therapeutic approaches. In this review a number of these potential therapeutic targets are highlighted.

Current Targets for TNBC Therapeutics

PI3K/Akt Pathway

<u>mTOR</u>

Eukaryotic translation initiation factor 4E (eIF4E1) along with EGFR have been

identified as proteins expressed in brain metastatic cells originating from breast cancer (Klein, 2009). Once eIF4E1 is activated it also activates hypoxia inducible factor alpha (HIF1 α), which then binds with HIF1 β , and together they function as transcription factors (TF) for genes involved in angiogenesis, namely matrix metalloproteinases (MMPs) and cyclooxygenase 2 (Cox-2; Dufour, 2011;see Figure 3).



Figure 3. Diagram of PI3K/Akt and Ras/Raf pathway (see text for details).

These proteins function together to remodel the extracellular matrix. HIF1α also acts as a TF for the growth hormone VEGF which when bound to its receptor, VEGFR also aids in angiogenesis (Dufour, 2011). The mammalian target of rapamycin (mTOR) is a serine/threonine kinase functioning as a main effector downstream of the phosphatidylinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway (Dufour, 2011). It is involved in many cellular processes including cell growth, survival, and invasion (Hudis, 2011; Paranjape, 2011; Populo, 2012). mTOR exist in two complexes mTORC1 and mTORC2 (Paranjape, 2011; Populo, 2012). mTORC2 phosphorylates Akt at S473 allowing phosphoinositide-dependent kinase 1 (PDK1) to phosphorylate Akt at T308 (Populo, 2012).

Both phosphorylations are needed for Akt activation. Akt is then able to phosphorylate and inhibit tuberous sclerosis 2 (TSC2) keeping it from forming a complex with TSC1 (Populo, 2012). This drives the GTPase Ras homolog enriched in brain (Rheb) into the GTP bound state. Upon activation, Rheb phosphorylates mTOR at the S2448 position (Paranjape, 2011; Populo, 2012). mTORC1 as well as Erk1/2 phosphorylates 4EBP1, the inhibitor of eIF4E1. Phosphorylation of 4EBP1 keeps it from inhibiting eIF4E1 and allows eIF4E1 to promote angiogenesis. mTORC1 also phosphorylates S6K1 which leads to the activation of

S6 the small ribosomal subunit. S6 association with mTORC2 allows it to phosphorylate Akt upstream of the mTORC1 complex (Dufour, 2011). Activated S6K1 is a key protein in the negative feedback loop to insulin receptor substrate (IRS-1). When mTOR is inhibited S6K1 does not inhibit IRS-1. IRS-1 can activate both the Ras/Raf and PI3K-Akt pathways as well as activating other receptor tyrosine kinases (RTK), such as VEGFR. In TNBC, KRAS mutations have been reported resulting in constitutive activation of the Ras/Raf pathway (Paranjape, 2011). In addition, TNBC patients may also carry gain-of function BRAF mutations further making it harder to effectively target the Ras/Raf pathway (Solit, 2006). Because rapamycin and its analogues partially inhibit mTORC1 the unobstructed feedback loop eventually overcomes the inhibition and cell proliferation continues again (Thoreen, 2009). Rapamycin and its analogs are partial inhibitors of the mTORC1 complex and do not inhibit mTORC2 at all Liu, 2010). However, in some cancers the rapamycin analogs have been shown to be very effective at inhibiting cell proliferation at greater than 24 h treatments (Populo, 2012; Liu, 2011; Sarbassov, 2006). Such results have not been seen with the TNBC cell lines. A recent study on the TNBC cell line MDA-MB-231 has shown that while 72 h of rapamycin

treatment induced apoptosis; this effect did not increase above the level of the untreated control (Sarbassov, 2006). Moreover, combination treatment of rapamycin and the drug indole-3-carbinol actually decreased the level of apoptosis achieved by indole-3-carbinol on its own (Sarbassov, 2006).

A novel ATP-competitive inhibitor of mTOR, Torin1, has been reported to inhibit cell proliferation more effectively than rapamycin (Populo, 2012). Indeed, studies show that a 10-day treatment of U87 primary glioblastoma multiforme, (GBM) xenografts with Torin 1 resulted in a robust activation of the PI3K/Akt/mTOR pathway and tumor growth suppression by over 99% (Liu, 2009). Though the signaling mechanism that connects mTOR to autophagy is yet unclear, Torin 1 has been shown to induce autophagy in mouse embryonic fibroblasts (MEF) and HeLa cells (Ozes, 2001). Torin1 has also been shown in decrease protein translation and cause a G1/S cell cycle arrest in MEF cells. Despite its performance Torin 1 is limited in its therapeutic use due to its low bioavailability and half-life of only 0.5 h with *i.v.* administration (Liu, 2009).

EGFR

EGFR is one of the receptor tyrosine kinases (RTK) that are activated by the substrate Insulin receptor substrate 1(IRS1), as a result of mTOR inhibition (see Figure 3). IRS1 is phosphorylated at S636/639 by the mTOR pathway (Ozes, 2001), keeping it from activating RTKs and further activating the PI3K pathway.

When mTOR is inhibited this negative feedback loop is disrupted and IRS1 is free to bind with EGFR and other RTKs. EGFRs involvement in cancer growth is well documented (Agrawal, 2005; Tsutsui, 2002). Tumors over-expressing EGFR tend to have higher proliferation rates, inhibition of apoptosis, chemoresistance, increased angiogenesis, and invasive and metastatic tendencies (Inoue, 2012). Sixty percent of basal-like tumors over-express EGFR and ~seventy percent of TNBC tumors (Dogu, 2010; Irvin, 2008; Peddi, 2012). These finding make EGFR a reasonable target. 173 patients were treated with cisplatin alone or in combination with cetuximab, an anti- EGFR antibody. The response rate was 20% with those treated with the combination vs. 10% with those treated with cisplatin alone (Ueno, 2011). Similar results were seen with the drug combination vs. carboplatin alone in a randomized phase II clinical trial of TBCRC001 (Schneider, 2008).

IGF1R

The Insulin Growth Factor 1 Receptor (IGF1R) has been associated with the growth, invasion, and metastasis in breast cancer patients and is over-expressed in 50%–75% of TNBCs (Grunstein, 2012). IGF1R has been reported to aid in metastasis by allowing the cancer cells to adapt to anchorage-independent growth (Baserga, 2003; Sell, 1994). Indeed pre-clinical trails have shown that over-expressing IGF1R induces tumor formation and metastasis (Jones, 2007; Lopez, 2002). IGF1R has also been shown to inhibit apoptosis induced by

chemotherapeutic drugs in the HBL100 breast cancer cell line inferring chemoresistance to the cancer cells (Plymate, 2007).

DNA Repair

<u>PARP</u>

BRCA1 (a gene involved in homologous DNA repair) mutations are seen in both basal-like and TNBC type breast cancers. Both subtypes are reported to have a high degree of genetic instability (Peddi, 2012; Natrajan, 2009; Bergamaschi, 2006). Seventy-five- eighty percent of all BRCA1 mutations have been reported to be basal-like by GEP (Anderson, 2002; Cheang, 2008; Rakha, 2007). BRCA1 mutations have been found in $\sim 60\%$ of the TNBC patients tested (Atchley, 2008; Foulkes, 2004; Prat, 2008; Sorlie, 2003). However, BRCA1 silencing due to promoter methylation has also been shown (Peddi, 2012). BRCA1 TNBC patients are among the minority of those who benefit from anthracycline-based chemotherapy, and are also susceptible to platinum based agents (Gonzalez-Angulo, 2011) BRCA1 mutations clear the way for alternative DNA repair mechanisms like base excision repair, which relies on Poly(ADP-ribose) polymerase (PARP) (Dent, 2007; Peddi, 2012; Sirohi, 2008). PARP activation leads to histone acetylation by histone acetyltranferases (HAT) of lysine residues on the N-terminus tail of the histone (Grunstein, 2012), allowing access of the repair machinery to the damaged DNA. PARP inhibition leads to an accumulation

of unrepaired DNA damage that would normally be repaired by homologous recombination mediated by BRCA1 (Chalmers, 2009; Guo, 2011; Peddi, 2012). The resulting abundance of DNA damage induces cell death. Cell death as a result of PARP inhibition and BRCA1 deficiency is known as synthetic lethality (Au-Yong, 2009; Bryant, 2005; Farmer, 2005; Fong, 2009). Nonetheless, PARP inhibition is not effective on cancers that lack the BRCA1 mutation.

Src Kinases

Finn *et al.* evaluated safety and efficacy of dasatinib, an effective Src-family kinase inhibitor with confirmed preclinical anti-proliferative, anti-metastatic, and anti-osteoclastic activity against TNBC (Finn, 2011). In a phase II clinical trial of 45 patients with advanced TNBC, as a single agent dasatinib had limited activity; however, the potential benefit of combining dasatinib with various chemotherapeutic drugs is under investigation. In a group of 39 human breast cancer cell lines characterized by gene microarray, basal-type breast cancer cell lines demonstrated the most substantial growth inhibition with dasatinib treatment (Gnoni, 2011). Preclinical findings by Tryfonopoulos *et al.* suggest substantial synergy when dasatinib is combined with other agents (specifically, cisplatin and FUDR) in TNBC cell lines (Tryfonopoulos, 2011).

Heat Shock Protein 90

Over-expression of the heat shock protein (HSP) 90 isoforms correlated with a

poorer prognosis in certain subtypes of breast cancer including TNBC (Caldes-Lopez, 2009; Chinosis, 2006) indicating that Hsp90 inhibitors could be used as therapeutic targets against TNBC. This category of agents prevents the protein folding function of the chaperone protein Hsp90, resulting in the degradation of client proteins (Patel, 2011). A preclinical assessment by Caldas-Lopes *et al.* of the Hsp90 inhibitor PU-H71 in TNBC xenografts indicated substantial antitumor activity (Modi, 2011). In another study, a combination of Hsp90 inhibitors, tanespimycin and trastuzumab, were shown to be well tolerated and exhibited antitumor activity in patients with breast cancer (Modi, 2011).

Combined Targeted Therapy

As a single agent IGF1R inhibitors have shown limited success against most cancers (Grunstein, 2012; Haluska, 2007; Ryan, 2008), combining IGF1R treatment with other targeted therapies may offer an improved therapeutic outcome. Current mTOR inhibition causes the upregulation of the Ras/Raf pathway and inhibition of the negative feedback loop of IRS1 while inhibition of IGF signaling has been shown to inhibit growth and induce death of cancer cells with upregulated PI3K. This effect is, due to a PTEN loss of function mutation, and/or gain of function mutations of the Ras/Raf pathway (Rochester, 2005; Yeh, 2006). Combining IGF1R inhibition with mTOR inhibition may thus prove to be effective at inducing cell death in TNBC where the Ras/Raf and/or PI3K pathways are up regulated. Similarly, IGF1R inhibition in combination with EGFR inhibition in EGFR over-expressing cancer cells or IGF1R inhibition in combination with HER2 inhibition in HER2 positive cancer cells have also shown improved results over the use of single agents only (Haluska, 2008; Jerome, 2006; Lu, 2005). EGFR inhibition has been shown to sensitize malignant tumors to chemotherapy with cisplatin or carboplatin (Oliveras-Ferraras, 2008), while combining EGFR inhibition with PARP inhibition has also produced encouraging findings. EGFR inhibition can reduce the expression of the BRCA1 protein, thereby making the cancer cells vulnerable to PARP inhibition (Nowsheen, 2012). This treatment can allow those TNBC patients without BRCA1 mutations to benefit from PARP inhibition.

As reported on the website clinicaltrials.gov, there are currently 67 clinical trials for TNBC in the U.S. at the time of this review. 53 of these trials are using a combination therapy. Directing combination therapy to the above targets, especially PARP and EGFR, can be effective, as reported by Nowsheen *et al.* (Nowsheen, 2012). Nevertheless, combination drug therapy can increase the probability of adverse side effects. To circumvent the peripheral toxicity of a combination of chemotherapeutic agents several types of nanoparticles have been developed as drug delivery vehicles (Lee, 2005; Nowsheen, 2012;

Oliveras- Ferraros, 2008).

Nanoparticle as a Drug Delivery Vehicle to Treat TNBC

Early onset of chemoresistance, a hallmark of TNBC tumors (Rakha, 2009), contributes to the fact that only 1/3 of TNBC patients have shown a pathological complete response (pCR) after anthracycline or anthracycline + taxane based neoadjuvant chemotherapy (Rakha, 2009). One of the major barriers to successful cancer chemotherapy is the development of multidrug resistance (MDR) within the cell (Lee, 2005; Patel, 2011), often due to the over-expression of the ATP-binding cassette transporter glycoprotein (P-gp) also known as MDR1 (Patel, 2011). P-gp is an ATP dependent transmembrane drug efflux pump that transports drugs across the cell membrane and out of the cell (Li, 2012; Patel, 2011). A number of drugs are substrates for P-gp including the anthracyclines and taxanes that are often used in TNBC treatment (Oliveras-Ferras, 2008). Consequently, drug accumulation in the tumor is limited, underscoring the need for advanced drug delivery vehicles to provide effective alternatives to traditional therapy. Some of these novel drug delivery approaches have been reported to increase the therapeutic index of cytotoxic drugs by prolonging circulating halflife and increasing drug accumulation in the tumor, in addition to reducing the risk of off target effects (Lee, 2010; Li, 2012). Lipoprotein based drug carriers have also shown to deliver drugs directly to the cytoplasm of cancer cells (Ding, 2014) and thus would be anticipated to avoid the drug resistance pumps.

With an alternate approach, Patel et al. have developed a non-targeting, long circulating liposome to encapsulate tariquidar (XR9576), a P-gp inhibitor that has been used to combat the MDR mechanism (Patel, 2011), along with the microtubule stabilizer paclitaxel. The liposome (with a diameter of 180 to 200 nm) enters the tumor through passive diffusion taking advantage of the increased permeability of the tumor cell environment (Patel, 2011). Testing their nanoparticle on a taxol-resistant ovarian cancer cell line (SKOV-3TR), Patel and his colleagues showed a decrease in the IC_{50} value of paclitaxel from 2743 nM to 34 nM when treated with the loaded tariquidar liposome versus the free drug (Patel, 2011). Using pH-sensitive folate-targeted micelles loaded with doxorubicin, Lee et al. have also been able to overcome MDR in the doxorubicinresistant breast cancer cell line MCF-7/DOX^R (Oliver-ferrars, 2008). With an average size of 65 nm, these micelles exhibit an improved doxorubicin release at acidic pH of 6.8, though some doxorubicin release was observed at pH as high as 7.3 (Oliver-ferrars, 2008).

Using yet another approach, As_2O_3 precipitate loaded PEGylated 100 nm liposome developed by Ahn et al. termed nanobin NB (Ni, As) produced similar effects as to the free drug concerning cell survival, invasion, and migration of TNBC cells *in vitro* (Ahn, 2010). The effects exerted by the NB (Ni, As) were mediated partially through caspase activation (Ahn, 2010). Using athymic nude mice as an orthotopic

model of TNBC for *in vivo* studies, however, showed the vast difference between the free drug and the drug encapsulated in the liposome. Using 4mg/kg of the nanobins versus free drug given twice weekly by i.p. injection, Ahn et al. were able to report that no effect was seen with the free drug, while the nanobin significantly inhibited tumor growth, and that doubling the free drug concentration to 8 mg/kg did not inhibit tumor growth *in vivo* (Ahn, 2010; Lee, 2010). The effect of the nanobins was attributed to their ability to infiltrate and remain in the tumor longer than the free drug (Ahn, 2010; Lee, 2010). This drug depositing liposome has shown some promise for the treatment of TNBC.

Anti-EGFR morpholino antisense oligonucleotides (AON) nano-bioconjugate, designed to target the breast tumors, was developed by Inoue et al. (Inoue, 2012). These nanoparticles have an anti-transferrin receptor mAb covalently conjugated to a poly (β -L-malic acid) (PLMA) foundation (Inoue, 2012), which allows for passage across the membrane. The nanoconjugate is targeted to the cancer cell by the nucleosome-specific 2C5 mAb (Inoue, 2012). Testing on the highly overexpressing EGFR TNBC cell line MDA-MB-468, the 13 nm nanoconjugate was reported to inhibit the expression of EGFR at 5 μ M more significantly than the naked AON at 10 μ M (Inoue, 2012). *In vivo* studies with athymic nude mice revealed a 56% reduction in tumor volume after 45 days resulting from a robust decrease in EGFR expression and pAkt expression (a downstream target of the

pathway) as shown by western blot (Inoue, 2012). Multiple biomolecules can be conjugated to the PLMA platform at the same time to reduce tumor size, angiogenesis, invasion, and metastasis (Inoue, 2012). Previous reports of these nanoconjugates have stated their ability to inhibit glioma tumor growth and angiogenesis (Inoue, 2012), while a variant of the nanoconjugate has also been shown to inhibit tumor growth in HER2 positive breast cancers (Inoue, 2012). This study has also demonstrated the potential of using this nano-conjugate drug delivery system for the treatment of TNBC.

Metabolic Profile of TNBC Cells Could Provide New Treatment Opportunities via Biocompatible Nanoparticles

Cancer cells proliferate at a higher rate than normal cells creating a need for malignant cells to acquire sources of energy and cell building constituents far in excess of normal cells. Cancer cells accomplish these tasks via mutating growth factor receptors resulting in constitutive signaling of key metabolic pathways (Vander Heider, 2009). In addition to basic nutrients, cancer cells have an excessive need for cholesterol for membrane biogenesis (Vander Heider, 2009). One of the mechanisms that cancer cells use to meet this requirement is by overexpressing the high-density lipoprotein (HDL) SR-B1 receptor (Sabnis, 2010). Drug delivery strategies can take advantage of the excessive SR-B1 receptor function in cancer cells and tumors, by utilizing reconstituted high-density lipoproteins (rHDL) that carry anti-cancer agents, instead of cholesterol as their targeted payload (Lacko, 2006). The drug carrying rHDL nanoparticles thus function as a "Trojan Horse" and enhance the therapeutic efficacy of the enclosed drugs toward malignant tumors including TNBC (Lacko, 2006). The overexpression of the SR-B1 in malignant tissues (Lacko, 2006), has the potential to facilitate the enhanced selective delivery of anti-cancer agents to tumors (Lacko, 2006; Sabnis, 2012), thus providing a marked improvement of the current chemotherapy regimens, including limiting off target toxicity (Sabnis, 2012). Though the above-mentioned nanoparticles have potential, at the time of the review none of them are in clinical trials.

Conclusion

TNBC is a heterogeneous group of cancers with diverse histology, molecular profile and response to treatment (Rakha, 2009). Characterized by loss of expression of the estrogen receptor (ER), progesterone receptor (PR), and a lack of over-expression of the human epidermal growth factor receptor 2 (HER2), TNBC is a highly aggressive and metastatic disease with a very overall poor prognosis with a current five-year survival rate of less than 30%. Because the current therapeutic modalities for TNBC have only limited effectiveness, alternative therapies are urgently needed to improve the prognosis for TNBC patients. This review focuses on the potential of nanoparticles as effective enhancers of treatment for TNBC tumors. Some of the current nano-delivery formulations should have the ability to vastly improve the response rate of TNBC

patients by transporting the anti-cancer agents selectively to the tumors while bypassing MDR mechanisms. In addition, some of these nanoparticles have the capacity to reduce the exposure of potentially harmful drugs to the non-malignant surrounding tissues and thusmarkedly reduce the off target effects of chemotherapy.

Summary of Current Treatments of TNBC and Justification for the Need of Nanoparticle Based Therapy Development

TNBC is frequently associated with a poor prognosis often as a result of recurrence, and visceral and brain metastases within the first 5 years of the initial diagnosis (Bos, 2009; Hudis, 2011; Mezi, 2014), as well as the lack of targeted therapies (Chitnis, 2008; Grill, 2008). TNBC patients' initial response to chemotherapy is positive; however, their overall response to treatment is poor in comparison to HER2 positive patients. Thus TNBC patients have lower survival rates than HER2 positive patients (Lehmann, 2011). Below is a list of a few of the open United States clinical trials for TNBC involving combination therapy (see Table 3). A few of these trials are using antibodies against receptors, as well as FDA approved nanoparticles.

Clinical ID #	Target tested	Nanoparticle	Intervention	Phas
NCT01997333	gpNMB DNA	No	Glembatumumab Verdotin (antibody conjugated to the drug, verdotin, inhibits gpNMB - invasion and migration), Capecitabine	II
NCT02393794	HDAC DNA	No	Romidepsin Cisplatin	I/II
NCT02657889	PARP PD-1	No	Niraparib, Pembrolizumab (antibody against the programmed cell death 1 receptor- induces T- cell mediated immune response against tumor cells)	I/II
NCT01964924	Akt MEK	No	GSK2141795 Trametinib	II
NCT02425891	Microtubules PD-1	Yes	Nab-Paclitaxel (albumin bound pacliatxel nanoparticle), Atezolizumab (antibody against programmed cell death receptor 1)	III
NCT02593227	CD4 T DNA	Yes	Folate receptor α peptide with GM-SCF vaccine, Cyclophasphamide	II
NCT02322814	MEK Microtubules	No	Cobimetinib Paclitaxel	II
NCT02222922	PTK7 fungal cytochrome P450 enzyme	No	PF-06647020 (antibody against Protein tyrosine kinase receptor 7- inbiting metastasis), Fluconazole	Ι
NCT02158507	EGFR PARP	No	Lapatinib Viliparib	Pilot
NCT02315196	DNA DNA DNA Microtubules	Yes	Pegylated Liposomal Doxorubicin hydrochloride, Epirubicin hydrochloride, Carboplatin, Paclitaxel	Π

Table 3. Open clinical trials for TNBC with combination treatment (clinicaltrials.gov)

The preferred treatment for women with early-stage or locally advanced TNBC is taxane-based or anthracycline regimens; because they are associated with high rates of pathological complete response (Brown, 2014). Taxane-based drugs such as paclitaxel or docetaxel disrupt the microtubule function (Hagiwara, 2004). Anthracyclines are an antibiotic derived from *Streptomyces peucetius*. Anthracyclines—e.g., doxorubicin and its analog valrubicin, epirubicin, and idarubicin—are widely used anti-cancer drugs. They have been used against leukemia, lymphomas, uterine, ovarian, bladder, lung, and breast cancers (Osheroff, 1986). Despite their effectiveness with various types of cancer, anthracyclines are limited in their use because of the cardiotoxicity that they can cause (Oshheroff, 1986). Valrubicin in particular targets the topoisomerase II of the cardiac muscle; thereby blocking DNA transcription and replication (Pommier, 2010). For those patients suffering from metastatic disease, sequential single agents or combination therapies are used, however, no particular approach improves survival; however, combination therapy is associated with longer time to progression, more patients achieved an overall response and increased toxicity than sequential single agents (Brewster, 2014). Alternative targeted therapies are urgently needed to improve the prognosis for TNBC patients.

Because TNBC is a heterogeneous group of diseases with various biomarkers and

response to treatment the fight against TNBC may require subtype specific anticancer drugs, surgeries, and/or radiation treatments. However, one universal uniting characteristic in the fight against TNBC is the need to direct neoadjuvant, chemotherapeutic or post-surgical anti-cancer drugs to the site of infection. Scientists and clinicians have attempted to address this issue with the use of nanoparticles.

Nanoparticle Background

Nanomedicine has become a topic of intense scientific research because of its many applications. According to the national science foundation (NSF). In 2011, there were approximately 250 nanomedicine devices in pre-clinical and clinical development with mass production expected in 2020 (Marshall, 2011). Nanomedicine (the application of nanotechnology) requires the convergence of many disciplines including: medicine, molecular biology, pharmacology, and physics to manipulate atoms and molecules for the engineering of devices that are within the range of 1-100 nm (nanoparticles; see Figure 4) for the purpose of improving patient care (Bobo, 2016; McNeil, 2005). Nanotechnology has allowed for the production of nanoscale particles with many applications including bioimaging, contrast imaging, disease marking and tracking, and drug delivery (see

Figure 5; McNeil, 2005). With the advances in biophysics and systems biology, and because nanoparticles have relatively the same size has biological molecules the clinical applications may be limitless.



Figure 4: Comparison of nanoparticle size. The above figure compares the size of various nanoparticles about the size of other well-known objects. (Image taken from <u>www.wichlab.com/research;</u> Kamaly, 2012)

One of the principal obstacles clinicians faced with drug administration was maintaining the therapeutic level of the drug within the body over an extended period of time with the purpose of achieving a positive therapeutic outcome (Coelho, 2010). As drugs enter the body, they become vulnerable to attack and can be altered or removed before they reach the site of injury. When a drug reaches the cell, it must avoid the efflux pumps embedded in the cell membrane, because if it is a substrate for those pumps, it will be expelled out of the cell. Resultantly, drug accumulation at the active site of the disease was a challenging task for clinicians to accomplish efficiently. Therefore, dosages were increased to obtain a therapeutic effect, which leads to greater toxicities in most cases (Blanco, 2015). Emerging as early as the 1960's with the liposome (though not approved for clinical application until 1995), and polymer-drug conjugates (Coelho, 2010; Kamaly, 2012) nanoparticles as drug delivery systems provided solutions to enhancing positive therapeutic outcomes while limiting side effects. There are several advantages to the use of nanoparticles as drug delivery vehicles: (1) the ability of improving the potency of the drug without altering the drug itself, (2) enhance the efficacy of the drug by targeting the nanoparticle through active targeting, (3) allowing the drug to escape efflux pumps found in the cell membrane; thereby increasing the drug level within the tumor without increasing the dose, (4) the ability to minimize side effects associated with the drug alone, (5)

the ability for a controlled release of the drug into the tumor (Burgess, 2010; Coelho, 2010; Farokhzad, 2009). NPs as drug delivery vehicles provide an instrument for drug formulations to overcome limitations without altering the drug itself (Blanco, 2015).



Figure 5: Application of nanoparticles. Flow diagram depicting a few of the many possible clinical applications for nanoparticles.

Active and passive targeting nanoparticles

There are two approaches a nanoparticle can use to deliver its payload: either through passive or active targeting (Kamaly, 2012). Passive targeting involves NPs that contain no affinity ligand but do instead accumulate at the tumor as a result of their size, shape and charge in conjunction with the enhanced permeability and retention effect (EPR) (McNeil, 2005). EPR is a product of the excessive branching of the tumor vasculature, and the expanded space between endothelial cells resulting from a loss of tight junctions and a collapse of the basal membrane. Also, an impaired lymphatic system consequentially results in a clearance delay of macromolecules (Matsumura, 1986). Many of the passive targeting NPs have a polyethylene glycol (PEG) coated outer shell (Kamaly, 2012). A PEG coating allows the NP to avoid immediate phagocytosis by macrophages, dendritic cells and other white blood cells of the mononuclear phagocytic system (MPS; Torchilin, 1994; Weissleder, 2014) as well as avoid non-specific protein binding, resulting in prolonged blood circulation time of the drug. However there are a number of disadvantages to using passively targeted NPs: (1) Due to size heterogeneity of NPs some of these may be too large to enter the center of the tumor thereby releasing their payload into the tumor microenvironment, (2) Not all tumors have the same leaky vasculature; therefore, NPs may not be as active among different tumor types, (3) The interstitial pressure present at the core of the tumor may keep

the NPs out and thereby limit the amount of drug that will accumulate within the tumor (Chrastina, 2011; Hobbs, 1998; Jain, 2001; Nagamitsu, 2009). To bypass these complications adjuvants involved in increasing vasculature permeability, and blood pressure (vascular epithelial growth factor (VEGF), prostaglandins, bradykinin, transforming growth factor beta receptor (TGFβ) inhibitors) have been coadministered to facilitate aggregation of passive NPs inside the tumor (Nagamitsu, 2009; Kano, 2007; Monsky, 1999). However, to avoid these additional processes, active targeting NPs could be administered for a more productive tumor targeting and confinement resulting in an enhanced drug efficacy (Kamaly, 2012). NPs with affinity ligands on their surface utilize active targeting mechanisms to reach their destinations. Affinity ligands are usually directed toward proteins or antigens that are up-regulated on the cell surface or in the microenvironment of the tumor (Kamaly, 2012). Short peptides have also been used as ligands in targeted NPs with the advantage of being smaller in size, more stable, and thus less immunogenic, although their lower affinity for the target site represents a disadvantage versus other drug carriers. Active targeting NPs can be internalized via many methods including: "clathrin-coated pits, caveolin-assisted, cell adhesion molecules, or lipid raft-associated mechanisms all leading to endosomal formation" (Bareford, 2007). NPs developed into a more targeted system through the addition of fragment or whole antibodies, peptides, aptamers, carbohydrate moieties and other small molecules (Allen, 1987; Leserman, 1980;

Warenius, 1981). A timeline of the evolution of NPs is depicted in (see Figure 6) beginning with the first liposome approved for clinical use, doxil. Doxil was FDA approved in 1995, loaded with doxorubicin; this nanoparticle was utilized for the treatment of Acquired Immune Deficiency (AIDS) related Kaposi's syndrome. Though the therapeutic index for doxorubicin increased and cardiotoxicity reduced, doxil had a small value for the maximum tolerated dose (MTD) at 50mg/m^2 compared to free doxorubicin (MTD = 60mg/m^2 ; Kamaly, 2012). Though many of these targeting ligands have been conjugated to drug molecules or radioisotopes, conjugating antibodies to NPs has produced a 10^3 greater targeted drug delivery than ligand-drug conjugates (Graham, 2011; Qian, 2002; Sudimack, 2000).

NPs represent a drug delivery system that facilitates the solubility of otherwise lipophilic drugs, and thus increase their bioavailability and enhance their preferential tissue distribution toward cancer cells and tumors. The application of nanoparticles for drug delivery has advanced the development of safer and potentially more efficient drugs without the need for modifications of the drug payload.


Figure 6: Timeline of clinical stage nanomedicine firsts. The above diagram depicts a sample of clinically tested nanoparticles. (With permission, image taken from Kamaly, 2012)

In collaboration with MD Anderson Cancer Center, Houston, Texas, we have previously published the mRNA levels of the SR-B1 receptor in several human cancer cells and tumors (see Figure 7; Shahzad, 2011) were substantially overexpressed compared to that of normal tissues. Drug delivery strategies could benefit from the up-regulation of the SR-B1 receptor in cancer cells, by using HDL mimic carriers to transport anti-cancer agents to malignant cells and tumors instead of their targeted cholesterol ester payload (Lacko, 2006; McConathy, 2006). The rHDL nanoparticles can thus function as a "Trojan horse" (Lacko 2006) improving the efficacy of the encapsulated drugs toward malignant tumors including TNBC (Johnson, 2013). SR-B1 overexpression in cancer cells and tumors thus has the potential to facilitate the selective delivery of anti-cancer agents to tumors (Sabnis, 2012; Vander-Heiden, 2009) providing a marked improvement of the current chemotherapy regimens, via limiting off-target toxicity. In these studies, we have used combination treatment against TNBC with SR-B1 targeted nanoparticles loaded with lapatinib and valrubicin.



Figure 7: Expression of SR-B1 mRNA in different human organs and tumors using RT-PCR. Actin is used as a loading control (With permission, image taken from Shahzad, 2011).

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Lapatinib ditosylate

Lapatinib ditosylate (C₄₃H₄₂CIFN₄O₁₀S₃, CAS # 388082-77-7) also called tykerb, tyverb, and GW-572016 is a yellow powder at room temperature. It is soluble in dimethylsulfoxide (DMSO), and slightly soluble in water and ethanol. Lapatinib ditosylate has a 24H biological half-life and a molecular weight of 925.46 g/mol, a density of 1.381g/cm³, and a melting range of 240-2420 °C. (see Figure 8; EMA, 2008). Lapatinib is a 4-anilinoquinazoline reversible kinase inhibitor with a dissociation half-life of \geq 300 minutes (EMA, 2008). It works by competing with the ATP binding site on receptor tyrosine kinase (RTK), thereby preventing autophosphorylation and activation of downstream signaling of the receptors. Lapatinib is specific for EGFR and HER2 (EMA, 2009; see Figure 9) with IC₅₀ values of 0.1µM and 3µM, respectively. Because lapatinib's effects are reversible, the drug is cytostatic. It is a target of the efflux pump permeability glycoprotein (P-gp); therefore, resistance does develop with continued use. It is metabolized by the cytochrome P_{450} 3A4 and 3A5 (Cyp3A4/5) and excreted through the feces (EMA, 2009).



Figure 8: The chemical structure of Lapatinib.

In 2007, the FDA approved Tykerb, a product of GlaxoSmithKline

Pharmaceuticals, lapatinib for women with metastatic breast cancer that is overexpressing HER2, who have previously received anti-cancer drug treatment, but only in combination with xeloda (active ingredient capecitabine; NCI, 2011). Lapatinib has also been shown to be beneficial when used in combination with letrozole (an aromatase inhibitor) trade name femara, a product of Novartis Pharmaceuticals, in post-menopausal women with hormone positive metastatic breast cancer who overexpress HER2 in whom hormone therapy is indicated (NCI, 2011). FDA approval for combination treatment of lapatinib with letrozole was given in 2010. Lapatinib is prescribed at $1250 \text{mg/m}^2/\text{day}$ in a 21-day cycle of combination therapy for the first 14 days, then Lapatinib alone for the next seven days (NCI, 2011). The cycle is repeated until the patient is disease free or an unacceptable level of toxicity has been reached (EMA, 2008). It has been indicated, but further study is needed to confirm that lapatinib can reduce the incidence of brain metastasis in HER2 positive breast cancer patients. Nine percent of those receiving lapatinib alone or in combination with capecitabine experienced \geq 50% reduction in their brain tumor size. In the United States, lapatinib is not currently FDA approved for use against TNBC. This drug comes with two warnings of major concern: hepatotoxicity and a decrease in left ventricular ejection fraction (LVEF). Although diarrhea occurred in \geq 58% of patients tested, it was not severe enough to stop treatment (EMA, 2008).

A.



Figure 9: EGFR and HER2 structure and therapeutic targets. The above image depicts the structure of both the epidermal growth factor receptor (EGFR) and the second member of the family human epidermal growth factor receptor 2 (HER2). There are currently, no known ligands for the HER2. HER2 forms heterodimers with the other members of the EGFR family. Once a ligand binds to EGFR it undergoes homo or heterodimerization and autophosphorylation at its tyrosine residues located in the cytoplasmic domain of the receptor. The red boxes list some of the antibodies and tyrosine kinase inhibitors FDA approved for use against EGFR and HER2. Lapatinib binds both EGFR and HER2 and acts as a competitive inhibitor for the ATP binding sites on both HER2 and EGFR. (With permission, image taken from Tebbutt, 2013).

Clinical ID	Target	Nanoparticle	Intervention	Phase	Purpose
NCT01875666	HER2 HER2 HER2	No	Pertuzumab (antibody against heterodimerization) Trastuzumab (antibody against homodimerization) Lapatinib (against ATP binding)	Pilot	Kinome identificatio
NCT02650752	HER2 DNA	No	Lapatinib Capecitabine	Ι	CNS metastasiso HER2+ brea cancer inhibition
NCT02073487	HER2 Microtubules Microtubules HER2 HER2/ Microtubules	Yes	Lapatinib (against ATP binding) Abraxane (paclitaxel nanoparticle) Paclitaxel Trastuzumab (antibody against homodimerization) Pertuzumab (antibody against heterodimerization) Trastuzumab Emtansine (antibody-drug conjugate)	Π	Combinatio Therapy for HER2+ brea cancer
NCT01283789	EGFR mTOR	No	Lapatinib Everolimus	II	Combinatio Therapy for TNBC

Table 4. Open clinical trials for Lapatinib in combination therapy (clinicaltrials.gov)

Valrubicin

Valrubicin ($C_{34}H_{36}F_3NO_{13}$, CAS # 56124-62-0), also called N-

trifluoroacetyladriamycin-14-valerate, and Valstar is a reddish –orange powder at room temperature (OSHA, 2012). It is soluble in DMSO, ethanol, and acetone. Valrubicin has a molecular weight of 723.64 g/mol and a melting range of 116-136 °C. It is a second-generation anthracycline, a class of drugs known as antitumor antibiotics (OSHA, 2012). Valrubicin is the semisynthetic analog of doxorubicin, sold as anthramycin; however, it is more lipophilic (Sabnis, 2012). Both drugs are products of Anthra Pharmaceuticals (see Figure 10). In 1998, the FDA approved Valrubicin for use in patients where in situ carcinoma of the bladder that has been confirmed by biopsy, Bacillus Calmette-Guerin (BCG) immunotherapy is ineffective, and surgical removal of the bladder is not recommended. Valstar is sold as a 200mg/5ml sterile solution for intravesical treatment. The recommended dose is 800mg once a week for a six-week period. If within three months, cancer cells are still detected, then cystectomy is reconsidered (OSHA, 2012). Valstar is effective in only one of five patients.

Unlike doxorubicin, valrubicin passes through the cell membrane and accumulates in the cytoplasm. Within the cell, valrubicin is rapidly metabolized into AD41 (N-trifluoroacetyladriamycin), which localizes to the nucleus where it functions as an anti-tumor agent by interfering with the incorporation of nucleosides into Deoxyribose nucleic acid (DNA) and Ribose nucleic acid (OSHA, 2012; RNA). It binds topoisomerase II after it has initiated cleavage of the nucleic acid; thereby preventing ligation and causing chromosomal damage, decreasing cell proliferation and survival (Anderson, 2010). Valrubicin is not currently FDA-approved for use in breast cancer; however, rHDL encapsulated valrubicin and its effects on ovarian and prostate cancer have been previously described (Sabnis, 2012). Unfortunately, it is believed that valrubicin targets topoisomerase II in the cardiac muscle; therefore, valrubicin's dose and usage are limited as a result of its cardiotoxicity making it a worthy candidate for SR-B1 targeted nanoparticle therapy.





Valrubicin

Doxorubicin

Figure 10: Chemical structure of the drug doxorubicin and its semisynthetic analog valrubicin. The green circles indicate the two moieties that were changed on doxorubicin to make valrubicin.

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CHAPTER II

PROJECT HYPOTHESIS AND SPECIFIC AIMS

Our synthetic/rHDL nanoparticles contain the same constituents as biological HDL particles. Cholesterol esters and free cholesterol are in the core along with the drug of choice. Phospholipids coat the outside embedded with free cholesterol and the apo-AI protein (see Figure 12). The average size of our nanoparticles range from 10-50nm (Sabnis, 2012). The natural composition and small size of our rHDL nanoparticles make them biocompatible and non-immunogenic which is an advantage over similar drug delivery systems (Sabnis, 2012). We have previously reported the remarkable stability of our rHDL nanoparticles after ultracentrifugation and storage up to 6 months at 4°C (McConathy, 2008). Made through sodium cholate dialysis, our rHDL nanoparticles are spherical. We have previously published the effectiveness of our rHDL particles encapsulated with drug. In McConathy *et al.* has shown that rHDL nanoparticles loaded with paclitaxel were more effective at inhibiting cell survival of MCF-7 breast cancer cells (80% kill) than the free paclitaxel (30% kill; McConathy, 2008). The IC_{50} values for the rHDL loaded paclitaxel nanoparticles against MCF-7 cells were 15 fold lower than the free paclitaxel (McConathy, 2008)). Without question some

of the most challenging dynamics influencing efficacy of cancer chemotherapy treatments includes poor bioavailability, toxic side effects and drug resistance of cancer cells (Kamaly, 2012). Our rHDL nanoparticles have the potential to increase the efficacy of effective hydrophobic drugs against cancer by delivering them directly to the cancer cell without obstruction from the immune system. In this aim, we have attempted to answer the question of which drugs to select for loading into the rHDL nanoparticle to effectively inhibit cell survival and metastasis of TNBC. The majority of patients diagnosed with breast cancer have access to targeted therapies. HER2+ patients may receive trastuzumab, while ER+ and/or PR+ patients may receive endocrine therapy (Hudis, 2011). In contrast, no targeted therapy is currently available for TNBC patients. **The**

TNBC subtype represents an unmet medical need, and there is currently an increasing interest to develop targeted therapies for this subtype (Hudis, 2011).

At initial diagnosis, TNBC often presents as stage III ductal carcinoma (Bauer, 2007; see Figure 11). At this juncture, there will be lymph node involvement with the potential for brain and lung metastasis (Bauer, 2007). Tumors overexpressing EGFR have high cell proliferation rates, inhibition of apoptosis, chemoresistance, increased angiogenesis, invasion, and metastasis (Inoue, 2012). Sixty percent of basal-like tumors overexpress EGFR as do ~70% of TNBC tumors (Peddi, 2012;

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Doru, 2010). EGFR has also been shown to promote migration of prostate and renal carcinoma, and the TNBC cell MDA-MB-231 through the PI3K/Akt pathway (see Figure 3; El Guerrab, 2011). EGFR has therefore become a key target for the treatment of TNBC (Novy, 2012; Nowsheen, 2012). We anticipated that reconstituted high-density lipoprotein (rHDL) nanoparticles containing lapatinib or valrubicin would have the ability markedly to improve the effectiveness of the individual drugs and drug combinations by transporting the anti-cancer agents selectively to the tumors while bypassing the multi-drug resistance (MDR) mechanisms.



Cancer in 1 to 3 lymph nodes in the axilla or near the breastbone

Figure 11: Stage IIIA breast cancer. Female breast depicting the possible medical condition associated with Stage IIIA breast cancer. National Cancer Institute 2012, Terese Winslow LLC. U.S. Gov't has certain rights.

The overall goal of this research was to determine the effectiveness of reconstituted high-density lipoprotein (rHDL) nanoparticles as a drug delivery system against triple-negative breast cancer (TNBC). There are several advantages to rHDL nanoparticles: (1) optimum size for *in vitro* and *in vivo* studies (40-50nm; Jiang, 2008), (2) biocompatibility (3) the particle is not endocytosed as some nanoparticle, (4) because of their small size and biocompatibility they have not been shown to illicit an immune response, (5) targeted to the HDL/ (SR-B1) receptor through their apo-AI (Lacko, 2002). The rHDL nanoparticles were loaded with hydrophobic, anti-cancer drugs that (as free drugs) had been shown to limit therapeutic effectiveness, due to toxicity or lack of bioavailability (see Figure 12). Targeting these drugs to pre-malignant and malignant cells also restrict the exposure of potentially harmful drugs to the surrounding healthy tissue. Consequently, the studies presented here fill a void in the treatment of TNBC via the use of rHDL nanoparticles. To determine the effectiveness of the rHDL nanoparticles, we have conducted studies using TNBC cells (MDA-MB-231) and because of the cardiotoxicity of valuation mentioned earlier we tested the healthy cells (cardiomyocytes, H9C2), and compared the impact of the free drugs with that of the rHDL-encapsulated drugs on the respective cell lines.

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Figure 12: rHDL nanoparticles. The above is an illustration of the similarity between the endogenous HDL molecule and the rHDL nanoparticle. The image indicates the main difference is the core of the particles.

Several drugs and drug combinations were evaluated for their efficacy against TNBC and their suitability for the rHDL nanoparticle. Those drugs that had an effect on the EGFR pathway were targeted, and lapatinib was ultimately chosen (see Figure 13). Consequently, the combination of valuation valuation and lapatinib was selected for these studies. The drugs used (lapatinib and valrubicin) have yet to be approved by the FDA for TNBC treatment, though lapatinib is in a phase II clinical trial in combination therapy with everolimus for TNBC treatment (see Table 4). Valrubicin is not currently in a clinical trial for any disease in the U.S. However, because of its previous success in ovarian cancer pre-clinical assessment within our lab (Sabnis, 2012), It was evaluated in these studies. To date, there has been no published pre-clinical data on the combination of these two drugs. Consequently, this study is unique regarding the use of these drugs, alone or in combination, and when encapsulated into rHDL nanoparticles.



Figure 13: Drugs attempted for combination therapy: The above diagram depicts the various therapeutic drugs that were evaluated for their potential effectiveness at killing TNBC cells alone and in combination.

Hypothesis: Lapatinib and valrubicin encapsulated in rHDL nanoparticles will increase the efficacy of these drugs alone and in combination against TNBC. Simultaneously, lower cytotoxicity against normal cells is anticipated upon encapsulation of the particular drugs in rHDL nanoparticles.

Specific Aim 1: To determine the expression level of SR-B1 in TNBC and cardiomyocyte cell lines MDA-MB-231 and H9C2, respectively.

Specific Aim 2: To evaluate the effectiveness of valuation loaded rHDL nanoparticles against TNBC cell line MDA-MB-231 and its protective abilities against cardiomyocytes.

Objective 2.1: To determine if SR-B1 is binding the rHDL nanoparticles in TNBC cell lines.

Objective 2.2: To determine if the free drug and rHDL formulation of valrubicin will inhibit cell proliferation and survival in TNBC cells.

Objective 2.3: To determine the mechanism of action of lapatinib and valrubicin rHDL encapsulated nanoparticles in the TNBC cells.

Specific Aim 3: To determine if combination therapy can be more effective than single drug therapy against TNBC while remaining protective to healthy cells.

Objective 3.1: To determine if the free drug lapatinib alone will inhibit cell proliferation and survival in TNBC cells.

Objective 3.2: To determine if the rHDL form of lapatinib is more efficient than the free drug.

Objective 3.3: To determine if the rHDL formulation of valuability and lapatinib can work together as a targeted therapy to inhibit TNBC cell proliferation.

CHAPTER III

MATERIALS AND METHODS

<u>Cell culture and chemicals</u>: MDA-MB-231 TNBC (Subik, 2010; ATCC), were grown in 75-cm² flasks with DMEM high glucose media with phenol red (Invitrogen Life Technologies, USA), supplemented with 10% FBS. H9C2 cardiomyocyte cells are ER+ (Al-khlaiwi, 2005; Urata, 2001), were grown in 75cm² flasks with DMEM with phenol red (Invitrogen Life Technologies, USA), supplemented with 10% FBS, at 37°C in a humidified incubator at 5% CO₂. Lapatinib ditosylate at 5mg/ml (Selleckchem, USA) and valrubicin at 5mg/ml (Koning, China) were kept at -20° C.

Determination of IC50 doses (cell viability): The effect of lapatinib and valrubicin in the free drug was determined using the cell counting kit-8 (CCK-8) kit (Dojindo Molecular Technologies, China). H9C2 and MDA-MB-231 were counted using the automatic counter cellometer (Nexicom, USA) and 6000 cells/ well were seeded into 96-well microtiter plates and incubated at 37°C in 5% CO₂ overnight. Free drug formulations were diluted in serum-free media to yield the concentrations (0.5μ M – 8μ M) for the cell viability tests. Controls include cells not treated with the drug. Cells were incubated at 37°C in 5% CO₂ for 72h. After incubation, 10 μ L of a tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium,

monosodium salt] stock solution (Dojindo) was added to all wells. After 3h of incubation at 37°C, the absorbance at 450nm was measured using a Power Wave 340 microplate reader (Biotek, USA). There were six replicates for each concentration. Each experiment was repeated at least 3 times. The same procedures were followed for combination drug assays.

Western blotting analysis (data found in Chapter VI): MDA-MB-231 cells were plated at 250,000/cells in a six-well plate and treated following the same process as cell viability. Cells were lysed in 1X NP-40 lysis buffer containing Nonidet P40 (NP-40) 1.0% NaCl, 150mM, Tris-Cl- 50mM (pH 8.0), 0.1% Sodium Dodecyl Sulfate (SDS) 1mM Phenylmethanesulfonyl fluoride (PMSF), 5mM ethylenediaminetetraacetic acid (EDTA), and 2µg/ml aprotinin. The protein concentration of the lysates was measured with a bicinchoninic acid reagent (BCA; Thermo Scientific, USA) at 540nm. Lysates were boiled at 75°C in 4X sample buffer containing 40% glycerol, 8% SDS, 2.5% 1 mM Tris–HCl (pH 6.8), 20% 2-mercaptoethanol, 0.8% bromophenol blue slurry (5mg/ml in water) for ten minutes. Proteins were resolved by SDSpolyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) transfer membrane using the iBlot2 system (Life Technologies, USA) for western blotting. Membranes were probed with monoclonal rabbit LC3B, H2AX, pH2AX (Cell Signaling, USA) at a 1/1000

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dilution and rabbit polyclonal β -actin (Cell Signaling, MA) at a 1/1000 dilution as a loading control. Anti-rabbit IgG HRP (Cell Signaling, MA) at a 1/5000 dilution was used as secondary antibodies. Western blots were developed using the West Femto enhanced chemiluminescence detection kit (Thermo Scientific, USA).

Flow cytometry analysis of SR-B1 expression. Cells were plated at 750,000 cells in a 100mm Petri dish. Cells were collected and washed with 1X PBS twice followed by a wash with fluorescence-activated cell sorter (FACS) buffer (1X PBS, 2% FBS, 0.1% sodium azide). Next cells were seeded in a 96 round bottom plate at 1 X 10^6 cells/well. Cells were blocked with anti-goat serum for 30 minutes at room temperature, and then treated with 1/50 dilution of SR-B1 antirabbit monoclonal antibody in FACS buffer for 30 minutes at room temperature. Cells were then washed in FACS buffer 2 times. Followed by incubation with the secondary goat anti-rabbit Alexa fluor 488 antibody at 1/2000 dilution for 30 minutes in the dark. Cells were pelleted and washed in FACS buffer. Cells were then analyzed using the Beckman Coulter FC500 Cytometer (Beckman Coulter, USA)._

<u>Nanoparticle Size Estimation</u>: rHDL/valrubicin and lapatinib nanoparticle size estimation was determined using Dynamic light scattering (DLS). Particle size analysis of the rHDL-valrubicin and rHDL-lapatinib nanoparticles was carried out using a Nanotrac system (Microtrac Inc, Montgomeryville, PA) as per manufacturer's instructions. Nanoparticle samples diluted in water were measured for particle size.

Flow cytometry analysis for apoptosis determination: Cells were plated at 750,000 cells in a 100 mm corning Petri dish. Cells were treated with lapatinib or rHDL-lapatinib at 2µM or left untreated. Four additional controls were used: hollow rHDL particles, propidium iodide (PI) only, Annexin V only, Annexin V and PI. Both floating and adherent cells were collected by trypsinization and washed with 1x PBS 3 times. Cells were given a final wash in 1x binding buffer. Cells were then incubated in the dark at room temperature with 5µl of Annexin V for 20 minutes, then pelleted at 3100g for 1 min 30 seconds. Cells were then washed in binding buffer and re-pelleted. Cells were then incubated in the dark at room temperature for 15 minutes with 5µl of 100µg/ml stock PI. Then pelleted at 3100g for 1 min 30 seconds. Cells were then washed in binding buffer and repelleted. Cells were then resuspended in binding buffer and placed on ice. Cells were analyzed using the Beckman Coulter FC500 Cytometer (Beckman Coulter, USA)._

<u>Preparation of rHDL/lapatinib and rHDL/valrubicin complexes</u>: A mixture of egg yolk phosphatidylcholine (PC; 7.5mg), cholesterol (1.75 x 10⁻¹ mg), and

cholesterol oleate $(7.5 \times 10^{-2} \text{ mg})$ in chloroform is prepared, and the chloroform evaporated under nitrogen gas to a thin film. 60µl DMSO for a 2 ml preparation is added to a mixture of apo A-I (2.5mg/ml), 140µl sodium cholate (from a stock of 100mM), and 1mg/ml of the individual drug. A final volume was made up to 2mL with sodium cholate buffer (10mM Tris, 0.1M KCl, 1mM EDTA pH 8.0). The cholate/lipid/protein mixture was then frozen in the -80°C freezer overnight. Preparations were then lyophilized until dry, followed by dialysis against 2L of PBS, for 48h, with three buffer changes in the first 6h. After dialysis, the preparations were then centrifuged at 3300g for 1 minute, and the supernatant kept. The nanoparticles are then syringe filtered through a .45µm pore filter (Millipore, USA). The preparations were kept at 4°C until used.

Determination of Drug Entrapment Efficiency (DEE): for valuability of Determination of Drug Entrapment efficiency (DEE): for valuability of valuability of drug in the nanoparticles before the cholate dialysis step mentioned earlier (initial drug) and after filtration (final drug). In a 96 well plate, a range of rHDL-valuability valuability of the power wave 340 plate reader the absorbance was read at 490nm. rHDL-lapatinib nanoparticles (5-20µl) were mixed with PBS/1% Triton 100 and read using a spectrophotometer at 362nm. DEE = {Drug concentration after dialysis/Drug concentration before dialysis} ×100.

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<u>Preparation of rHDL/³H-Cholesteryl oleate complex</u>: A mixture of egg yolk phosphatidylcholine (PC) in chloroform with labeled cholesterol oleate (³CE), and cholesteryl oleate (CE) were prepared with a molar ratio of apo-A1: ³CE: CE: PC = 1:5:1.3:1.15 M. Chloroform was evaporated from (PC, ³CE, and CE) under nitrogen gas to a thin film and rehydrated in 60µl DMSO for a 2ml preparation. To this mix, apo A-I (2.5mg/ml) and 140µl sodium cholate (from a stock of 100mM) were added with Sodium cholate buffer (10mM Tris, 0.1M KCl, 1mM EDTA pH 8.0) to make a volume of 2mL. The cholate/lipid/protein mixture was then frozen in the -80°C freezer overnight. Preparations were then lyophilized until dry, followed by dialysis against 2L of PBS, for 48H, with 3 buffer changes in the first 6H. After dialysis, the preparations were then centrifuged at 3300g for 1 minute. The preparations were kept at 4°C until used.

<u>rHDL Nanoparticle Characterization</u>: Determination of chemical composition of the rHDL nanoparticles: BCA protein assay kit (Pierce, USA) was used to determine protein concentration. Cholesterol and phospholipid contents were determined by respective enzymatic reagent kits (cholesterol E and phospholipid C; Wako chemicals, USA) using microtiter plate assays as per manufacturer's suggestions. All assays were read on the Power Wave 340 microplate reader (Biotek, USA) at the appropriate wavelength. <u>Uptake studies</u>: rHDL nanoparticles were made using ³H labeled cholesteryl oleate 1mCi/ml (American Radiolabeled Chemicals, USA). MDA-MB-231 cells were treated with a range of block lipid transport (BLT-1) concentrations (0.005mg-0.5mg). Cells were plated in 12-well plates (75,000 cells/well) overnight. The cells were then washed twice with PBS, pH 7.4 and incubated at 37°C with serum-free media for 90 minutes. Cells were washed with PBS and incubated with a single concentration of the ³H /rHDL complex for 90 minutes. The cells were washed once with 1x PBS, pH 3.0 followed by a wash with 1x PBS, pH 7.4. The cells were lysed with lysis buffer and protein concentration determined using the BCA reagent. The ³H /rHDL content was measured using a scintillation counter.

<u>Confocal Microscopy.</u> MDA-MB-231 cells (7.5 X 10⁴ per well) were seeded onto poly-lysine coated 12mm coverslips (Corning, USA) in a six-well plate (Fisher Scientific, USA) and incubated overnight at 37 framework at mosphere of 5% CO₂. The Zeiss LSM 510 took the confocal image of the cells at 40x magnification, at excitation/emission wavelengths of 488/518nm for SR-B1 and 488/535nm valrubicin, and 405/461nm for DAPI, and 650/668nm for actin phalloidin. *SR-B1 images* were obtained from MDA-MB-231 cells that were seeded overnight as previously mentioned. The cells were then washed 3 times with PBS then blocked with 5% anti-goat serum at room temp for 1hour. Cells were then incubated with a 1/50 dilution of the SR-B1 antibody (Abcam, USA) at

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room temperature for 1h. Following incubation cells were washed 3 times with PBS containing 1% FBS followed by incubation with the Alexa fluor 488 secondary antibody (Thermo Fisher, USA), for 1h in the dark at room temperature. Cells were then washed 3 times with PBS containing 1% FBS. The coverslip was removed from the six-well plate and mounted on microscope slides with DAPI antifade mounting serum (Molecular Probes, USA). BLT-1 uptake *images* MDA-MB-231 cells were pre-treated with a range of block lipid transport (BLT-1) concentrations (0.005mg-0.5mg) for 30 minutes. After pre-treatment, the cells were then treated with rHDL valrubicin or free valrubicin at 8µM and incubated at 37°C with serum-free media for 3H. Cells were then washed 3 times with PBS containing 1% FBS. The coverslip was removed from the six-well plate and mounted on microscope slides with DAPI antifade mounting serum (Molecular Probes, USA).

<u>Statistical analysis.</u> All experimental data were confirmed with three independent experiments. Experimental error bars display the standard error. All p-values were calculated at a 95% confidence interval. Differences in cell viability IC_{50} values were evaluated using the Graph Pad Prism software. Comparing rHDLlapatinib vs. the unencapsulated form, and valrubicin rHDL vs. free valrubicin and the combination of the two drugs in rHDL and unencapsulated forms. The comparisons were made in TNBC cells and cardiomyocyte. The unpaired twotailed student's t-test was used to evaluate IC_{50} values for cell viability test. P-
values were considered statistically significant when less than 0.05. The paired student's t-test was used to assess the difference in the radioactivity in the uptake study at all concentrations of BLT-1. Image J software (Wayne Rasband, NIH) was used to determine per cell fluorescence intensity of the confocal images. Fluorescence intensity was displayed as corrected total cell fluorescence (CTCF) CTCF= (Integrated density-(area of the cell * Integrated density of the background)). The Student's test was used to compare the differences in fluorescence intensity in valrubicin rHDL treated TNBC and un-encapsulated valrubicin. As well as in comparing rHDL-valrubicin treated TNBC vs. rHDLvalrubicin + BLT-1 treated TNBC. Survival statistics were determined and depicted as mean and median survival. Survival percentage was determined using a linear regression for all TNBC cells treated with rHDL nanoparticle vs. cells treated with free drug formulations. Data is graphed as percent survival vs. concentration.

References for methods

Sabnis, N.; Nair, M.; Israel, M.; McConathy, W.; Lacko, A. Enhanced solubility and functionality of valrubicin (AD-32) against cancer cells upon encapsulation into biocompatible nanoparticles. Int. J. Nanomed. 2012, 7, 975-983

CHAPTER IV RESULTS

SR-B1 Expression

As a result of the work done at the University of Texas MD Anderson Cancer Center on the mRNA expression levels of SR-B1 in various tissues and cancer cell lines (see Figure 7), the triple-negative breast cancer cell line, MDA-MB-231, was chosen for these experiments. However, mRNA levels do not always equal protein levels. Therefore, to determine the protein expression levels of SR- B1 in TNBC and healthy cells not previously tested, the MDA-MB-231 cell line and the H9C2 cell line were probed for SR-B1 (see Figure 14), using the rabbit monoclonal antibody. In Figure 14, the purple line represents the level of auto- fluorescence that the cells emit naturally; the red line represents the mean fluorescence intensity as a result of the SR-B1 probe. The amount of fluorescence is equivalent to the amount of SR-B1 protein present in the cell. As a result, the fluorescence intensity shifts to the right. The cardiomyocytes show almost no variation in fluorescence intensity, while the MDA-MB-231 cell line does show a significantly (t= 17.26; p < 0.01) greater shift in fluorescence intensity indicating that the TNBC cell line, unlike the cardiomyocytes, expresses the SR-B1 protein. SR-B1 expression was further analyzed in the MDA-MB-231 cell line using confocal microscopy (see figure 15) to certify the previous results. The green color indicates SR-B1 protein expression the blue-stained nucleus was done by using DAPI. The results show not only the presence of SR-B1 but also that it is present in the cell, not localized to one area. 94



Figure 14: Flow cytometry analysis of SR-B1 expression in malignant and

normal cell lines. The figure on the left represents mean fluorescence intensity. A mAb for SR-B1 at a 1/100 dilution was used to determine SR-B1 protein expression in both cell lines. The purple line represents the auto-fluorescence the cells emit and the red line represents the concentration of SR-B1 in the cell. The bar graph on the right shows the quantitative mean n=3. Error bars indicate a significant difference. *Indicates (t=17.26; p < 0.01). Statistical analysis was performed using the student's t-test with free version of Graph Pad software.



Figure 15: Confocal analysis of SR-B1 expression. SR-B1 expression was also assessed in the MDA-MB-231 cell line using a mAb for SR-B1 at a 1/50 dilution. The blue stain of the nucleus is done with DAPI. The green fluorescence indicates the SR-B1 protein present in the cell line.

Characterization of the SR-B1 targeted rHDL- value val Because SR-B1 protein expression was present in the MDA-MB-231 cells, we proceeded to make the rHDL nanoparticles (see Figure 16). Valrubicin was chosen as a drug to encapsulate into the particle because value value value into the particle because value value into the particle because value value into the particle because value va of a very successful chemotherapeutic drug, doxorubicin. Doxorubicin has also been previously encapsulated into a liposome and is currently FDA approved for use (Kamaly, 2012). However, the doxorubicin liposome (Doxil) can still cause congestive heart failure one of the reasons being the liposome is not targeted. Therefore, because of previous pre-clinical success valubicin rHDL has had with ovarian cancer (Sabnis, 2012), we have chosen it to begin these studies. Valrubicin nanoparticles were made using cholate dialysis. The chemical composition of the rHDL-valrubicin nanoparticle was determined using luminescent assays for cholesterol and phospholipid. Protein and drug content were determined using the power wave 340 plate reader. Figure 16 and Table 5 demonstrate the relative percentages of each compound within the nanoparticle. Dynamic light scattering indicates that the majority of the nanoparticles are of the same size, and that size is within the acceptable range for *in vitro* work with nanoparticles (Jaing, 2008). Results show a negative zeta potential and an incorporation efficiency of 86%. These data indicate that the construction process for rHDL-valrubicin is efficient and because of its negativity it may escape opsonization once inside the body. To visualize the actual nanoparticles,

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an image of rHDL-valrubicin was taken (see Figure 17) using a fluorescent microscope under 40X magnification. The image reveals the red color of the nanoparticles due to the red color of the free valrubicin drug. The image also shows that the particles are spherical in shape. In some cases the form of nanoparticles is discoidal or non-spherical. However, spherical nanoparticles are better suited for SR-B1 targeting (Liadaki, 2000).

Туре	rHDL components mg/mL (%)				
of particle	Protein	Phospholipid	Cholesterol	Drug	
rHDL/valrubicin	2.46 (32)	3.72 (49)	0.15 (2)	1.29 (17)	

Table 5. Chemical composition of rHDL nanoparticle



Valrubicin

В.

A.

Valrubicin rHDL	
Incorporation efficiency	85%± 3.45
Zeta Potential	96.43mV ±30.6
Dynamic Light Scattering	52nm

Figure 16:rHDL valrubicin nanoparticle composition and size. A. Shows the chemical composition of the nanoparticles encapsulated with valrubicin. The composition of the nanoparticles was determined using colorimetric assays for cholesterol and phospholipids. Drug concentration was measured using a power wave plate reader at 490nm; protein was measured using a BCA kit. **B**. Figure B is a chart showing the relative size of the nanoparticles as determined by dynamic light scattering, the intensity of the negative charge and the efficiency of the nanoparticle construction process.



A.

Figure 17: Fluorescent image of valrubicin rHDL nanoparticle. A 40X image of valrubicin rHDL nanoparticles showing the spherical shape and red color of the particles. The relative size of the particles ranges from 20nm to 60nm.

Cytotoxicity of the rHDL-Valrubicin Nanoparticle against TNBC and Cardiomyocytes

To determine the effectiveness of valrubicin against TNBC cell viability assays were executed using free and encapsulated valrubicin. Because valrubicin is a known cardiotoxic drug and is thereby limited in its use and delivery route, we wanted to test the protectiveness of rHDL-valrubicin vs. valrubicin alone. Therefore, we have also tested the drug valuation on cardiomyocytes. Untreated cells were used as controls. The cell viability was determined over a 72h period using a cell counting 8 kit (CCK8). The IC_{50} values for the drug alone vs. encapsulated for the TNBC cell line and the cardiomyocytes are displayed in (Table 6). IC_{50} numbers were calculated using the Sigma Plot software (Systat, USA). In the TNBC cell line, the IC_{50} for rHDL-valrubicin vs. free valrubicin was 2.29µM, and 3.47µM respectively. These values are significantly different (t=2.99, p<0.05) indicating the greater potency of the rHDL-valrubicin over the free drug (see Figure 18). The rHDL-valrubicin reached IC_{50} at a lower concentration than the free drug indicating that the encapsulated form of the drug is more effective than the free version the opposite was observed in the cardiomyocytes. The free drug reached an IC_{50} at a lower concentration than the encapsulated form. Indicating that the rHDL-valrubicin maybe more efficient than the free drug in treating TNBC while still limiting side effects. The IC_{50}

values for rHDL-valrubicin vs. free valrubicin in cardiac cells were significantly different (t= 13.43, p < 0.001).



Figure 18: Valrubicin treatment of MDA-MB-231 TNBC cell line at 72H valrubicin at 4μ M in free versus encapsulated drug. Viability was determined by using a CCK8 assay. A. Graph depicting empirical data of 72H valrubicin treatment in the MDA-MB-231 cell line. There were six samples for each dose. Three independent experiments were performed. Statistical analysis was performed using the student's t-test with the free version of Graph Pad Software.

Cell Line	IC50 rHDL (µM)	IC50 free (µM)	Drug Used
H9C2	7.53 ± 0.01	4.67 ± 0.39	Valrubicin
MDA-MB-231	2.29 ± 0.39	3.47 ± 0.06	Valrubicin

Table 6. Cytotoxicity of valuation vs. encapsulated form on TNBC and cardiomyocytes

Validation of SR-B1 method

With the significance of the viability results from the 72H cytotoxicity experiments above, the question arises whether these SR-B1 targeted nanoparticles are exerting their effects through the SR-B1. To further validate the use of the TNBC cell line MDA-MB-231 and to determine if the drugs encapsulated into the rHDL nanoparticles are delivered to the cell as a result of uptake with the SR-B1, we used an SR-B1 chemical inhibitor, Block lipid transporter (BLT-1; see Figure 19). BLT was added at increasing $(0.5-5\mu g \mu l^{-1})$ concentrations to the TNBC cell line MDA-MB-231 along with rHDL particles containing radiolabeled cholesterol oleate replacing the drug. The uptake was allowed to continue for 90 minutes. As shown in Figure 19, there is a significant decrease of radioactivity detected in the cells at a concentration of 0.005mg/protein indicating that the effect seen in MDA-MB-231 cells with the encapsulated drugs may be mediated through the SR-B1.

Flow cytometry analysis was used to determine to what degree non-drug carrying rHDL nanoparticles might contribute to apoptosis (see Figure 20). TNBC cells were stained with Annexin V and propidium iodide dye after treatment with hollow rHDL nanoparticles for a 72h period. Untreated cells were used as a control. Results show that the hollow particles had little effect on the TNBC cell line at the 72h mark.

To investigate the rHDL mechanism, further confocal images were taken of rHDL treated MDA-MB-231 cells vs. free drug-treated cells. Before making confocal images of treated MDA-MB-231 cells, an untreated confocal image, (see Figure 21) of the cell line was taken as a control. The images were taken with a Zeiss LSM 510 confocal microscope. The cells were stained for F-actin shown in magenta. The image demonstrates the epithelial to mesenchymal transition (EMT), a spindle-like shape of the cells. An image of MDA-MB-231 cells after being treated with the drug valrubicin or the rHDL preparation of valrubicin at 8µM for a three-hour period is depicted in Figure 22. The fluorescent intensity of the cells was measured using the Image J software (NIH, USA). The fluorescence intensity is a measure of the amount of drug that has entered the cell. There was a significantly higher fluorescence intensity observed in the rHDL treated cells than in the cells treated with the free drug (t=3.45, p < 1000.05) indicating that more drug accumulated within the cell when treated with rHDL-valrubicin than with the free drug. The rHDL nanoparticle is only a vehicle to deliver drugs to the targeted tissue. Because the drug is not altered in the preparation of the nanoparticle the mechanism of action of the drug remains the same. To determine how far the drug migrated within the cell, a confocal zstack image was taken of the three-hour rHDL or free drug treated MDA-MB-231 cells. Both the free and rHDL treated cells show similar peri-nuclear

migration within the three-hour period. The image also indicates that free valrubicin may enter the cell faster than the rHDL-valrubicin as evidenced by the valrubicin present at the top of the rHDL treated cells as opposed to little to no valrubicin at the upper part of the free drug-treated cells. To show that the rHDL nanoparticles are interacting with the SR-B1, a chemical inhibitor, block lipid transporter-1 (BLT-1) at 0.05mg/ml was used. Simultaneously, the cells were also treated with rHDL Valrubicin or the free drug at 8µM concentration. After three hours, the experiment was stopped, the cells were washed, and confocal images were taken. Figure 24 illustrates the results of the rHDL treated cells. Figure 25 depicts the results of the free drug-treated cells. As expected, the fluorescence intensity as measured by Image J software (NIH, USA) is significantly decreased in cells treated with rHDL plus BLT-1 as opposed to just rHDL alone (see Figure 24; t = 6.35; p < 0.01). Figure 25, which depicts the cells treated with the free drug valrubicin and BLT-1 or valrubicin alone, shows no significant decrease in fluorescence intensity. These results indicate that the rHDL nanoparticles may indeed work through the SR-B1 receptor to exert their effect.

А.



Figure 19: Radioactive rHDL uptake in the presence of a chemical inhibitor.

BLT-1 was used to block the activity of the SR-B1 protein in MDA-MB-231 cells. The cells were then treated with radiolabeled rHDL nanoparticles. There were four samples per dose. Three independent experiments were performed. Statistical analysis was performed using the student's t-test with the free version of Graph Pad Software. *Indicates (t = 49.51; p < 0.001).



Figure 20: Flow cytometry analysis of apoptosis in MDA-MB-231 cells. The data show Annexin V-PI staining results of TNBC cells before and after a 72h treatment with non-drug containing rHDL nanoparticles. The figure on the right is an analysis of untreated cells. The figure on the right shows the small amount of death observed when using empty particles.



Figure 21: Confocal microscope images MDA-MB-231 cells. A. Image depicting the morphology of the MDA-MB-231 cells. The nucleus has been stained with DAPI (blue); the rest of the cell is stained with phalloidin dye at a 1/100 dilution for F-actin (magenta). The image was taken using a Zeiss LSM 510 confocal microscope.



В.





Free



Figure 22: Confocal microscope images of valuabicin in MDA-MB-231 cells.

The images depict the amount of valrubicin that has entered the cell after a threehour period. **A.** Unencapsulated valrubicin at 8μ M in MDA-MB-231 cells after three-hour uptake. **B.** rHDL-valrubicin at 8μ M in MDA-MB-231 cells after three-hour uptake. Valrubicin has a natural fluorescence at 535-590nm. The image was taken using a Zeiss LSM 510 confocal microscope. The bar graph below the picture shows the corrected total cell fluorescence at n=3. Error bars indicate a significant difference. *Indicates (t=3.45; p < 0.05). Statistical analysis was performed using the student's t-test with the free version of Graph Pad Software.

•





Figure 23: Confocal Z-stack image of valrubicin migration in MDA-MB-231 cells. A. MDA-MB-231 cells treated with rHDL-valrubicin or free valrubicin at 8µM. The images depict the area of the cell where the valrubicin has traveled within a three-hour period. Images were taken with a Zeiss LSM510 confocal microscope. The image indicates that rHDL and free valrubicin migrate similarly.



rHDL

B.







Figure 24: Confocal microscope images of BLT-1 and rHDL valrubicin treatment in MDA-MB-231 cells. A. rHDL valrubicin at 8µM and BLT-1 drug at 1.5 mg ml⁻¹ in MDA-MB-231 cells after a three-hour uptake and **B**. Free valrubicin at 8µM in MDA-MB-231 cells after three-hour uptake. Valrubicin has a natural fluorescence at 535-590nm. The bar graph below the picture shows the quantitative mean n=3. Error bars indicate a significant difference. *Indicates (t= 6.35; p < 0.01). Statistical analysis was performed using the student's t-test with the free version of Graph Pad software.





Free







Figure 25: Confocal microscope images of BLT-1 and free valubbility treatment in MDA-MB-231 cells Confocal. A. Free valubbility in MDA-MB-231 cells after a three-hour uptake. **B.** Free valubbility at 8µM and BLT-1 drug at 0.05 mg ml⁻¹ in MDA-MB-231 cells after three-hour uptake. Valubbility has a natural fluorescence at 535-590nm.

Lapatinib as a Candidate for Combination Treatment

TNBC is a difficult disease to treat. It is standard practice for clinicians to use combination therapy either administered the same time or in sequential steps (see Tables 4 and 5). This treatment approach usually allows the patient more time before the disease inevitably progresses. However, as a result of combined treatment, the patient experiences greater toxicities (Brewster, 2014). We proposed here that combination therapy might be a more viable option when the drugs are encapsulated into our rHDL particles. To that end, a search was conducted for a suitable drug to use in addition to valrubicin in combination therapy. Because EGFR is up-regulated in 60% of TNBC tumors, the drugs that are involved in regulating that pathway were targeted (see Figure 13). Lapatinib is an inhibitor of the EGFR. To determine if lapatinib will have an effect on the TNBC cell line, MDA-MB-231, apoptosis analysis was performed by flow cytometry using Annexin V and propidium iodide staining. Flow cytometry analysis shows (see Figure 26) early apoptosis beginning to appear after 48h of lapatinib treatment. The incidence of apoptosis increases after 72h, late apoptosis is also becoming evident. As a result, lapatinib was finally chosen because of its ability to cause apoptosis in the TNBC cell line within 72 hours. Table 7 and Figure 27 demonstrate the relative percentages of each compound within the nanoparticle. Dynamic light scattering indicates that the majority of the

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nanoparticles are of the same size, and that size is within the acceptable range for *in vitro* work with nanoparticles (Jaing, 2008). Results show a negative zeta potential and an incorporation efficiency of 56%. These data indicate that the construction process for rHDL-lapatinib is efficient and because of its negativity it may escape opsonization once inside the body.

To compare the effectiveness of the rHDL-lapatinib vs. the free drug, a 72h cytotoxicity experiment was performed (see Figure 28). In the TNBC cell line, the IC₅₀ for rHDL-lapatinib vs. free lapatinib was 0.818μ M, and 2.24μ M respectively. These values are significantly different (t= 18.21, p < 0.01) indicating the greater potency of the rHDL-lapatinib over the free drug (see Figure 28). These results lead to the further experiments with lapatinib and valrubicin in combination therapy.



A.

Figure 26: Flow cytometry analysis of apoptosis with un-encapsulated lapatinib

at $2\mu M$. MDA-MB-231 cells treated with $2\mu M$ of lapatinib, which is the IC_{50} , for 24-72h. Cells were stained for apoptosis with Annexin V and propidium iodide. Three independent experiments were performed. Results show some an increasing apoptosis level as time increases.

Table 7. Chemical composition of rHDL-lapatinib
nanoparticle

Туре	rHDL co nponents mg/mL (%)			
of particle	Protein	Phospholipid	Cholesterol	Drug
rHDL/lapatinib	2.94 (25)	6.1 (52)	0.4 (4)	2.31 (19)



Lapatinib

Lapatinib rHDL	
Incorporation ef-iciency	56%± 2.33
Zeta Potential	98.88mV ±12.6
Dynamic Light Scattering	48.1nm

Figure 27: rHDL lapatinib nanoparticle composition and size. A. Shows the chemical composition of the nanoparticles encapsulated with lapatinib. The composition of the nanoparticles was determined using colorimetric assays for cholesterol and phospholipids. Drug concentration was measured using a spectrophotometer at 362nm; protein was measured using a BCA kit. B. Figure B is a chart showing the relative size of the nanoparticles as determined by dynamic light scattering, the intensity of the negative charge and the efficiency of the nanoparticle construction process.



A.

Figure 28: Cytotoxic results of lapatinib treatment on MDA-MB-231 TNBC cell line at 72 hours. Lapatinib at $2\mu M$ in free versus encapsulated drug (t=18.21, p < 0.01.) A. Graph depicting empirical data of 72H lapatinib treatment on the MDA-MB-231 cell line. There were six samples for each dose. Three independent experiments were performed. Statistical analysis was performed using the student's t-test with the free version of Graph Pad Software.

Combination Treatment Cytotoxicity

Lapatinib and valrubicin both have rHDL incorporations of at least 50% with valrubicin being as high as 86% on average (see Figure 29). This data indicates the procedure to make both particles is efficient. Therefore, we moved forward to combination studies. Lapatinib is currently FDA approved for HER2+ treatment in conjunction with xeloda or letrozole (NCI, 2011). However, lapatinib can cause left ventricular ejection fraction (LVEF) decrease in some patients (EMA, 2008). As mentioned earlier, valuation targets topoisomerase II of the heart causing cardiac damage. Because the objective is combination therapy, and both of these drugs have potentially harmful side effects to the heart, lapatinib was tested on cardiomyocytes together with valrubicin to determine the effect it may have on the cells (see Figure 30). Cell viability assays were executed using free valrubicin, free lapatinib, and encapsulated valrubicin and lapatinib. Untreated TNBC and cardiomyocyte cells were used as controls. The IC_{50} values for the drugs alone and in combination for cardiomyocytes and TNBC are displayed in (see Table 8). IC₅₀ numbers were calculated using the Sigma Plot software (Systat, USA). In the H9C2, cardiomyocyte cell line, the rHDL form of the both drugs provided protection against the effects of the free drug. In the case of single free lapatinib vs. rHDLlapatinib, the IC₅₀ value for the rHDL form was found to be greater than 20μ M. 138

At 20 μ M the concentration is already 2x as strong as the free drug yet was still protective. In the TNBC cell line, the combination of the two drugs valrubicin (4 μ M) and lapatinib ditosylate (.5 μ M- 4 μ M) had greater efficacy than valrubicin as a single agent (see Figure 30). The rHDL encapsulated combination treatment of valrubicin and lapatinib had the best effectiveness of all drugs formulations, either free or encapsulated. In all cases, the encapsulated form of the drug had greater efficacy than the free drug formulation. All together these data do suggest that the combination treatment is effective against TNBC while limiting side effects (see Figure 31).



Figure 29: Drug entrapment efficiency. Figure A depicts the percentage of the drug that was encapsulated into the particle. The results indicate that valrubicin incorporates better into the rHDL than lapatinib.



Figure 30: Combination treatment of MDA-MB-231 and cardiomyocyte cell lines at 72*h* value value in free vs. encapsulated drug (p < 0.05). *A*. *Graph depicting empirical data of 72<i>h* combination treatment in the MDA-MB-

231 cell line. There were six samples for each dose. Three independent experiments were performed. Statistical analysis was performed using the student's t-test with the free version of Graph Pad Software.



Figure 31: rHDL Combination treatment of MDA-MB-231 and cardiomyocyte

cell lines. (t= 3.82, p < 0.05). *A*. *Graph comparing empirical data* of 72*H combination treatment in the MDA-MB-231 cell line vs. cardiomyocytes. Results demonstrate the efficacy of the combination treatment against TNBC while still protecting the cardiomyocytes. Statistical analysis was performed using the student's t-test with the free version of Graph Pad Software.*

Cell Line	IC50 rHDL (µM)	IC50 free (µM)	Drug Used
H9C2	7.53 ± 0.01	4.67 ± 0.39	Valrubicin
	>20	9.68 ± 3.40	Lapatinib
	12.33 ±3.07	2.40 ± 0.15	Combination
MDA-MB-231	2.29 ± 0.39	$3.47{\pm}0.06$	Valrubicin
	$\boldsymbol{0.818 \pm 0.06}$	2.24 ± 0.05	Lapatinib
	$\textbf{0.585} \pm \textbf{0.14}$	2.95 ± 0.54	Combination

Table 8. Cytotoxicity of valrubicin and lapatinib in combination and alonevs. their encapsulated forms on TNBC and cardiomyocytes

Cytotoxicity Survival Statistics

Survival statistics of the free drug valrubicin and lapatinib as a single agent or in combination treatment versus their rHDL formulations are shown in Figures 32 and 33. Percent survival data were normalized to the untreated control and plotted as percent survival against concentration. Linear regression lines were used to determine mean, median and lowest survival percentage. The curved line in the graph of Figure 32 shows the relationship between survival and concentration. At low concentrations the survival drops steeply (see Figure 32), however the survival begins to level off at higher dose concentrations. Survival comparisons of free drug vs. encapsulated drug formulations (see Figure 32) reveal that regardless of the drug (lapatinib or valubicin) used or if that drug is in combination treatment or alone, the rHDL formulation is more effective than the free form at killing TNBC. Statistical analysis also suggests that rHDL lapatinib was the most efficient drug when compared with valrubicin. Furthermore, lapatinib alone was more effective at inhibiting survival than the combination of valrubicin and lapatinib. The lowest survival percentage was achieved by rHDL lapatinib at 4µM which allowed only 15% survival (see Figure 33). IC₅₀ summary table (see Figure 34) shows the IC₅₀ values for the TNBC cell line alone and the significance of the cytotoxicity data. However, though the IC_{50} value for combination treatment is lower than

rHDL lapatinib, it is not significantly different.



Overall Survival by Percent			
p < 0.001	Median	Mean	
rHDL	58	58 ± 0.33	
Free Drug	73	68 ± 0.27	

Figure 32: Scatter plot analysis of all cytotoxic data. The graph on the left is of data collected from TNBC cells treated with rHDL combination or the individual rHDL formulations vs. TNBC cells treated with free drug combination or the single drug alone. The chart on the right states statistical mean and median of rHDL treatments vs. free drug therapies. Graph shows a curved lined depicting the relationship between concentration and survival. Statistical graphs were done by Dr. Brad Cannell Dept. of Biostatistics and Epidemiology. (t= 23.45; p< 0.001)



Figure 33: Observed survival statistics of all data. The graph is of data collected from TNBC cells treated with rHDL combination or the individual rHDL formulations vs. TNBC cells treated with free drug combination or the single drug alone. The graph indicates that the rHDL-lapatinib at 4μ M has the lowest survival percentage out of the formulations tested. Statistical figures were done by Dr. Brad Cannell Dept. of Biostatistics and Epidemiology.

A.

Cell Line	IC ₅₀ rHDL (µM)	IC ₅₀ Free (µM)	Drug Used
MDA-MB-231	2.29 ±0.39	3.47 ± 0.06	Valrubicin
	0.82 ± 0.06	2.24 ± 0.05	Lapatinib
	0.58 ± 0.14	2.95 ± 0.54	Combination

B.

rHDL Drug	P Value at 95 CI	5%
Lapatinib vs. Valrubicin	t(4) = 3.73 p < 0.05	significant
Combination vs. Valrubicin	t(4) = 4.11 p < 0.05	significant
Combination vs. Lapatinib	t(4) = 1.53 p > 0.05	not significant

Figure 34: Comparison of IC_{50} numbers on cardiomyocytes and TNBC and their significance. A. IC_{50} summary table for TNBC IC_{50} table comparing free drug versus rHDL drug alone and in combination. B. IC_{50} comparison among rHDL treatments showing statistical significance of the IC_{50} for these drugs.

CHAPTER V

DISCUSSION

While the current average five-year survival rate for breast cancer patients is 89.2% (NCI, 2013), the five-year survival for patients with metastatic TNBC is < 30% (Liedtke, 2008). This slight percentage of survival impacts African American women disproportionally as approximately 50% of African American women with breast cancer (under the age of 40) present with the TNBC type and thus with a poorer prognosis (Liu, 2011). Overall, African-American women tend to develop a more aggressive form of breast cancer than other ethnic groups (Siegel, 2015). Early diagnosis and treatment have proven to be useful in treating cancer of any type; however, screen-film mammography (SFM), the standard for breast cancer screening and detection, is largely ineffective at detecting tumors in the breast of young women (dos Anjos, 2014). Because, mammogram machines work best on breasts with sparse or thinly packed breast tissue, the U.S. Preventive Services Task Force mammogram guidelines recommends women begin screening at age 50 (USPSTF, 2009), and the American Cancer Society recommends screening to start at age 45 (Oeffinger, 2015). Consequently, this screening approach is of

little help to women under the age of 50 with TNBC, especially to African American women who are at risk around the age of 40. Current options for TNBC treatment do not uphold favorable clinical outcomes, and alternatively, frequently results in poor treatment response and overall survival rate (OS; Lin, 2012). This problem is partly due to a lack of prognostic biomarkers to predict which patients are likely to respond to particular chemotherapeutic and targeted therapies. Also, there is a lack of consistent diagnostic biomarkers for screening purposes (Shin, 2015). Consequently, predictive biomarkers for diagnosis that could identify patients with this aggressive and metastatic disease are urgently needed. In 1996, the human mammaglobin (h-MAM) gene was discovered to code for Mammaglobin A (MGBA), a 10-kd protein that is higher in the serum of breast cancer patients than in healthy controls, which indicated its potential as a breast cancer biomarker (dos Anjos, 2014). It can help to determine the stage of cancer, and when used in combination with gross cystic disease fluid protein-15 (GCDFP-15) another biomarker, it can be diagnosis lymph node metastasis by immunohistochemistry with 83% accuracy (dos Anjos, 2014).

Mammaglobin A, however, is expressed differently in different breast cancer tissues. ER+ breast cancer cells express higher levels of the mRNA, while ERnegative and high-grade tumors have lower expression levels per cell (dos Anjos, 2014). Using miRCURY LNA array platform and validation by real-

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time PCR on plasma samples from patients with TNBC, with non-TNBC breast cancer and from patients without breast cancer or a history of the disease (Healthy; n=90), Shin et al. selected five miRNAs—miR92a-3p, miR342-3p, miR-16, miR-21, and miR199a-5p—that could be potential biomarkers for TNBC (Shin, 2015). After further validation with pre- and post-operative plasma from patients (n=252) with 67 TNBC, 95 non-TNBC, and 90 Healthy, Shin et al. were able to identify miR199a-5p as having a significantly differential expression among non-TNBC and healthy groups (Shin, 2015).

SR-B1 has been associated with the selective uptake of cholesteryl esters from the HDL (Liadaki, 2000). Recently, Yuan et al. showed that 54% of breast cancer patients' tumors had high expression of the SR-B1 receptor, of the 54%, 75% were of the TNBC subtype (Yuan, 2015). Yuan et al. also developed a prediction algorithm that indicated a poorer outcome for breast cancer patients whose tumors showed a high expression of the SR-B1 receptor (Yuan, 2015). The present study aimed to fill a void in TNBC treatment by using rHDL nanoparticles that are targeted to the TNBC tumor via the SR-B1 receptor.

Chemotherapeutic drugs today with high potency and yet toxic side effects directly diffuse throughout the body resulting in limited concentrations at the

site of the disease thereby limiting their effectiveness (Johnson, 2013). Cancer chemotherapy faces significant challenges, including off-target toxicity, causing damage to healthy tissues, chemo-resistance leading to relapse and often metastatic complications, and limited access to tumor targets due to poor bioavailability (Kamaly, 2012). Nanoparticles have provided promising alternatives to traditional therapy by enhancing the therapeutic index and thus strengthening the impact of the drug on the tumor (Kamaly, 2012). The rHDL nanoparticles loaded with chemotherapeutic agents appear to have highly desirable characteristics needed for improved cancer chemotherapy (Lacko, 2006). During this study, several suitable drugs were employed that have the potential to contribute to tumor selective breast cancer therapy. Valrubicin has been reported to enter the targeted cells and localize in the cytoplasm where it interacts with PKC α (Laugesen, 2013). These studies have illustrated the potential for rHDL nanoparticle treatment against TNBC without increasing but limiting cardiotoxicity. The rHDL nanoparticles are an active targeting nanoparticle. The ability to target SR-B1 in cancer cells and deliver the payload through the cholesterol uptake mechanism was illustrated in radioactive cholesterol ester uptake studies as well as confocal images of rHDL-valrubicin (see Figures 19, 22-24). The SR-B1 is known to remove cholesterol esters from the HDL particle without damaging or endocytosing the particle. The

rHDL nanoparticles are a delivery vehicle rHDL encapsulated, and the free drug should behave the same once inside the cell. Z-stacking images (see Figure 23) of rHDL-valrubicin vs. free valrubicin do depict peri-nuclear localization within the 3H timeframe. The images also reveal rHDL-valrubicin present at the top of the cell while microscopic amounts were present at that location in free drug treated MDA-MB-231 cells. These data could suggest that valrubicin may enter the cell faster than rHDL-valrubicin. However, as shown in Figure 22, the accumulation of rHDL valrubicin is higher than the free drug. Taken together these results may suggest that valrubicin, a substrate for P-gp, may enter the cell but be subject to the efflux pump thereby limiting its accumulation within. The rHDL formulation may bypass these efflux pumps in the cell membrane and thereby have the ability to accumulate at greater quantities within the cell. To further validate the role of the SR-B1 receptor in discharging the payload of the rHDL nanoparticles the rHDL nanoparticles were formulated with labeled ³Hcholesterol oleate. These particles were incubated with MDA-MB-231 in the presence and absence of the BLT-1 inhibitor, a significant decrease in cholesteryl oleate was noted in BLT-1 treated samples versus the rHDL alone, p < 0.01. Taken together these findings show that the MDA-MB-231 cells acquire drugs and cholesterol from rHDL nanoparticles and thus validate the proposed experimental approach to be eventually developed for TNBC therapeutics.

These results are in agreement with work done in 2013 by Lin et al., demonstrating cytosolic (SR-B1 mediated) delivery of the payload transported via rHDL nanoparticles (Lin, 2013).

It has been noted in several publications that although nanoparticles have increased the biological half-lives of chemotherapeutic drugs and increase nonspecific accumulation at the site of injury they have only marginal success in increasing progressive free survival (Blanco, 2015; Bobo, 2016). It has been noted that the size of the nanoparticle plays a role in how the nanoparticle is eliminated from the body. If it is > 10nm then it will be excreted by the liver (Choi, 2007; Rolfe, 2014). Because most nanoparticles are larger than 10nm (Jaing, 2008; excluding antibody-drug conjugates), this may be a problem for the liver. Some of the other disadvantages with nanoparticle treatment are: (1) opsonized and sequestered by MPS, which leads to spleen and liver accumulation and not the site of injury, (2) non-specific accumulation, (3) the pressure gradients within the center of a tumor may be too high keeping the nanoparticle from reaching the center and delivering the payload, (4) or the particle may be subject to the drug efflux pumps (Blanco, 2015). The rHDL nanoparticles overcome many of these obstacles with their small size and spherical shape, and the fact that they are targeted to SR-B1 makes them one of the individual particles specific for cancer therapy. It has been noted that

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discoidal nanoparticles are preferable over the spherical shape. Spherical particles flow through the middle of the blood stream and do not have enough contact points, while discoidal particles have a flat surface, many contact points, and larger sizes. As a result, they will tumble around the blood vessel and have more contact with the cell wall giving the particle a better chance to be extravasated through the vessel to the site of injury (Blanco, 2015). However, spherical rHDL nanoparticles are ideally suited for contact with SR-B1. It has been noted that discoidal rHDL particles targeted to the SR-B1 interact with LCAT. The LCAT will esterify the cholesterol on the surface and cause a change in shape of the particle and causes apo-AI mutations as the CE move into the center of the particle resulting in drug leakage (Liadaki, 2000).

However, these problems were not associated with spherical SR-B1 targeted nanoparticles.

Cytotoxicity studies

Cytotoxicity studies with MDA-MB-231 cells (see Figure 13) further supported the suitability of the rHDL nanoparticles as drug delivery agents for breast cancer treatment as the rHDL-valrubicin as well as the rHDL-lapatinib were more efficient in killing the MBA-MB-231 cells than the free drug, both (p < 0.05; p < 0.01). The addition of the lapatinib nanoparticle for combination therapy provided somewhat unexpected results. While the lapatinib-rHDL

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nanoparticles were also more effective in inhibiting cancer cell proliferation than the free drug (see Figure 34), p < 0.05, the combination of the two rHDL formulations together did not significantly enhance the cytotoxicity of the combination versus rHDL Lapatinib alone (see Figure 34). Combination therapy using both rHDL formulations together, however, was considerably more efficient than the combination of the free drugs (see Figure 34). It may be possible that the competition of the two different rHDL nanoparticles for the same type of receptor may have hindered their effectiveness. When conducting combination experiments with the unencapsulated forms of lapatinib and valrubicin, the effective dose was a 50:50 mixture. When both rHDL particles are competing for SR-B1, then it is possible that the concentration of the drug that enters the cell is not 50:50, though both rHDL particles are administered. Consequently, optimization of one or both of the nanoparticles, using folate conjugation, may enhance the drug delivery by allowing interaction with a second target (the folate receptor) in addition to the to the SR-B1 target (Mooberry, 2010). Alternatively, a dual incorporation preparation of both lapatinib and valuation into just one nanoparticle could be explored and thus alleviated the competition for the same receptor site by two separate particles. These data suggest that the rHDL particles are targeted to the cancer cells via the overexpressed SR-B1 receptor and could thus have the potential to

achieve tumor-selective drug delivery combined with markedly reduced offtarget toxicity seen with chemotherapeutic drugs.

In the future, an *in vivo* study of these particles could further validate their usefulness to fight TNBC while still providing some protection to the cardiac tissue.

CHAPTER VI

FUTURE DIRECTIONS

Future *in vivo* studies of the rHDL nanoparticles could help to validate the potential of the rHDL nanoparticle in limiting off-target effects as well as determine the level, if any, of liver toxicity. Because SR-B1 is the receptor for HDL, *in vivo* studies could also help to elucidate how active the rHDL particles would be against its natural competitor for SR-B1, HDL.

In addition to *in vivo* studies, further, *in vitro* studies on the mechanism of action of the rHDL-lapatinib and valrubicin may be helpful. Preliminary Western blot results show that the rHDL lapatinib has little effect on the phosphorylation of H₂AX (an indicator of double-stranded DNA damage; see Figure 35A), while rHDL valrubicin clearly phosphorylates the histone. Flow cytometry apoptosis analysis of free lapatinib (see Figure 26), previously shown also indicates a modest amount of apoptosis after 72h. It may be possible that rHDL-lapatinib induces death in additional mechanisms in addition to apoptosis. In Figure 36B, shows a preliminary Western blot depicting the LC3B levels (a protein involved in autophagosome formation), in MDA-MB-231 cells treated with both lapatinib and valrubicin rHDL

formulations. In the completion of autophagy, LC3B will be degraded, and therefore their levels will be low. When apoptosis is the primary mechanism of cell death, the LC3B levels are high. Data from Figure 36B indicates a greater level of LC3B expression over the control in rHDL-valrubicin treated cells. rHDL-lapatinib-treated cells reveal no significant increase in protein levels above the control. However, the levels are consistently lower than the valrubicin treated cells. Together these preliminary data indicate that valrubicin-rHDL may induce apoptosis in TNBC cells through possible DNA damage. Further study is needed on rHDL-lapatinib to determine if they inhibit survival through induction of autophagy. Though some apoptosis does occur after 72h, it is possible that lapatinib may induce that apoptosis as a result of prolonged autophagy.
А.



B.



Figure 35. LC3B protein levels in rHDL treated MDA-MB-231 cells.

The western blot above depicts the protein levels of **A**. H_2AX and $_PH_2AX$, which indicate double strand DNA damage, and **B**. the autophagy protein, LC3B in TNBC cells treated with lapatinib or valrubicin rHDL at the IC₅₀ values of the prospective drugs. Actin used as a loading control. The phosphorylation of H_2AX is pronounced in the valrubicin treated cells not in the lapatinib treated cells. The LC3B levels appear to increase slightly when compared to the control over time; however the LC3B levels are lower in the lapatinib treated samples. Together these preliminary results may indicate that lapatinib may induce autophagy.

CHAPTER VII

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