A NUTRACEUTICAL APPROACH TO COMBATING CHEMORESITANCE IN ACUTE LYMPHOBLASTIC LEUKEMIA

by

OLIVIA IDOM

(Under the Direction of Jason Zastre)

ABSTRACT

Patients with acute lymphoblastic leukemia (ALL) often face relapse and death due to chemoresistance to standard chemotherapy treatment. Based on studies that have evaluated benfotiamine's anticancer properties, BFO increases intracellular thiamine pyrophosphate (TPP) levels and compromises cancer cell viability. Cancer cell metabolism is dependent on endogenous TPP, and it would not be anticipated that thiamine uptake and subsequent TPP formation would be susceptible to acquired or intrinsic chemoresistance mechanisms. Therefore, we hypothesize that benfotiamine will produce similar anticancer activity in chemoresistant cancer cells as in wildtype acute lymphoblastic leukemia. The results of this study showed that twice the dose of BFO was required to compromise cell viability in the chemoresistant ALL cell line, as compared to the chemo-naïve parental line. Therefore, there is great therapeutic potential for higher concentrations of benfotiamine to be employed in the treatment of chemoresistant cancers.

INDEX WORDS: Benfotiamine, Cancer, Chemoresistance, Doxorubicin, Drug Resistance, Thiamine

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by

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CHAPTER 1

1. Acute Lymphoblastic Leukemia:

Leukemia is a cancer of the peripheral blood that affects all ages, but the highest incidence is in the two extremes of age, young children and elderly adults. Leukemia has four different subtypes classified as acute myeloid leukemia (AML), chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL), and acute lymphoblastic leukemia (ALL).

Acute lymphoblastic leukemia is the rapid progression of immature, malignant lymphocytes in the bone marrow (Onciu, 2009; American cancer society, 2023). According to Winslow, normal hematopoiesis begins with a blood stem cell that differentiates into a myeloid or lymphoid stem cell (Winslow, 2007, figure 1.1). Hematopoietic stem cell differentiation begins in the bone marrow, where lymphoid-primed multipotent progenitors and early lymphoid progenitors regulate lymphoid specification (Pelayo et al. 2012; Purizaca et al., 2012). Multipotent progenitors produce oligopotent progenitors, that can develop into a natural killer cell, T cell, or B cell (Purizaca et al., 2012). If a cell does not enter the natural killer cell pathway, it continues downstream along the lymphoid pathway, where committed precursors are created to aid in cell maturation (Purizaca et al., 2012). Phenotypic primitive characteristics in lymphocytes may cause developing cells to be vulnerable to intrinsic and extrinsic stimuli that factor into developing malignant cells (Espinoza-Hernandez et al., 2001; Cox et al., 2004). A study conducted by Cox et al., examined long-term cultures of bone marrow and concluded that B cell ALL targets immature cells in the lymphoid pathway that have not become B cell committed precursors in the lymphoid pathway (Cox et al., 2004; Malouf et al., 2017). These

malignant cells are further classified as precursor B lymphocytes that rapidly over-proliferate, accumulate in the bone marrow, and spill into the peripheral blood (Campos-Sanchez et al., 2011; Pelayo et al., 2012; National Cancer Institute, 2022).

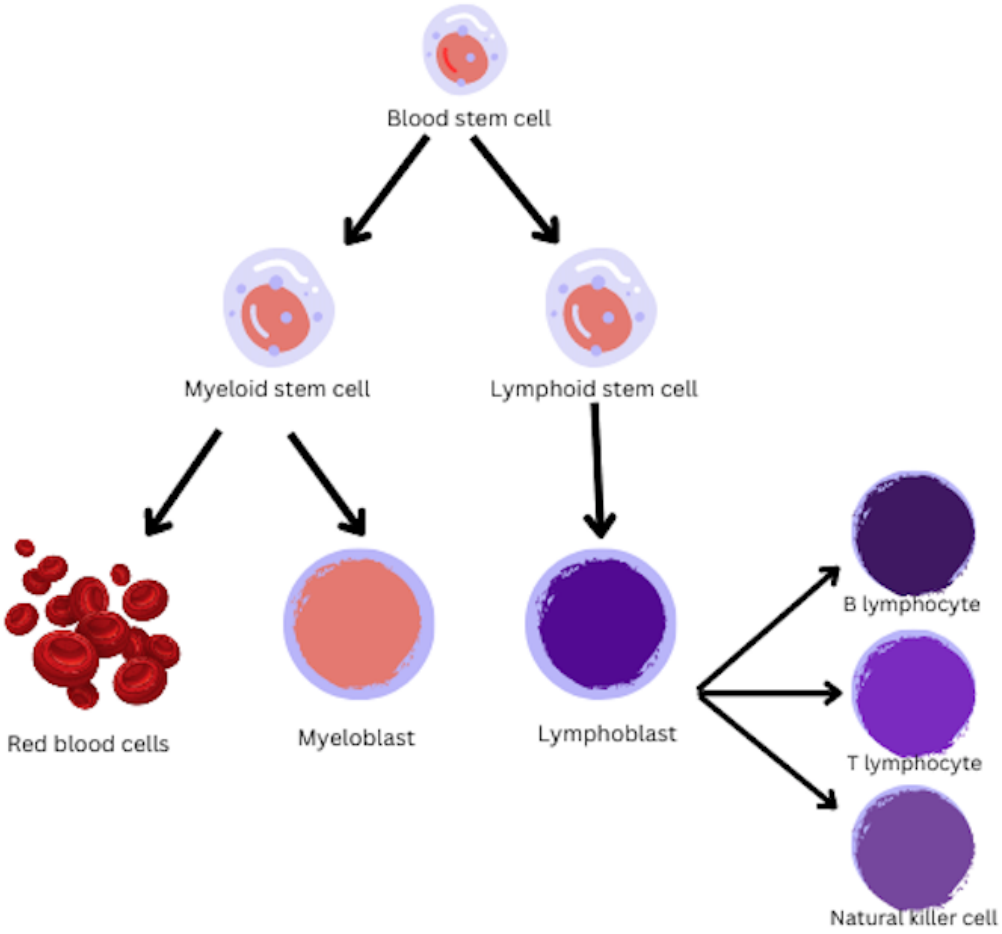

Figure 1.1: Normal hematopoietic differentiation of a blood stem cell in the bone marrow, adapted from Winslow, 2007.

1.1 Prognosis and Incidence:

In acute lymphoblastic leukemia, the main prognosis factors evaluated to determine the best course of treatment in patients are age, central nervous system (CNS) involvement, and chromosomal abnormalities (National Cancer Institute, 2023). Children typically have a better prognosis than adults, primarily based on age and lack of underlying health conditions (National Cancer Institute, 2022). Underlying conditions in older adults can limit the range and effectiveness of treatment, leading to an unfavorable prognosis. CNS involvement is the most common extramedullary involvement in patients with ALL (Lazarus et al., 2006; Kuo et al., 2018). If a patient's leukemia blasts spread to the central nervous system, they can build up in the spine and brain. Spread of leukemia blasts would cause a patient to have related symptoms and overall inferior survival rates (Kuo et al., 2018). A study showed that the 5-year survival rate for patients with CNS involvement is 28% compared to patients without CNS involvement. Patients with CNS involvement also had a high relapse rate (Kuo et al., 2018). Chromosomal abnormalities such as, translocations between specific chromosomes, amplification of chromosomes, complex karyotypes, or hypodiploidy, in leukemic cells can negatively affect a patient's prognosis (American Cancer Society, 2018).

The disease state of a patient's leukemia before, during, and following treatment substantially affects short and long-term survival. A patient has an active leukemic disease when leukemia accounts for 5% or greater of the bone marrow (American Cancer Society, 2018). Minimum residual disease is when leukemia cells are still present in the bone marrow, but it cannot be detected with standard lab tests. These cells can only be detected with polymerase chain reaction (PCR) and flow cytometry lab tests, and these patients are more likely to experience a disease relapse (Kruse et al., 2020). Quantitatively, remission is classified when

over 95% of the bone marrow contains normal hematopoietic cells. Prognosis factors and disease states are the most effective way medical providers can predict survival rates for their patients.

2. Treatment:

To date, four types of established treatment options exist for patients with acute lymphoblastic leukemia. FDA-approved treatment options include chemotherapy, radiation therapy, chemotherapy with stem cell transplant, and targeted therapy (Adult Treatment Editorial Board, 2002). Treatment options vary for different stages in adult ALL. These stages are untreated adult ALL, adult ALL in remission, and recurrent ALL. Two standard treatment options for patients with untreated ALL are remission induction therapy and central nervous system prophylaxis therapy (Adult Treatment Editorial Board, 2002). Remission induction therapy includes combination chemotherapy, imatinib mesylate, imatinib mesylate combined with chemotherapy, and supportive care (Adult Treatment Editorial Board, 2002). A remission induction study showed that patients have a 60-80% chance of reaching complete remission with remission induction therapies if complications do not arise (Larson et al., 1995; Adult Treatment Editorial Board, 2002). Combination chemotherapy treatment is the most utilized treatment for ALL patients and has three stages: induction, consolidation, and long-term maintenance (Terwilliger et al., 2017). The most heavily used drugs in combination chemotherapy are prednisone, vincristine, and anthracyclines (Adult Treatment Editorial Board, 2002). CNS prophylaxis therapy includes cranial radiation therapy with intrathecal (IT) methotrexate, high-dose systemic methotrexate, and IT chemotherapy (Adult Treatment Editorial Board, 2002). The criteria for determining if ALL patients are in remission is when the bone marrow has less than 5% blasts, no signs or symptoms of ALL, no signs of CNS prophylaxis, and blood counts are within normal ranges (Adult Treatment Editorial Board, 2002). In addition, patients in remission

receive post-remission therapy and CNS prophylaxis therapy (Adult Treatment Editorial Board, 2002). Post-remission therapy includes allogenic bone marrow transplant and long-term therapy that utilizes lower doses of treatment than used to treat patients with remaining active ALL (Adult Treatment Editorial Board, 2002). Treatment options for patients with recurrent adult ALL are reinduction chemotherapy, blinatumomab followed by allogenic bone marrow transplant (BMT), inotuzumab ozogamicin followed by BMT, palliative radiation therapy, and Dasatinib (Adult Treatment Editorial Board, 2002). Ongoing clinical trials are investigating novel targeted treatments for acute lymphoblastic leukemia in adults and children, but the FDA has not yet approved these.

A study in 2011, involving untreated ALL adult patients, evaluated the treatment outcome of cyclophosphamide, vincristine, dexamethasone/methotrexate, cytarabine, and doxorubicin. These drugs were employed in induction therapy, CNS prophylaxis, supportive therapy, and post-induction therapy (Morris et al., 2011). This study's treatment regimen included the combination chemotherapy of these drugs, and complete remission (CR), relapse, refractory disease, and early induction death were measured. In the assessment of patients that underwent induction therapy, 86% of patients achieved complete remission (Morris et al., 2011). The induction mortality rate was 8%, and the remaining patients did not achieve complete remission due to refractory disease, severe infection, and high combination chemotherapy toxicity levels (Morris et al., 2011). Of the 86% of patients that achieved complete remission in induction therapy, 54% received post-induction therapy (Morris et al., 2011). Median survival rates for patients with event-free survival was 37%, and overall survival was 48% (Morris et al., 2011).

Doxorubicin (DOX) is a standard anthracycline drug utilized in ALL treatment, but resistant phenotypes are often produced in response to DOX treatment, causing chemoresistance. Doxorubicin is usually administered to patients through an intravenous injection and is usually given in combination with vincristine, prednisolone, and L-asparaginase, to produce a maximum response and decrease toxicity (Thorn et al., 2011; Lee et al., 2017).

Doxorubicin has two mechanisms of action within a cancer cell: intercalation into DNA and inhibition of the topoisomerase II enzyme (Gewirtz, 1999; Thorn et al., 2011; Figure 1.3). In vivo, doxorubicin undergoes a process where it is oxidized to the unstable metabolite semiquinone and converted back into doxorubicin, where a reactive oxygen species (ROS) is released (Doroshow, 1986; Thorn et al., 2011). The ROS can cause lipid peroxidation, membrane damage, DNA damage, oxidative stress, and trigger cell death through apoptotic pathways (Thorn et al., 2011). Through the intercalation mechanism, doxorubicin inserts itself into a cancer cell and binds to the DNA structure, where DNA damage occurs and interferes with DNA replication. Doxorubicin also inhibits cancer cell proliferation by entering the cellular nucleus and inhibiting topoisomerase II (Micallef and Baron, 2011; Thorn et al., 2011). Topoisomerase II cuts a double-stranded DNA helix to allow for DNA passage, and when it is inhibited by doxorubicin, DNA replication is halted (Taymaz-Nikerel et al., 2018). Doxorubicin is very efficacious due to its rapid distribution into tissues, but if chemoresistance is acquired against doxorubicin, very challenging treatment issues arise.

2.1 Chemoresistance:

Despite current treatment options, the National Institute of Cancer reported that since 1975, the incidence rate for new cases of acute lymphoblastic leukemia in patients of all ages has increased, and the death rate has not decreased (Adult Treatment Editorial Board, 2002; Figure

1.2). Based on studies conducted on patients who have relapsed or failed to respond to treatment, the most significant obstacle in ALL treatment is combating chemoresistance (Kuo et al., 2018; Aberuyi et al., 2020). Drug resistance can develop within weeks or months of treatment and cause over 90% of drug treatments to fail in metastatic cancers (Longley and Johnston, 2005; Garrett and Arteaga, 2011; McGuirk et al., 2021). Treatment resistance often causes disease relapse or death for the patient. In addition, drug resistance often results in multidrug resistance to a large variety of chemotherapy drugs, not all similar in structure. (Yusuf et al., 2003; Szakacs et al., 2006; Wang et al., 2017).

Chemoresistance mechanisms can be intrinsic or acquired. Intrinsic resistance is an innate resistance existing before the patient is administered drugs (Yang et al., 2015; Wang et al., 2019). Patients can have an initial therapeutic response to treatment but may face relapse due to the proliferation of resistant subclones (Liang et al., 2010; Mansoori et al., 2017; Wang et al., 2019). Intrinsic resistance is mediated by endogenous variants such as genetic mutations, amplifications, deletions, and chromosomal rearrangements (Mansoori et al., 2017). The formation of these variants leads to alterations within genes that intrinsically cause drug resistance (Mansoori et al., 2017).

Acquired drug resistance occurs when the sensitivity of the cancer cell to the drug gradually decreases over time. Acquired resistance occurs not only with chemotherapy drugs but can also be acquired to targeted cancer drugs. Resistance to targeted drugs is developed when the specific target develops mutations or the expression levels change (Baudino, 2015; Mansoori et al., 2017; Wang et al., 2019). Chemotherapy acquired resistance can result in the activation of a second proto-oncogene that becomes the newly emerged driver gene, mutations and altered

expression levels of the drug target, and tumor microenvironment changes after treatment (Wang et al., 2019).

In ALL treatment with anthracyclines, P-glycoprotein mediated resistance is an extremely common acquired drug resistance mechanism (Callaghan et al., 2014). Cancer stem cells can have an overexpression of P-glycoprotein (P-gp), ABCG1, ABCG2, Bcl-2, or survivin, which are all involved in multidrug chemoresistance in cancer cells (Altenberg, 2004; Zheng, 2017). In the evaluation of a group of ALL patients, 33.3% of patients had an over expression of P-gp in leukemic cells (El-Ghaffar et al., 2006).

P-gp is part of the adenosine triphosphate binding cassette (ABC) of transporters and is encoded by the multidrug resistance 1 gene (MDR1) (Callaghan et al., 2014; Yeldag et al., 2018). The ABC family of transporter proteins functions to translocate substrates across the cellular membranes using an ATPase (adenosine triphosphate) transporter (Altenberg, 2004; Zheng, 2017). These ABC transporters are on the plasma membrane of cells and mediate chemoresistance by transporting chemotherapy drugs out of the cell before they can elicit a response (Yeldag et al., 2018). P-gp specifically causes multidrug resistance by promoting the efflux of hydrophilic and hydrophobic drugs, such as anthracyclines, out of the cell (Yeldag et al., 2018). It prevents the accumulation of anticancer drugs within the cell by utilizing ATP-dependent efflux of anticancer drugs against concentration gradients and across the plasma membrane (Callaghan et al., 2014). When malignant and normal tissues are compared, the levels of ATP are much higher in malignant cells to fuel rapid proliferation (Wang et al., 2019). These increased levels are hypothesized to be due to the Warburg effect, where glucose transport and aerobic glycolysis are upregulated in cancer cells (Qian et al., 2014; Wang et al., 2019). Molecularly, the downstream effect of ATP binding to the cytoplasmic domain of the p-

glycoprotein is ATP hydrolysis, which causes multidrug chemoresistance through drug efflux out of the cell (Karthika et al., 2022).

Knowing P-gp confers multi-drug chemoresistance, there has been in vitro research focused on creating molecules to inhibit P-gp. To date, all developed P-gp inhibitors have failed clinical trials. The p-glycoprotein mediates drug resistance in cancer cells but also has critical regulatory effects in the human body. P-gp facilitates the efflux of toxic substances out of the cell making it a crucial transporter for survival (Ahmed Juvale et al., 2022). It is not only expressed in cancer cells, but in tissues in the brain, liver, gastrointestinal tract, intestines, testis, and placenta (Ahmed Juvale et al., 2022). Creating a drug molecule that would block p-glycoprotein could cause immense damage to human organ systems unless it specifically blocks p-glycoprotein's expression in cancer cells. Unfortunately, no inhibitor for that purpose exists to combat chemoresistance, so alternative research ideas and novel drugs should continue to be studied and developed.

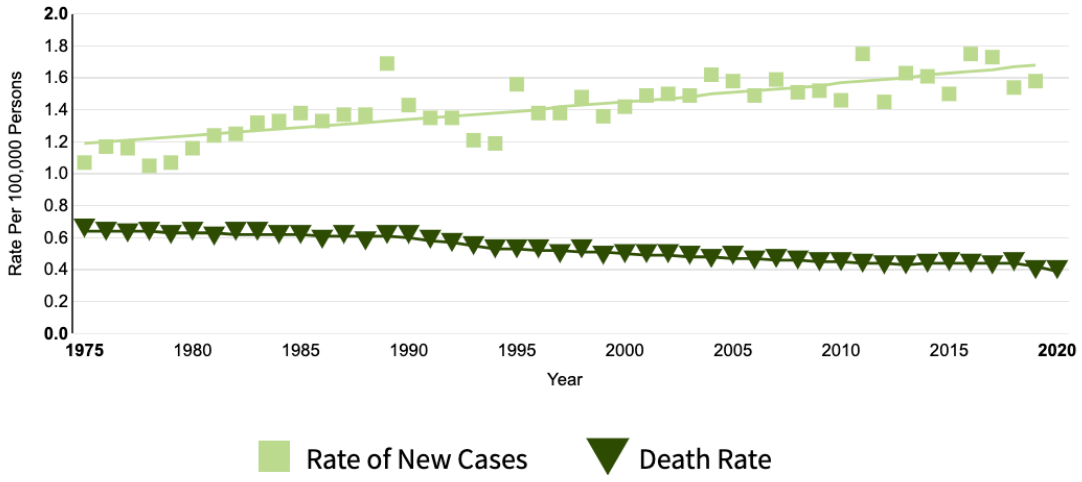

Figure 1.2: Data from the National Cancer Institute: rates of new acute lymphoblastic leukemia cases and deaths per 100,000 since 1975. National Cancer Institute, 2022.

2.2 Doxorubicin:

Anthracyclines are employed in first line chemotherapy treatment of acute lymphoblastic leukemia (Terwilliger, 2017). Doxorubicin (DOX) is a standard anthracycline drug utilized in ALL treatment, but resistant phenotypes are often produced in response to DOX treatment, causing chemoresistance. Doxorubicin is usually administered to patients through an intravenous injection and is usually given in combination with vincristine, prednisolone, and L-asparaginase, to produce a maximum response and decrease toxicity (Thorn et al., 2011; Lee et al., 2017).

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Chemoresistance mediated by the P-glycoprotein is the primary mechanism leading to the emergence of a doxorubicin chemoresistant phenotype in B Acute Lymphoblastic Leukemia

(Mansoori et al., 2017). Precursor B ALL cancer cells have an overexpression of P-gp, allowing a quicker convergence of chemoresistance, leading to chemotherapy treatment failure in patients (Callaghan et al., 2014).

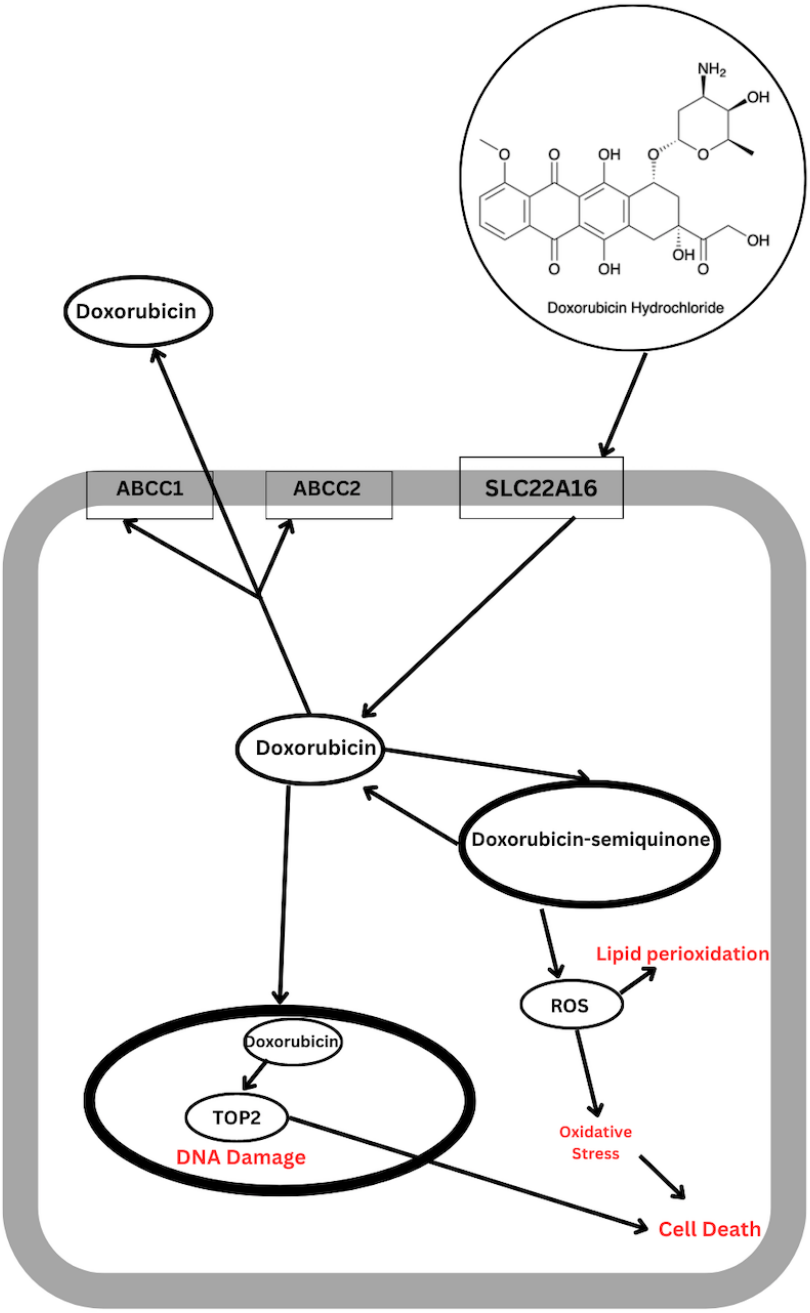

Figure 1.3: Doxorubicin anticancer mechanism of action in a stylized cancer cell, adapted from Thorn et al., 2011.

3. Nutraceuticals:

A nutraceutical is any substance that is food or part of a food that provides medical or health care benefits, including the prevention and treatment of disease (Kalra, 2003). Nutraceuticals have been studied in the context of many diseases to determine if they can produce therapeutic effects with minimal side effects. Nutraceuticals have been found to have anti-inflammatory, antibacterial, antifungal, chemo-preventive, and neuroprotective pharmacological effects (Ishak et al., 2017; Calvani et al., 2020).

3.1 Vitamin C:

Vitamin C is an example of a nutraceutical with significant anti-cancer potential. In 1972, vitamin C deficiency was observed in many cancer patients, and was further explored through a clinical trial conducted by Ewan Cameron and Linus Pauling (Cantley and Yun, 2020). This trial administered vitamin C to an experimental group of terminal cancer patients intravenously and orally (Cantley and Yun, 2020). The experimental treatment group had a significantly greater quality of life and four-fold higher survival time than the placebo group (Cantley and Yun, 2020).

Recently, clinical trials and case reports have been conducted evaluating the safety and therapeutic potential of high-dose vitamin C. A phase I clinical trial was conducted on metastatic colorectal cancer patients with combination treatment of high-dose vitamin C and chemotherapy (Wang et al., 2019; Mussa et al., 2022). It was concluded that high-dose vitamin C increased the efficacy of the anti-cancer drug in colorectal cancer, and the patient had reduced side effects (Wang et al., 2019; Mussa et al., 2022). Another study utilized combination treatment of vitamin C and chemotherapy in malignant multiple myeloma (Qian et al., 2020). A control group of patients treated solely with chemotherapy was employed in this study (Qian et al., 2020). In the

combination treatment group, the median survival was 44.2 months in comparison to 17 months for the control group (Qian et al., 2020; Mussa et al., 2022). The results of these studies show a positive therapeutic outcome of the nutraceutical, vitamin C, in cancer treatment.

3.2 Thiamine:

In addition to vitamin C, thiamine has also been studied to determine the extent of its potential therapeutic properties in humans. Thiamine, also known as vitamin B1, is an essential part of the human and animal diet since they cannot synthesize it naturally (Jong et al., 2004). Mammals naturally receive thiamine through their diet, including but not limited to grains, fish, meat, eggs, beans, and potatoes (Zastre et al., 2013). Vitamin supplements can be a source of thiamine for humans, containing over 100 times the recommended daily intake (RDI) (Zastre et al., 2013). Thiamine is readily excreted from the human body, so a regular intake of thiamine through a balanced diet is essential to maintain normal B1 levels in the blood (Martel et al., 2022). Vitamin B1 can be administered orally, intravenously, or intramuscularly. The drug use indications of thiamine are mainly for thiamine deficient linked diseases such as beriberi and Wernicke-Korsakoff syndrome (Jong et al., 2004).

The structure of thiamine contains a thiazole ring connected by a methylene to an aminopyrimidine ring (Figure 1.4). Thiamine is hydrophilic due to its polar, primary alcohol and quaternary nitrogen, and at physiological pH it exists as a cation (Zastre et al., 2013). Overall thiamine very low bioavailability which reduces its clinical effectiveness (Jonus et al., 2020).

For thiamine to elicit a response, it must be phosphorylated into its active cofactor, thiamine diphosphate (TDP or TPP) (Zastre et al., 2013; Figure 1.5). Thiamine has two additional phosphorylated forms, thiamine monophosphate (TMP) and thiamine triphosphate (TTP), but these do not function as enzyme cofactors (Zastre et al., 2013; Jonus et al., 2020;

Figure 1.4). Thiamine transporters 1 (THTR1) and 2 (THTR2) are encoded by solute carrier genes SLC19A2 and SLC19A3, respectively (Lindhurst et al., 2006; Zastre et al., 2013). These carriers are responsible for the uptake of free thiamine (Zastre et al., 2013). Thiamine pyrophosphokinase 1 (TPK1) converts thiamine into its active cofactor, thiamine pyrophosphate (TPP) (Zastre et al., 2013). The thiamine pyrophosphate carrier transports TPP into the mitochondria, where it is a cofactor for pyruvate dehydrogenase and alpha-ketoglutarate (α-KGDH) dehydrogenase (Zastre et al., 2013; Figure 1.5).

One small molecule, dichloroacetate functions on a TPP-dependent enzyme pathway. Dichloroacetate selectively targets and interferes with the metabolism of cancer cells through the inhibition of pyruvate dehydrogenase kinase (PDK). PDK triggers the deviation of glycolysis into oxidative phosphorylation (Tataranni et al., 2019). Pyruvate dehydrogenase kinase inhibits pyruvate dehydrogenase (PDH) through the phosphorylation of an alpha subunit. When DCA inhibits PDK, PDH is activated. The activation of PDH causes mitochondrial oxidation of pyruvate, which interferes with the metabolic activity within cancer cells (Kankotia and Stacpoole, 2014; Tataranni et al., 2019). Specifically, when pyruvate enters the mitochondria, organelle remodeling occurs (Tataranni et al., 2019). Organelle remodeling results in the upregulation of reactive oxygen species and the efflux of apoptotic-inducing factors such as cytochrome c (Michelakis et al., 2008; Tataranni et al., 2019). There is a regulatory relationship between thiamine and DCA, in terms of PDH activation. Because DCA inhibits PDK, it inadvertently causes the activation of PDH. Studies have shown that the activation of PDH disrupts cancer metabolism, leading to cell death. Therefore, there is anti-cancer potential for thiamine because its phosphorylated form, TPP, is a cofactor for the PDH enzyme.

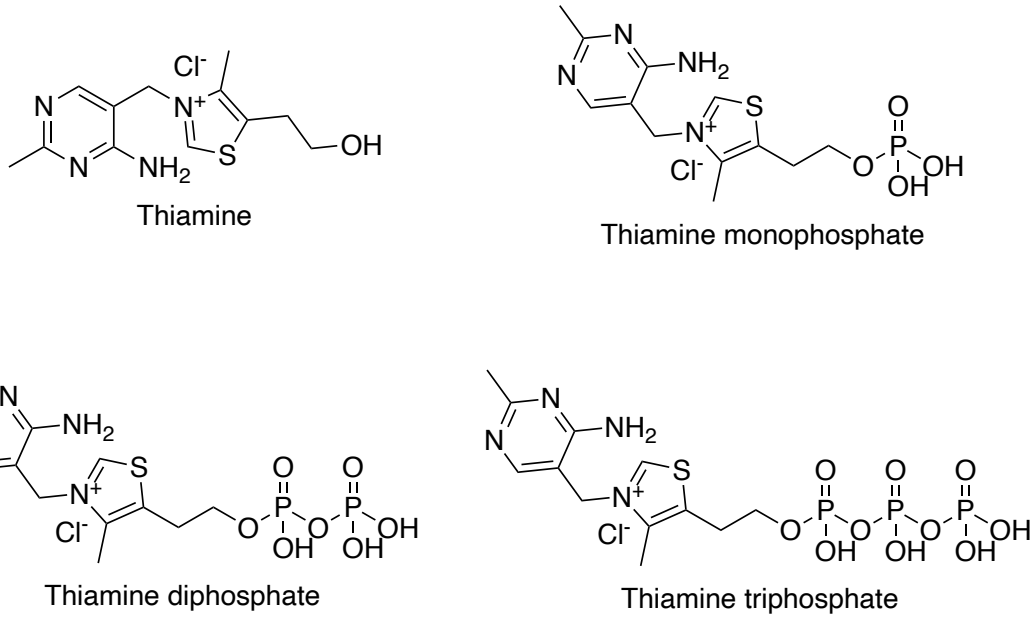

Figure 1.4: Chemical structures of thiamine and its phosphorylated forms, thiamine monophosphate, thiamine diphosphate, and thiamine triphosphate, generated by ChemDraw 19.1.

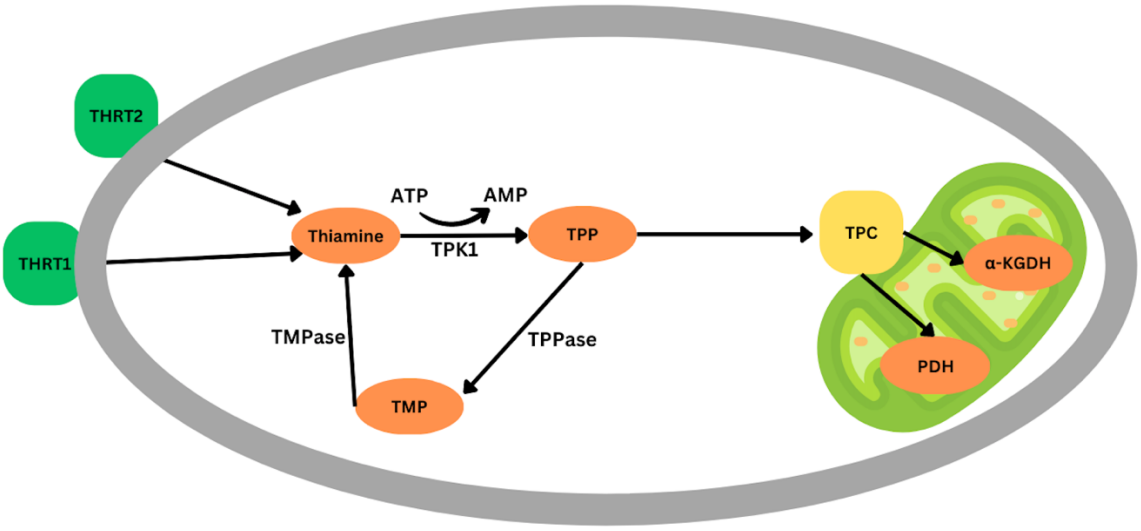

Figure 1.5: Transport of thiamine intracellularly and its mechanism into the active cofactor, TPP, adapted from Zastre et al. 2013.

Intracellularly, thiamine is involved in the antioxidant system, fatty acid metabolism, fatty acid and nucleic acid synthesis, glucose metabolism, amino acid metabolism, and neurotransmitter balance (Lonsdale and Mars, 2017). Thiamine pyrophosphate is an essential cofactor for enzymes involved in carbohydrate metabolism in the pentose phosphate pathway (PPP), citric acid cycle, and glycolysis (Jonus et al., 2020). In the pentose phosphate pathway, transketolase utilizes TPP to generate DNA and RNA synthesis (Lonsdale, 2006). Thiamine is also involved in peroxisomal lipid metabolism. Peroxisomes function to shorten long-chain fatty acids that cannot be oxidized by the mitochondria (Lonsdale, 2006). Thiamine plays a role in peroxisomal lipid metabolism, because TPP is the cofactor for 2-hydroxyacyl-CoA lyase 1 (Lonsdale, 2006). Glucose undergoes glycolysis to break down into pyruvate, where TPP serves as a cofactor for PDH to convert pyruvate into acetyl CoA (Chaudhry and Varacallo, 2022). Thiamine is also an essential factor in the citric acid, due to the role of TPP in producing acetyl CoA. Acetyl CoA can be synthesized from glucose or fatty acid synthesis beta-oxidation (Chaudhry and Varacallo, 2022). If acetyl CoA is synthesized from fatty acid synthesis beta-oxidation, TPP serves as a coenzyme for the enzyme, alpha-ketoglutarate dehydrogenase's conversion to succinate CoA (Chaudhry and Varacallo, 2022).

Hanberry and colleagues (2014) conducted a study evaluating if high-dose thiamine has anticancer properties like DCA. Cell proliferation was measured in pancreatic cancer cells treated with 25 mM of thiamine and DCA. The IC50 values for thiamine treatment were lower than DCA, but both reduced cellular proliferation in Panc-1 cells (Hanberry et al., 2014). This study showed that thiamine possesses anticancer properties due to its involvement in the PDH complex. There is promising potential therapeutic use for thiamine in cancers that are highly dependent on metabolic pathways.

3.3 Benfotiamine:

Lipid-soluble, synthetic forms of thiamine, benfotiamine (BFO), and sulbutiamine (SLBT), have a higher bioavailability and therapeutic response at lower doses than water-soluble thiamine (Jonus et al., 2020). These mimetic forms have been studied to determine if they increase intracellular TPP when compared to thiamine (Jonus et al., 2020). Benfotiamine is an S-acyl derivative of thiamine and has current clinical use supplementation in diabetic complications, neuropathy, nephropathy, and retinopathy (Volvert et al., 2008; Balakumar et al., 2010; Figure 1.6). Sulbutiamine's chemical structure contains a disulfide bridge that connects two thiamine molecules (Starling-Soares et al., 2020). However, sulbutiamine is only used for dietary supplementation and currently does not have clinical use in the United States. Benfotiamine's intracellular conversion to thiamine begins with dephosphorylation into S-benzoylthiamine (Wada et al., 1961; Mizuhira et al., 1968; Volvert et al., 2008). S-benzoylthiamine crosses the cell membrane and then is converted into thiamine intracellularly (Wada et al., 1961; Mizuhira et al., 1968; Volvert et al., 2008; Figure 1.7). Similarly, Sulbutiamine can cross the cell membrane to enter the cell and is converted to thiamine intracellularly by thioesters (Jonus et al., 2020). These lipophilic derivatives can produce more TPP intracellularly than thiamine, at lower concentrations (Volvert et al., 2008).

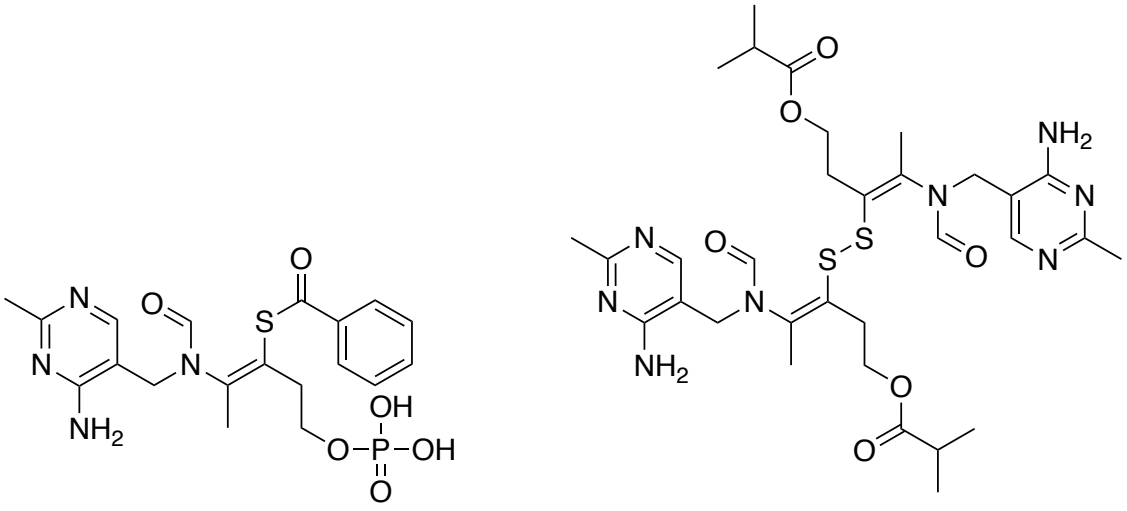

Figure 1.6: Chemical structures of synthetic thiamine analogs: benfotiamine and sulbutiamine, generated by ChemDraw 19.1.

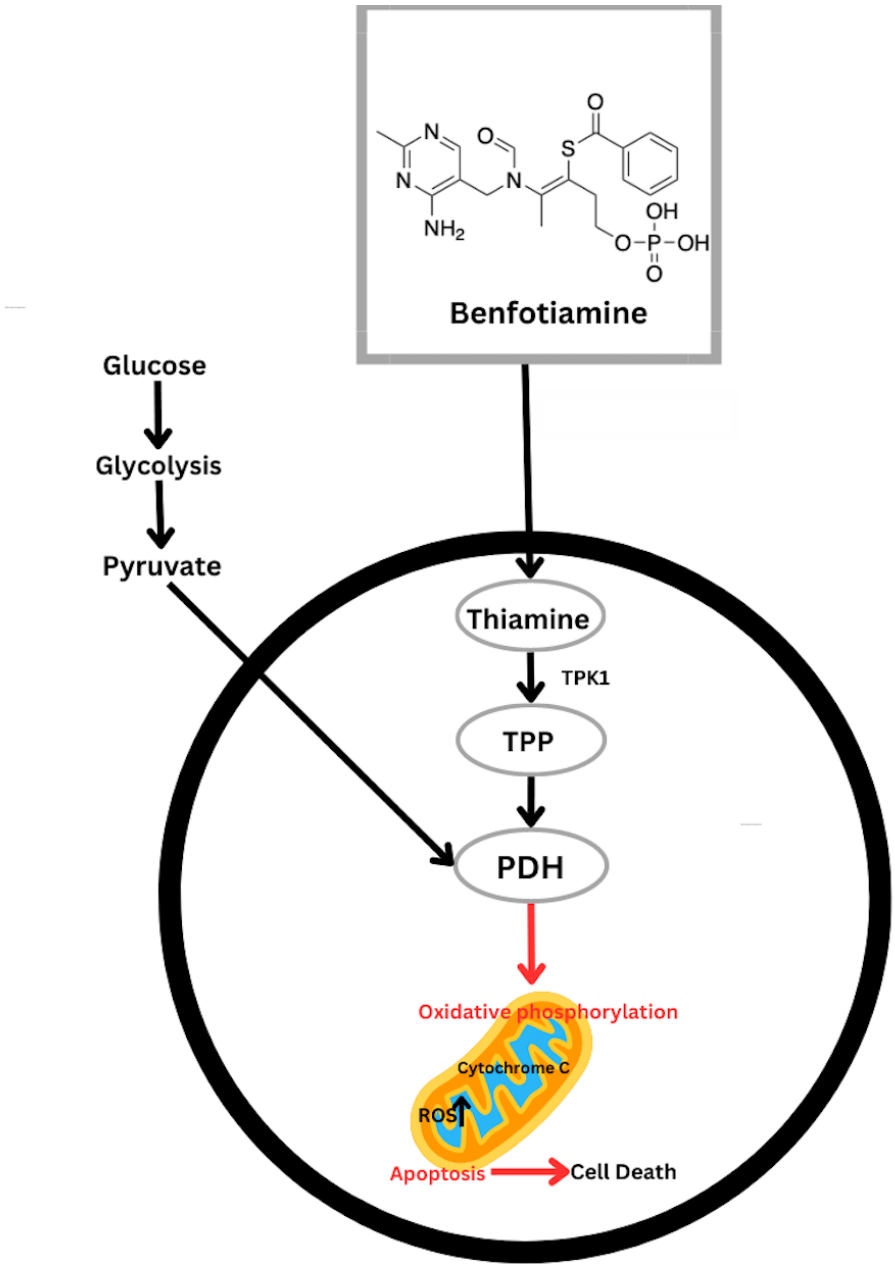

Figure 1.7: Anticancer activity of Benfotiamine Within a Cell, adapted from Tataranni and Piccoli, 2019.

A study conducted by Jonus et al. (2020) quantified intracellular amounts of thiamine, TPP, benfotiamine, and sulbutiamine in human cancer cell lines HCT 116 (colorectal carcinoma), U-87 MG (glioblastoma), and MDA-MB-231 (metastatic breast adenocarcinoma), and a FHC cell served as the control. The IC50 values for BFO and SLBT were 30-50-fold lower than thiamine at lower concentrations (Jonus et al., 2020). Cell viability was calculated to be 4-5-fold lower in cell lines treated with BFO and SLBT as compared to thiamine treatment and the control (Jonus et al., 2020). The concentrations used were 25 mM thiamine, 1 mM BFO, and 0.5 mM SLBT. TPP levels in cell lines treated with thiamine, BFO, and SLBT were increased by 2-fold. The thiamine treatment concentration was 100X higher when compared to concentrations of BFO and SLBT (Jonus et al., 2020). In terms of intracellular TPP levels, BFO and SLBT are more bioavailable and produce more TPP, at lower doses, than hydrophilic thiamine. Sugimori and colleagues (2019) designed a study to evaluate the therapeutic potential of BFO in primary AML, AML, B-cell leukemia, T-cell leukemia, BM-S blasts (collected upon diagnosis), PB-R blasts (collected upon relapse), and Burkitt's lymphoma. Cell viability, death mechanisms, and cytotoxicity were evaluated throughout this study. An MTT assay was utilized to assess the sensitivity of these cell lines to 100 uM of BFO treatment. Cell viability was compromised in BM-S blasts, AML cells, and in four out of the nine primary AML cell lines. It was concluded that BFO exhibited anticancer potential in various leukemia cell lines, at micromolar concentrations.

4. Hypothesis:

Based on studies that have evaluated benfotiamine's anticancer properties, BFO increases intracellular TPP levels and compromises cancer cell viability. Cancer cell metabolism is dependent on endogenous TPP, and it would not be anticipated that thiamine uptake and

subsequent TPP formation would be susceptible to acquired or intrinsic chemoresistance mechanisms. Therefore, we hypothesize that benfotiamine will produce similar anticancer activity in chemoresistant cancer cells as in wildtype acute lymphoblastic leukemia cells.

CHAPTER 2

1. Objective:

This study aimed to evaluate the anticancer activity of benfotiamine in N6/ADR chemoresistant cells vs. NALM-6 wildtype cells. Doxorubicin functioned as a control for the chemoresistant cell line.

2. Materials and Methods:

A precursor B cell acute lymphoblastic leukemia cell line was utilized as the in vitro model system to evaluate the research objectives. The wildtype NALM-6 cell line and the chemoresistant phenotype, N6/ADR, were kindly provided by Dr. Eugene Douglass' lab at the University of Georgia. NALM-6 cells were cultured with 21.4 nM of the anthracycline, doxorubicin, for 12 months to produce the Adriamycin-resistant cell line (ADR) phenotype, N6/ADR (Treichel & Olken, 1992).

2.1 Cell Culture:

The NALM-6 and N6/ADR cells were maintained in RPMI 1640 with L-glutamine, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured in T-75 flasks in a carbon dioxide (CO_2) incubator at 37°C, 5% CO_2, and 95% humidity.

2.2 Plating density:

Cells had a 95% viability or higher before being seeded into plates for experiments. The cells were seeded into sterile 96-well culture plates at different densities. The cell seeding density for NALM-6 was 4×10^5 cells/well and 2×10^5 cells/well for the N6/ADR cell line. Seeding densities for each cell line were based on plating experiments according to beer's law.

2.3 Drug Treatment:

NALM-6 and N6/ADR cell lines were treated with varying concentrations of benfotiamine prepared from a 1 mM stock of benfotiamine. A cell mix comprised of cells and RPMI 1640 media was seeded into each well at 100 uL per well. Increasing treatment concentrations were added to wells at 100 uL per well. The control cells were seeded in RPMI 1640 media at 200 uL per well. Cells were incubated for 72 hours with benfotiamine treatment.

NALM-6 and N6/ADR cells were treated with DOX to serve a control for BFO treatment. Cell lines were treated with varying concentrations of doxorubicin prepared from a 10 mM stock solution. A cell mix comprised of cells and RPMI 1640 media was seeded into each well at 100 uL per well. The control cells were seeded in RPMI 1640 media at 200 uL per well. Cells were incubated for 72 hours after doxorubicin treatment.

Table 2.1: Drug treatment concentrations of BFO and DOX in NALM-6 and N6/ADR cell lines.

NALM-6 + BFO	NALM-6 + DOX	N6/ADR + BFO	N6/ADR + DOX
0.001 mM	0.05 nM	0.001 mM	0.001 uM
0.005 mM	0.1 nM	0.005 mM	0.01 uM
0.01 mM	0.5 nM	0.01 mM	0.1 uM
0.05 mM	1 nM	0.05 mM	0.5 uM
0.1 mM	5 nM	0.1 mM	1 uM
0.25 mM	10 nM	0.25 mM	5 uM
0.5 mM	50 nM	0.5 mM	10 uM
0.75 mM	100 nM	0.75 mM	25 uM
1 mM	1000 nM	1 mM	50 uM

2.4 MTT Assay:

Cell viability, after drug treatment, was measured by an MTT assay. This assay involves a reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), to measure cellular metabolic activity (Ghasemi et al., 2021). MTT is reduced by nicotinamide adenine dinucleotide phosphate (NADPH) dependent oxidoreductase enzymes to insoluble formazan (Ghasemi et al., 2021).

In this study, 15 uL of 5 mg/mL MTT was added to each well and incubated for 3 hours at 37°C. The plate was spun down in a 5810 R Eppendorf centrifuge at 2610 Xg and 37°C for 5 minutes. MTT was removed from each well. Formazan crystals were solubilized by the addition of 100 uL of dimethyl sulfoxide (DMSO) to each well. The plate was placed on a low-speed shaker for at least 15 minutes. The absorbance of each well was measured at 560 nm by a SpectraMax M2 microplate reader.

Cell viability was calculated by averaging absorbance values for each concentration, the control, and the DMSO blanks. Blank absorbance values were subtracted from the control and concentration absorbance values. The formula used to calculate cell viability was $(treatment\ absorbance)/(control\ absorbance)\ x\ 100 = cell\ viability\ (\%)$. Standard deviation was additionally calculated for the control group and each treatment concentration. GraphPad Prism was used to create non-linear regression dose-response curves from DOX and BFO treatment in NALM-6 and N6/ADR cell lines.

3. Results:

The viability of NALM-6 and N6/ADR cells after 72-hour treatment with benfotiamine was reduced in a dose-dependent manner with IC50 values of 0.03 mM and 0.5 mM, respectively (Figure 2.1). In benfotiamine treatment, the wildtype cell line had lower cell viability than the

chemoresistant cell line at the same concentrations. The IC50 value between the two varied significantly, concluding that N6/ADR required a much higher concentration than NALM-6 to kill 50% of cells. After doxorubicin treatment in NALM-6 and N6/ADR cell lines, IC50 values were 50 nM and 500 nM, respectively (Figure 2.2). In doxorubicin treatment, the IC50 value for the chemoresistant cell line was also 10-fold greater than the wildtype. It was evident that the chemoresistant cell line only responded to high doses of doxorubicin, while the wildtype responded at a significantly lower dose.

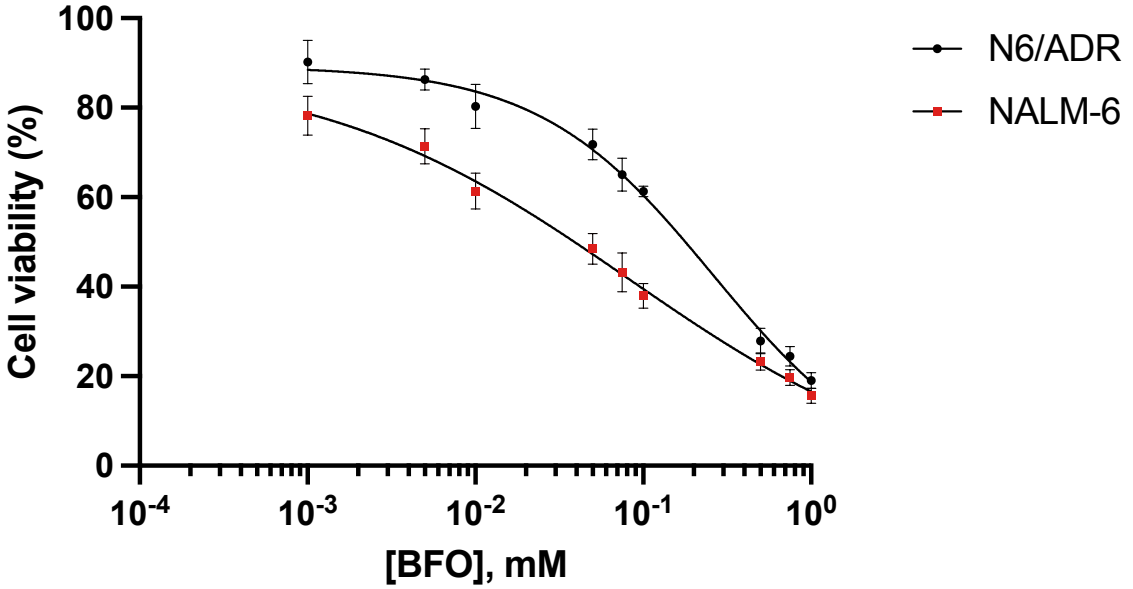

Figure 2.1: Dose-response curves of benfotiamine monotherapy in NALM-6 and N6/ADR cell lines. IC50 values for NALM-6 and N6/ADR were 0.03 mM and 0.5 mM. Experiments were performed in triplicate. IC50 values and standard deviation values were calculated for each value and graphed on non-linear regression dose-response curves, generated by GraphPad Prism 9.5.1.

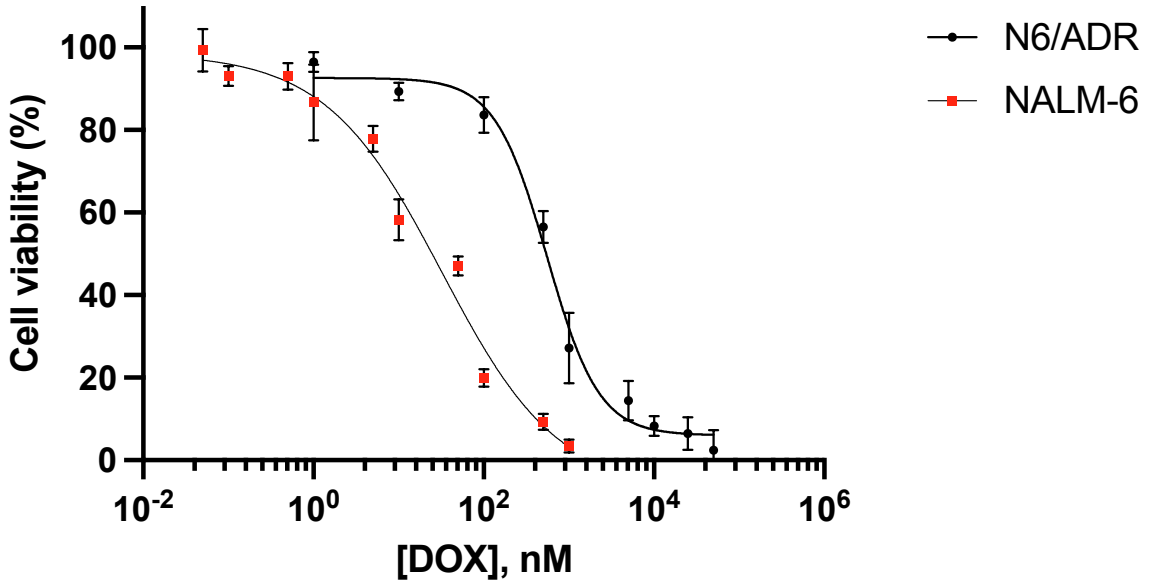

Figure 2.2: Dose-response curve of doxorubicin monotherapy in NALM-6 and N6/ADR cell lines. IC50 values for NALM-6 and N6/ADR were 50 nM and 500 nM. Experiments were performed in triplicate. IC50 values and standard deviation values were calculated for each value and graphed on non-linear regression dose-response curves, generated by GraphPad Prism 9.5.1.

4. Discussion and Conclusion:

We hypothesized that benfotiamine would be able to produce anti-cancer activity in a chemoresistant cell line. Due to the hydrophobicity of BFO, leading to more intracellular production of TPP, and its endogenous nature, we expected the same result in each cell line. This hypothesis was evaluated through an MTT cell viability assay comparing treatment in the NALM-6 cell line to the N6/ADR cell line.

In this study, doxorubicin was the control for the chemoresistant cell line, N6/ADR. We confirmed the chemoresistance of N6/ADR to doxorubicin through MTT assays. The IC50 of N6/ADR was 10X higher than NALM-6. Even though the chemoresistant cell line had a much lower sensitivity to doxorubicin than the wildtype, it still responded to high dose treatment.

Based on MTT assays, nonlinear regression dose-response curves showed that benfotiamine reduced cell viability in the wildtype and chemoresistant cell lines. The IC50 value for benfotiamine treatment in the chemoresistant cell line was significantly greater than the value for the wildtype. This data showed that a higher concentration of benfotiamine is needed to produce a therapeutic response in a doxorubicin resistant cell line.

There are a lack of studies investigating the anticancer activity of thiamine and benfotiamine in wildtype and chemoresistant cancers. In this study, we were able to confirm the anticancer properties of benfotiamine both cell lines. We are unsure as to why a higher concentration of benfotiamine is necessary to compromise cell viability in the chemoresistant cell line, but we believe the chemoresistant line has some type of resistance to BFO treatment.

CHAPTER 3

1. Discussion and Future work:

Data from dose response curves showed that a much higher concentration of benfotiamine was needed to produce the same result in the chemoresistant cell line. In comparing the drug treatments, the data was very similar. In each drug treatment the chemoresistant cell line required a much higher drug concentration than the wildtype. This result was not anticipated in BFO treatment, because we hypothesized that due to the endogenous nature of cofactor, TPP, it would not be susceptible to drug resistance mechanisms.

1.1 Cell Viability Assays:

An optimized MTT assay was the sole assay utilized to assess the cell viability of benfotiamine and doxorubicin in wildtype and chemoresistant cell lines. This assay can assess cell viability, cell proliferation, and cytotoxicity by measuring cellular metabolic activity. The wildtype and chemoresistant cell line could have significant differences in metabolic activity due to the cytotoxicity of treatment. Metabolic rate can vary due to treatment response in different cell lines, with increasing or decreasing concentration. Even though these assays were performed in triplicate, complimentary assays could be utilized to provide a more complete analysis of cell viability. Other in vitro assays that could have been utilized in evaluating the therapeutic potential of benfotiamine are a bromodeoxyuridine assay (BrdU), propidium iodide staining, and Alamar blue staining. A BrdU assay incorporates into newly synthesized DNA to detect cell viability (Crane and Bhattacharya, 2013). A propidium iodide staining assay detects cell viability by binding to DNA and measuring DNA content. Unlike the BrdU assay, propidium iodide does

not directly measure DNA synthesis. Propidium iodide staining can also measure cell cycle (Shen et al., 2017). This could have been used to evaluate if benfotiamine was cell cycle specific to maximize its therapeutic efficacy. Alamar blue staining would have been another useful assay to incorporate in this study as well. (Rampersad, 2012). An Alamar blue assay could be utilized as another metabolic cell viability assay because it is stable, non-toxic, and allows for the continuous monitoring of cells (Rampersad, 2012). Incorporation of additional in vitro assays would have provided additional data to compare with the cell viability data from the MTT assays.

1.2 Research Gaps:

Recent research from this study and others has shown that nutraceuticals, such as benfotiamine, interfere with cancer metabolism. Up to this point, there have been no published studies evaluating the therapeutic effect of benfotiamine on chemoresistant cancers. We were able to produce cell viability dose-response curves and IC50 values from BFO treatment in chemoresistant and wildtype cell lines. With BFO treatment, the chemoresistant cell line required a much higher drug concentration, similar to doxorubicin treatment in the chemoresistant cell line. Originally, we hypothesized that BFO would not be susceptible to a drug resistance mechanism because it is an endogenous molecule, but now believe that N6/ADR, the chemoresistant cell line, has some type of resistance mechanism against BFO treatment. Future work could be done to evaluate if there is a protective mechanism causing N6/ADR to require twice the dose of treatment as the wildtype. This resistance mechanism could be a P-gp mediated or a different mechanism. This could be determined by utilizing P-gp inhibitors in combination with BFO treatment, to evaluate if cell viability decreases or remains the same. In addition to

this, categorizing the mechanism could further lead to an answer on how to optimize BFO treatment in drug resistant phenotypes.

Our lab has previously conducted studies evaluating intracellular TPP levels in cancer cells following BFO treatment. In this study we did not quantify TPP levels in BFO treated wildtype and chemoresistant cells. The quantification of intracellular TPP could indicate the expression level of TPK1. A high TPP levels would be an indication that there are no TPP conversion issues. Conversely, low TPP values could indicate a low expression of TPK1, because TPP levels are limited to the amount of TPK1 present. Evaluating this would show if TPP conversion must be optimized in order to produce maximal therapeutic activity.

Given the endogenous nature of BFO's active form, TPP, it is possible that it could be used in combination with chemotherapy drugs, such as doxorubicin, and augment drug toxicity. In future work, it would be interesting to study if BFO has additive, synergistic, or antagonistic effects when used in combination with DOX. If BFO was found to have an additive or synergistic effect to DOX, DOX could be administered at a lower dose when used in combination with BFO. This could significantly decrease toxicity and adverse effects in patients, and furthermore increase patient compliance and tolerability.

1.3 Benfotiamine

It is widely known that chemotherapy treatment is extremely toxic to patients, leading to a plethora of adverse effects. Because of toxicity, chemotherapy is greatly limited by dose and patient compliance. The exogenous nature of chemotherapy drugs makes them very susceptible to drug resistance mechanisms that can greatly limit their effectiveness. On the other hand, benfotiamine's active cofactor form, TPP, is an endogenous molecule with very low toxicity and high safety profile. Low toxicity would not limit treatment in terms of dosage amounts. The high

safety profile of benfotiamine indicates that it would not cause serious or life-threatening adverse effects in patients. This in turn would lead to higher patient compliance, which is more likely to ensure a favorable outcome.

Based on our research, we discovered that the benfotiamine treatment outcome in the N6/ADR, chemoresistant cell line was not what we anticipated. Rather than BFO producing the same effect in wildtype and chemoresistant cells, the chemoresistant cells required twice the dose as the wildtype. Because of this, we believe that BFO is susceptible to a drug resistance mechanism, when employed in treatment of the N6/ADR cell line. Further research would provide more insight as to what the resistance mechanism is, and how it could be combatted to maximize BFO effectiveness in chemoresistant cancers.

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