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ANNEXIN A5 – CHLORAMBUCIL: A TARGETED THERAPEUTIC DRUG

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For my mom.

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Abstract

Breast cancer is the most common cancer in women worldwide and its incidence is increasing, particularly in developing countries. The survival rate of early stage breast cancer is 80% but falls to 24% for breast cancers diagnosed at a more advanced stage. Leukemia is a cancer that begins in the bone marrow and results in abnormal white blood cells or leukemia cells. The five-year survival rate is only 57% in the US. Current therapies affect every cell, healthy or not, and generate physical and psychological consequences in the patient.

Our approach is to target breast cancer cells and leukemia cells with a protein, annexin A5, and kill them specifically with an anti-cancer drug. Annexin A5 binds to phosphatidylserine, a marker of cancer cells expressed on the outer leaflet of the plasma membrane. Chlorambucil, or Leukeran (4-[bis(2-chlorethyl) amino] benzenebutanoic acid) is a drug used in chemotherapy treatment to treat leukemia; several molecules of this alkylating agent are linked by their carboxylic functional groups to primary amines of the protein by EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide).

The mass of the conjugate was compared to the mass of annexin A5, and the quantity of chlorambucil on the protein has been determined. In vitro cytotoxicity assays were made on two breast cancer cell types, EMT6 and 4T1, and two leukemia cell types, L1210 and P388. The cytotoxicity of the conjugate was compared to the cytotoxicity of chlorambucil alone, showing a significant improvement with a LD50 (median lethal dose or amount of the substance required to kill 50% of the cells) 10 or 100-fold times less than the free chlorambucil. In vivo experiments have been made with 4T1 and L1210 cells on BALB/c mice, and the survival and physiologic measurements were made. The results showed that the conjugate increased the survival time in both cancers.

Introduction

In 2015, 90.5 million people had cancer and 14.1 million new cases occur every year. Breast cancer is the 7th leading cause of death in women, creating a tumor with a possibility to metastasize causing 90% of solid tumor cancer death. Leukemia is the most common type of cancer in children with a very low rate survival.

Today, different kinds of treatments are able to treat cancers, and non-targeted therapies are one of them. Targeted therapies are developed to focus on a component only present on cancer cells and kill them without killing healthy cells, therefore reducing side effects. Our idea is to select cancer cells by the molecule expressed on their surfaces by conjugating a protein with an alkylating agent to kill them.

Cancer

With more than 100 different types of cancer that affect humans, each year cancer is responsible of 15.7% of deaths worldwide[1]. Colorectal, breast and lung cancer are the most common type of cancer in females. In children, acute lymphoblastic leukemia and brain tumors are the most common[2]. The risk of cancer increases significantly with age. Chemical, mechanical, and environmental factors are responsible for 90% of cancer cases, and inherited genes only 10% [3]. In 2010, the financial cost of cancer was estimated at $1.2 trillion USD[2].

Six biological capabilities are required by the cell to form a malignant tumor and constitute the hallmarks of cancer: sustaining proliferative signaling, resisting cell death, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Moreover, two emergent hallmarks are appearing: reprogramming of energy metabolism and evading immune destruction[4].

The genome controls the life cycle of the cell, and several checkpoints are dispatched through the life of the cell to assure the integrity of the DNA, the proper chromosome duplication and the attachment of the kinetochore to a spindle fiber during mitosis. Checkpoints examine internal and external cues and determine whether to move forward or not. Two central control nodes, RB (retinoblastoma associated) and TP53, regulate the cell and make the decision to let the cell proliferate or activate the apoptotic program. RB protein transmits growth inhibitory signals from an indicator outside of the cell. TP53, or "the guardian of the genome", transduces signals from stress within the intracellular operating system and can stop the cell-cycle progression to allow repairs or trigger apoptosis if damages are too significant[4,5]. A mutation into genes related to these two gatekeepers is common among multiple tumors and can lead to uncontrollable growth. Those several steps, known as malignant progression, will change a normal cell into a cancer cell[4].

The disease is due to the continuous division of cells in their environment with possibilities of metastasis, a step where cells are spreading and inducing tumors everywhere in the body[1,6]. This last step is responsible for 90% of death caused by cancer[7]. Cancer cells spread through the body by invading nearby normal tissue, moving through the walls of blood vessels or lymph nodes and travelling through the lymphatic system or the blood stream, stopping in small blood vessels and moving into surrounding tissue, and growing into this tissue until a tumor forms inducing new blood vessels to grow allowing the tumor to continue growing[8]. The phenomenon known as anoikis programs cell death when cells detach from the extracellular matrix (ECM) and is essential to put the cell in their correct anatomical location. During the metastasis, cells develop a resistance to anoikis and inhibit the activation of both death receptors and

mitochondrial pathways[9]. Cells go through an epithelial-mesenchymal transition, or EMT (changes in cell polarity, cell invasive and migratory property and cell-to-cell and cell-to-matrix adhesion), and become undifferentiated, isolated, and mesenchymal cells. Mutation is responsible for down regulation of E-cadherin, a protein responsible for the attachments on the ECM and up-regulation of N-cadherin, inducing a better movement through walls. They have new properties of invasion and migration and can survive detached from the ECM and reach new tissues through the blood or the lymph stream[10].

Breast Cancer

Breast cancer is the 3rd leading cause of death worldwide for females between 50-59 years old, and the number will increase in the next few years. Soon 1 in 8 women will be diagnosed with breast cancer[11,12]. An epidemiology study shows that the incidence of breast cancer is greatest in the more developed countries and lowest in the least developed countries[13].

Between 5 and 10% of breast cancer is due to gene inheritance, obesity, older age, and drinking alcohol[14]. Improvement of new treatment decreases the number of deaths, and today a patient with a breast cancer detected in its early stage has a five-year survival rate of 93%[15].

Breast cancer starts when cells in the breast grow out of control and form a tumor, most frequently in milk ducts or lobules[14]. More than 80% of breast cancer is discovered due to the first typical symptom: a lump in the breast which feels different during a palpation than the rest of the breast[16]. Occasionally the cancer can invade surrounding tissues and/or metastasizes[17]. Breast cancer can be treated by local and systemic treatments such as surgery, chemotherapy, hormonal therapy, targeted therapy and radiation therapy[14].

Leukemia

Known as the most common type of cancer in children, leukemia affected 2.3 million people in the world in 2015 and caused 354 000 deaths. The five-year survival is between 60 and 85% in children under 15, depending on the type of leukemia and the patient. However, in the US, in total, the average five-year rate survival is 57%[2]. Moreover, like breast cancer, leukemia occurs more in developed countries[2].

Exact causes are unknown, but it's a combination of inherited and environmental factors, the most common being smoking, some chemicals, chemotherapy, and down syndrome[18]. The bone marrow is the place where the stem cells of blood cells are made. When leukemia occurs, the number of red blood cells decreases at the expense of abnormal white blood cells called leukemia cells[19]. The cells are non-adherent and don't form a tumor, and they spread everywhere in the body by the blood and lymphoid stream. Because of the lack of red blood cells and abnormal white blood cells different symptoms occur, such as feeling tired, bruising, bleeding, fever, and increased risk for infections[20].

Four types of leukemia are known: Acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL)[2].

In acute leukemia, which means that it progresses quickly, bone marrow cells cannot evolve the way they should and stay immature, which causes death in only few months. However, many patients can be cured. In chronic leukemia (CL), cells are more mature, but not completely, and are less effective to fight infection than mature white blood cells. People with CML can live longer than acute leukemia, but it is harder to cure[21–24]. Lymphocytic, or lymphoid, leukemia is induced by a problem in the progenitor of lymphocytes B or T[21,25,26]. If cancer cells are in the bone marrow and blood, the cancer

is a lymphocytic leukemia; however if the cancer cells tends to be in other tissues like lymph nodes they are lymphomas[21]. Lymphomas can be two kinds: Hodgkin and non-Hodgkin lymphomas.

Hodgkin lymphomas start in B lymphocytes and turn into Reed-Sternberg cells, which are large, malignant, mature B cells. Non-Hodgkin lymphomas don't have Reed-Sternberg cells and can start both in B or T lymphocytes. Lymphomas and leukemia belong to the group of tumors of the hematopoietic and lymphoid tissues[27–30].

Chemotherapy, radiation therapy, bone marrow transplants and targeted therapy are treatments commonly used. The success depends on the age of the patient and the type of leukemia[31–34].

Therapies

Non-targeting and targeting treatments

To treat cancer, cancer cells must be killed faster than they replicate. Several treatments exist and are adapted to each case: surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplant and precision medicine[35]. Surgery is used to physically remove a solid tumor from the body, and a second treatment must be taken to kill remnant cancer cells. Chemotherapy and radiation therapy are not specifically aimed against cancer cells but against every cell in their pathway. Because healthy cells are also dying, side effects appear like fatigue, nausea, hair loss, and mood changes[36].

Passive targeting exists when nanoparticles like a drug or a protein accumulate in a tumor because of the enhanced permeability and retention in this tissue (EPR). EPR depends not only on the particle size but also on the impaired lymphatic drainage and leaky vasculature[37,38]. In effect, macromolecules around 40 kDa tends to accumulate in

neoplastic tissues after an intravenous administration because this range size is above the renal threshold[39–41]. When tumors grow up, they rapidly need vasculature to bring nutrients and oxygen to the cells. A tumor larger than 2 to 3 mm^3 will release angiogenic factors and the imbalance with matrix metalloproteinase (MMPs) within the neoplastic tissue will create new disorganized blood vessels allowing nanoparticles with a diameter superior to 600nm to penetrate into the tumor[42,43]. These new vessels are made rapidly, and a lack of tight junctions between endothelial cells results in a leaky and permeable vessel[44].

Immunotherapy and targeted therapy target only cancer cells. The first therapy is a type of biological therapy using certain components of the immune system of the patient to kill cancer cells. Targeted therapy can use antibodies, small molecules, or proteins to avoid harming normal cells.

Phosphatidylserine

Cells naturally have a membrane made of phospholipids, molecules with two hydrophobic tails and a hydrophilic head. Phosphatidylserine is the most abundant phospholipid negatively charged in membrane of eukaryotic cells. Discovered in the 40's by Folk et al., phosphatidylserine has a central glycerol where two fatty acids are attached on the first and the second carbon of glycerol by an ester linkage, and a serine is attached on the third carbon by a phosphodiester linkage as we can see on Figure 1.

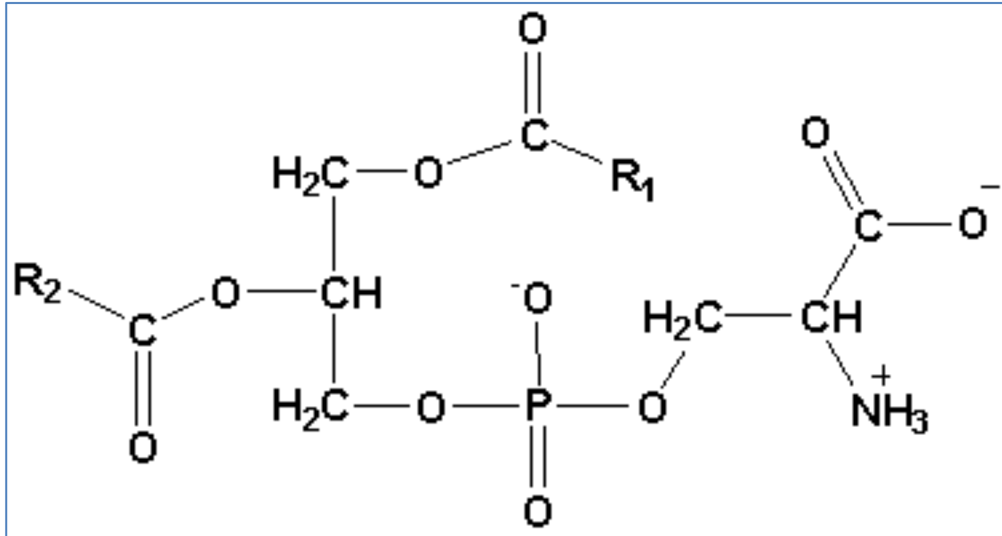

Figure 1: Structure of the phosphatidylserine

Between cell types and organelles, saturated fatty acids will differ to create a different phosphatidylserine[45].

A healthy cell will keep negatively charged amino-phospholipids like phosphatidylserine (PS) and phosphatidylethanolamine (PE) to the cytosolic face, while the cytoplasmic face has phosphatidylcholine (PC) and sphingomyelin. Lipids are exposed to the intracellular side due to an enzyme, the flippase, inhibited by the presence of calcium[46,47]. Flippase transports lipids like PE or PS from the exoplasmic to the cytoplasmic face while the floppase does it in the reverse direction. These two enzymes are ATP-dependent and uni-directional, and their activities balance the membrane composition and create a symmetric membrane. A third enzyme, the scramblase, which is energy-independent and bidirectional, can quickly modify the equilibration and exposes lipids on one face of the membrane[48–50]. The concentration of calcium inside a healthy cell is very low. A domain calcium dependent, named EF-hand-like Ca^{2+} binding domain, will activate the scramblase when the concentration of calcium intracellularly is high, signaling a problem. The activated scramblase transports lipids including PS to the

outer leaflets. These three different types of enzymatic groups of phospholipid transportation can be found in every eukaryotic cell.

When the phosphatidylserine flips to the extracellular surface, it can play a role in the coagulation cascade by activating the several clotting factors. Moreover, when a cell undergoes programmed death, phosphatidylserine molecules are extracellularly exposed, and it's one of the earliest indicators of apoptosis. In effect, an oxidative stress causes exposure of phosphatidylserine on the outer membrane due to the activity of the scramblase calcium dependant[51–54]. This stress can come from cancer development, and cancer cells will expose phosphatidylserine. Today this molecule is widely used as a marker for tumors[55].

Phosphatidylserine is one element responsible for the structural integrity of the cell membrane and plays two major roles in the coagulation and cell signaling. Because an injury will induce stress to the cells, phosphatidylserine on the surface of activated platelets will be expressed. It serves as a pro-coagulant surface by orienting tissue factor (TF) and factor VII, responsible of the clotting[56].

Moreover, when phosphatidylserine is expressed on the outer surface of apoptotic cells due to the activity of scramblase, the cell sends an "engulf me" message to macrophages[51]. Macrophages can respond to three different messages: "find me", "engulf me" and "eat me"[57]. The first message is made of cytokines and chemokines released in the environment by apoptotic cells. The macrophages will try to find the source of these molecules by following the gradient. The second signal is expressed by apoptotic cells, increasing by 300-fold the concentration of PS on the outer membrane, and macrophages will kill these cells by phagocytosis[58]. However, the opposite signal exists too, the "don't eat me" signal. In effect, cancer cells have found a way to avoid being killed by

macrophages. For example, CD47 is a protein expressed and a signal to the macrophages that the cell is healthy and does not need to be eaten. Other proteins and molecules exist and trick macrophages like CD31 or CD300A[57,59,60]. "Engulf me" is the last signal, expressed by apoptotic cells to avoid an immune response and kills targeted cells with precision.

Chlorambucil

During World War I, after soldiers were exposed to mustard gas, a physician discovered that the number of white blood cells had decreased significantly. The active ingredient in the mustard gas is due to the use of sulfur on the molecule. By reducing the toxicity by exchanging sulfur for nitrogen atoms, they were able to create a new chemotherapeutic drug. However, it was only in the 1950s that Everett et al. synthetized Leukeran or chlorambucil (CHL). With a less toxic alkylating agent, the molecule is able to react with the DNA slowly and kill cancer cells[61].

Chlorambucil (4-[bis(2-chlorethyl)amino]benzenebutanoic acid) is listed in the World Health Organization List of Essential Medicines[62]. It's an alkylating and antineoplastic agent of 304.2 g/mol, working by blocking the formation of DNA and RNA. The drug is used to treat Hodgkin lymphoma and non-Hodgkin lymphoma and is a preferred treatment for chronic lymphocytic leukemia (CLL)[63].

The molecule has one carboxylic function linked to an aromatic ring and a tail with a tertiary amine linked to two chloride. Due to the small electronegativity difference between C and H, the aromatic ring does not have polarity and induces a high hydrophobicity of the molecule increased by the four atoms of carbon linked to it (Figure 2)[64]. The alkylating agent has an appearance of a white crystalline powder and a melting point at 65°C. The water solubility of the drug is less than 0.1 mg/mL at pH 7 and 25°C

but soluble in an acidic environment[65]. The aromatic ring is also a good reactant to energy in the form of photons, creating a molecule sensitive to light or heat[66].

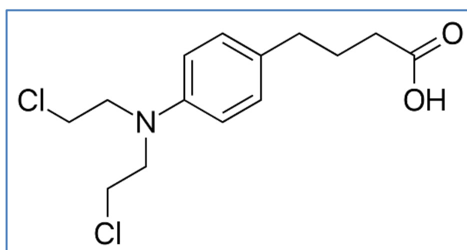

Figure 2: Semi-structural representation of the molecule of chlorambucil or Leukeran

Chlorambucil, by its property of light sensitivity, can be quantitatively measured in a solution. In effect, with a presence of dimethyl sulfoxide (DMSO) and ultraviolet light at 358 nm (ultraviolet range between 1 – 400 nm), chlorambucil is photoactivated, and the tail with the tertiary amine and 2-chlorethyl reacts with the DMSO. The two chlorides are replaced by the sulfoxide, and a ring forms a fluorescent component at 434 nm[67], the isocyanate of chlorambucil or 3-{4-[bis(2-chloroethyl)amino]phenyl}propyl-isocyanate.

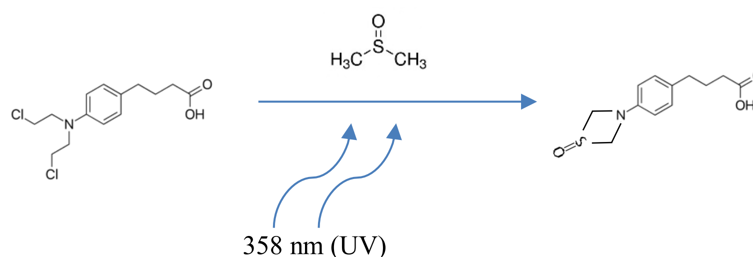

Figure 3: Photoactivation of chlorambucil into 3-{4-[bis(2-chloroethyl) amino] phenyl} propyl-isocyanate

The drug is supposed to be taken by mouth, go through the digestive system and reach the blood. The molecule will reach the tumor by the blood stream and enter into each cell that it meets. The small molecule will penetrate in the nucleus and by a reaction

of substitution, the two chlorine atoms (electrophilic sites) will react with the two azote atoms belonging to guanine in DNA or RNA and form two covalent adducts[63,68]. The two bases are linked together, and chlorambucil acts like a locker by forming interstrand cross-links in both DNA and RNA. They cannot be opened for transcriptions or translations, and the alkylating agent induces the death of the cell. The peak of the highest concentration is reached 1 hour after swallowing the pill, and the drug has a half-life between 40 and 110 minutes. The molecule is quickly metabolized in the liver in ester of phenylacetic acid, a highly active agent with a half-life twice as high as the chlorambucil. The drug is eliminated by 60 % in 24 hours through the urine[69,70,70–72].

A study of the effect of chlorambucil on HeLa cells showed that after 1 hour of treatment, cells were unable to divide but they were producing RNA, protein, and DNA. If cells are treated during the G1 phase, the S phase is longer and accompanied with a mitotic delay. Treatment during the G2 phase inhibits the DNA synthesis and delays the mitosis[73].

Nitrogen mustard plays an important role in modern chemotherapy, and chlorambucil, because of its two alkylating functions, is one of the most effective alkylating agents. A half-mustard analogue with just one alkylating agent is less effective to break chromosomes and cannot form interstrand cross-links. The major mutations induced by chlorambucil are deletions, and the molecule is more effective as an antitumor agent by forming a lot of interstrand cross-links[74]. This effectiveness leads to multiple breaks and mitosis pulverizations, in cultivated cells from hamster cell line V79 according to Speit et al.[75].

Moreover, chlorambucil is used in several studies to reach the nucleus by penetrating the nucleoplasm and study chromosome aberrations in rats or mice[76]. The pH

also plays a role in the cytotoxicity of the chlorambucil. Chlorambucil is a weak acid, and we see its cytotoxicity increased into a neutral intracellular compartment due to the ionization of the weak acidic function favorizing drug uptake. An intracellular acidification alters the distribution of the alkylating agent by reducing the pH gradient across the cell membrane. The cytotoxicity of chlorambucil is reduced in response to intracellular acidification[77].

The more common side effects are nausea, vomiting and change in menstrual periods. These side effects do not need medical attention and are due to the non-targeted treatment[63].

Annexin A5

With a weight of 36 kDa (kilo Dalton), annexin A5 is a protein which belongs to the annexin A5 super-gene group, a protein which is mostly found in eukaryotic organisms and can be found inside or outside the cell. Its subcellular locations are the cytoplasm, the extracellular and intracellular region, and the cytosol[78]. The protein is a non-glycosylated single chain protein. The 3-dimensional structure of annexin A5 is shown in Figure 4. The secondary structure of the protein is illustrated in Figure 5.

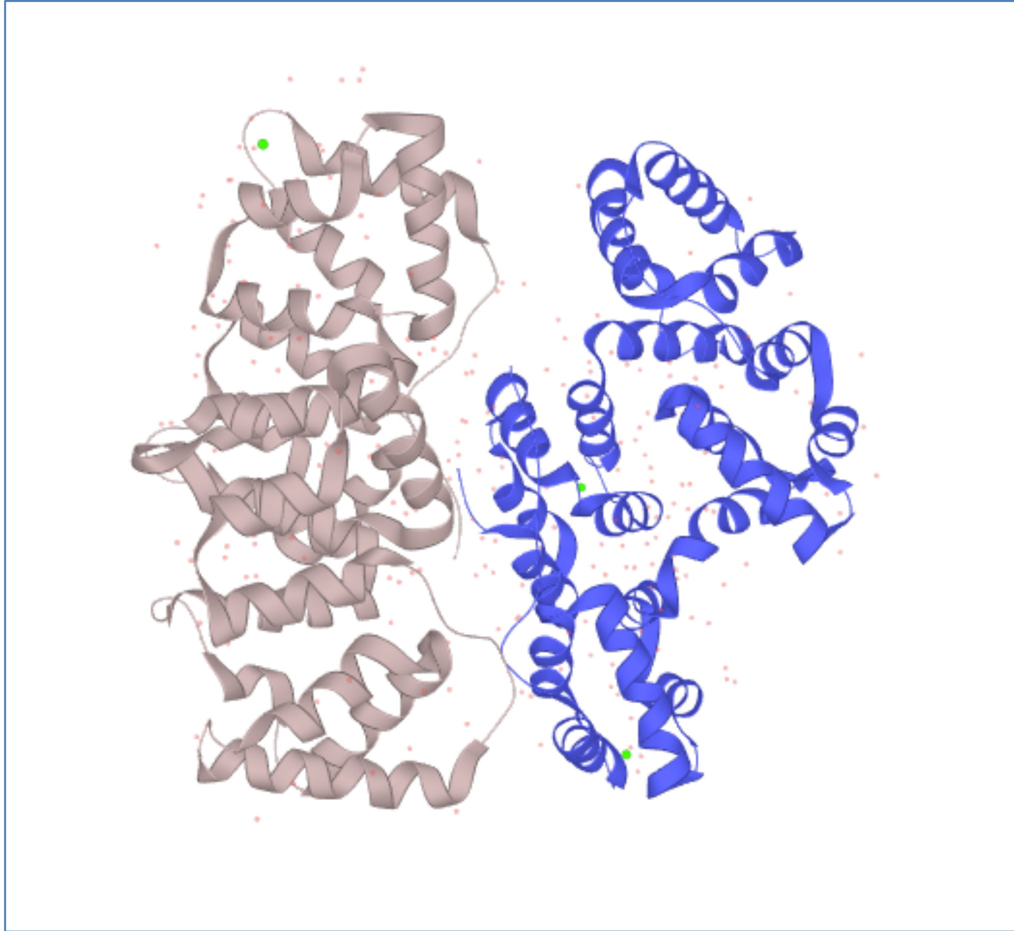

Figure 4: Structure of Homo Sapiens annexin A5. Structure made of alpha-helices and beta sheet.
Source: https://www.uniprot.org/uniprot/P08758

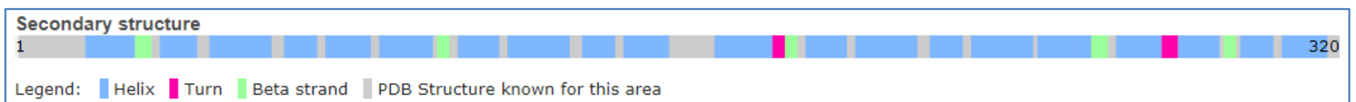

Figure 5: Secondary structure of Homo sapiens annexin A5, indicating helixes, turns, and beta strands.
Source : https://www.uniprot.org/uniprot/P08758

The precise function of the protein is unknown; however, a hypothesis by scientists is that annexin A5 plays a role in the inhibition of blood coagulation by inhibiting phospholipase A1 and binding to phosphatidylserine binding site and

competing with prothrombin. Then the prothrombin cannot be proteolytically cleaved to form thrombin in the clotting process. Thrombin converts fibrinogen into insoluble strands of fibrin; without thrombin the blood coagulation is inhibited.

Currently ANXA5 (annexin A5) is used in flow-cytometry; its ability to bind to phosphatidylserines is used to detect apoptosis, cancer cells and tumor vasculatures[55,79,80]. When exposed in the same environment with phosphatidylserine and calcium Ca^{2+}, the protein can bind to the PS by its convex part on the core. The core is made of alpha-helices, four domains contained in the C-terminal of the protein chain. The annexin A5-core has a calcium binding site, so calcium is necessary to bind to PS, and the ANXA5, rather than a monomer, will form a trimer. It has been demonstrated recently that annexin A5 is internalized in cells by a pinocytic pathway[81]. An annexin A5 protein network bonds the membrane patch nanomechanically into the cell and elicits budding, endocytic vesicle formation, and cytoskeleton-dependent trafficking of the endocytic vesicle. This finding has opened new avenues for targeted drug delivery and cell entry.

Conjugation for a targeted therapy

Conjugation with chlorambucil

Chlorambucil is originally a non-selective anti-cancer drug. Its action is directed against all rapidly dividing cells including cancer cells but also those of the normal hemopoietic tissues, gonads, and mucous membranes of the gastrointestinal tracts[82]. Moreover, Israel and Linford showed that the chlorambucil can bind without losing its alkylating activity, and its adsorption is increased by the presence of the chloroethyl group in the drug molecule[83].

Several experiments have successfully conjugated chlorambucil to antibodies or proteins to try to decrease the side effect of the chemotherapeutic treatment. Antibodies

have been shown to have a destructive effect on cancer cells but are not therapeutically effective. Conjugated to a chemotherapeutic or cytotoxic drug, immunoglobulins can be therapeutically effective and several are now in late-phase clinical testing[84]. Promising results are appearing when chlorambucil is conjugated to an immunoglobulin[85]. Especially to treat some forms of leukemia like Chronic Lymphocytic Leukemia (CLL), the association of chlorambucil with Rituximab, a CD20 targeted monoclonal antibody, is effective to improve considerably the survival of young patients[86]. Moreover, Obinutuzumab, another CD20 targeted monoclonal antibody appears to be more effective on the majority of CLL patients, usually older[87]. T. Ghose et al. showed that conjugated to chlorambucil, an immunoglobulin could increase mice survival for more than 200 days without developing any tumors on BALB/c mice with Erlich ascites carcinoma[88]. Moreover, conjugated to secreted immunoglobulin M or IgM of lymphoblastoid cells lines, chlorambucil selectively killed lymphoblastoid cells and caused cytolysis faster than the immunoglobulin or drug alone[89]. Against melanoma, the heterologous antibody against the mouse EL4 lymphoma conjugated with chlorambucil is also promising and more effective than the drug or antibody alone to inhibit the tumor[90].

To target breast cancer, the most common non-cutaneous cancer in women, molecules of chlorambucil can be bound to another amino-acid, L-methionine. Because the uptake of polyamine compounds is increased in breast cancer cells, a conjugation between methionine and chlorambucil led to a majority of the killed cells being cancerous ones. The conjugate shows better anticancer effect with less toxicity in comparison with free chlorambucil[91]. Spermidine is also a polyamine compounds and conjugated to chlorambucil the cytotoxicity against plasmacytoma cells was increased by 225-fold and the conjugate was 10,000 fold more active than chlorambucil at cross-linking DNA[92].

The use of a vector able to target neoplastic tissue has been studied by Miot-Noirault et al. The vector is not naturally found in healthy tissue and is named 2-fluoro-2-deoxyglucose (FDG). This compound is known to accumulate in solid tumors; by conjugating it to chlorambucil; researchers have obtained a targeted drug against cancer. Results are interesting with a high decrease in the toxicity of the drug against healthy cells and promising anti-tumor activity against the two mice models, melanoma and colon carcinoma tumors[93].

Chlorambucil can also be conjugated to proteins through multiple bond types which can affect the activity of the conjugate. A study revealed that chlorambucil bound to an albumin with an ester bound is less toxic than a conjugation with hydrazone bonds. The chlorambucil-asparagine conjugate shows to be targeted against cancer cells and cause less damage than free chlorambucil and yield better results[94]. Glutathione-chlorambucil also shows promising results against adenocarcinoma[95].

The alkylating agent can be conjugated to other drugs, like gemcitabine to increase the solubility and also the toxicity of the two drugs. The hydrophilic gemcitabine increases the solubility of the chlorambucil and allows a better distribution[96].

EDC/NHS conjugation method

The most common technique to conjugate a carboxylic molecule to another amine containing molecule is to use a carbodiimide. 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) is a zero-length carboxyl to amine crosslinker with a molecular weight of 191.7 g/mol.

This molecule is currently widely used to attach hapten to carrier proteins, crosslink proteins to carboxyl-coated beads or surfaces, or form amine bonds in peptide synthesis. EDC will form an amine reactive O-acylisourea intermediate when it reacts

with the carboxylic function of the first molecule (see Figure 6). The carboxylic acid group and amine containing molecule are linked by an amide bond, and isourea is released from this reaction. However, O-acylisourea intermediate is unstable in aqueous solutions, and a hydrolysis of the intermediate with a regeneration of the carboxyl can occur as we can see on the Figure 6 [97]. The most efficient conditions are an acidic environment with around a 4.5 pH and a buffer without any carboxylic group. However, using a phosphate buffer with neutral pH condition is possible but the efficiency is lower, which can be compensated by a higher amount of EDC.

N-hydroxysulfosuccinimide (sulfo-NHS) is a compound that can be added to the conjugation reaction to increase efficiency or create stable intermediates. EDC is a coupling reagent and with NHS, they will form a NHS ester, a highly reactive activated and less labile acid intermediate. The intermediate is stable and can be stored at low temperatures[98]. By forming a sulfo-NHS ester, the stability and solubility of the molecule increases but also the efficiency of the conjugation to primary amines at physiologic pH.

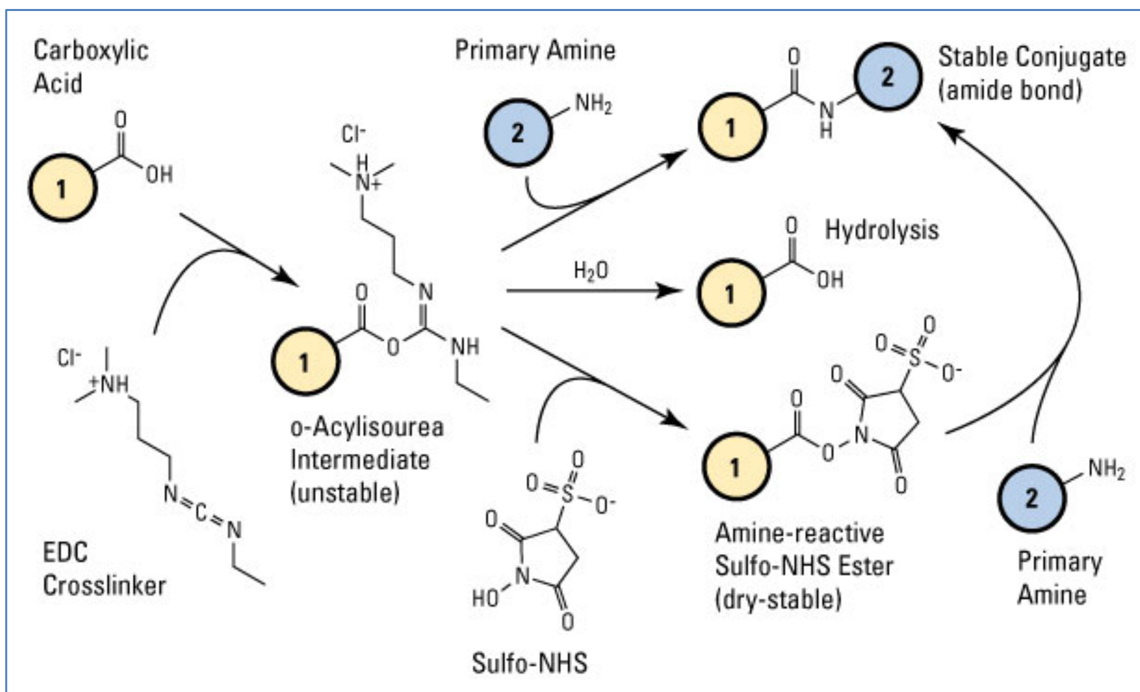

Figure 6: Scheme of the reaction of the conjugation between a carboxylic acid molecule (1) and a primary amine molecule (2) with EDC with or without sulfo-NHS. The molecules can be proteins, chemicals or peptides.
Source: https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/carbodiimide-crosslinker-chemistry.html

Lysine is the only amino acid with a primary amine where the EDC/NHS reaction can occur. They exist also at the end of each polypeptide chain. At physiologic pH, primary amines are positively charged and became more accessible to conjugation reagents. Moreover, they form a nucleophilic group, giving them the ability to be targeted for conjugation. The ANXA5 has an amino acid sequence with 22 lysines, so theoretically we can conjugate at least 22 molecules of chlorambucil if the steric configuration allows it, Figure 7 shows the FASTA file of human annexin A5.

However, the secondary, tertiary and quaternary structures of the molecule reduce this possibility. In effect, some of the functions would be hidden inside helixes and turns creating the tertiary shown on Figure 4.

```
>sp|P08758|ANXA5_HUMAN Annexin A5 OS=Homo sapiens OX=9606
GN=ANXA5 PE=1 SV=2

MAQVLRGTVTDFPGFDERADAETLRKAMKGLGTDEESILTLLTSRSNAQRQEISAAFKTL
FGRDLLDDLKSELTGKFEKLIVALMKPSRLYDAYELKHALKGAGTNEKVLTEIIASRTPE
ELRAIKQVYEEEYGSSLEDDVVGDTSGYYQRMLVVLLQANRDPDAGIDEAQVEQDAQALF
QAGELKWGTDEEKFITIFGTRSVSHLRKVFDKYMTISGFQIEETIDRETSGNLEQLLLAV
VKSIRSIPAYLAETLYYAMKGAGTDDHTLIRVMVSRSEIDLFNIRKEFRKNFATSLYSMI
KGDTSGDYKKALLLLCGEDD
```

Figure 7 : Amino acids of the human annexin A5. In yellow, the amino acids are indicated that can possibly have a primary amine able to bind the chlorambucil molecule with the EDC/NHS chemistry.
Source : https://www.uniprot.org/uniprot/P08758.fasta

Scope of Thesis

Hypothesis

Annexin A5 is naturally attracted by phosphatidylserines (PS) at the surface of cancer cells, and it has been shown to be internalized in cells. Chlorambucil can be internalized and kill a cell by blocking its DNA. In this project we hypothesize that the conjugation between those two molecules gives a new molecule with these two characteristics: a molecule that targets cancer cells and is internalized in these cells, leading to cell death. Those characteristics will be kept unmodified during the process of conjugation; the molecule will be less toxic to normal cells and more effective than free chlorambucil against breast cancer and leukemia.

1st Objective: Conjugation of the annexin A5 with chlorambucil

The conjugation of a protein with a chemotherapeutic agent uses current established techniques used in the biochemistry industry. The yield should be good enough after the chemistry, and the solution has to be pure to be used in vitro and in vivo and to avoid secondary effects (see the 2nd and 3rd objectives). The protein has to be undenatured and able to bind to the PS, and the chlorambucil has to keep its chemotherapeutic activity.

2nd Objective: In vitro studies on leukemia and breast cancer

Several cell lines are used to test the activity of the protein-drug molecule. Because PS is present in every cancer cell, the drug should be able to have an activity at least equivalent to the free chlorambucil. Cell lines of leukemia, non-adherent cancer cells, and breast adherent cancer cells are used to study the selectivity, targeting and cytotoxicity of the drug. The free drug and the conjugate activity and selectivity are compared statistically.

3rd Objective: In vivo studies on leukemia and breast cancer

Mice with breast cancer or leukemia shows us the consequence of an injection of our drug and help us study the evolution of the disease. The new drug will be compared to the free chlorambucil and a saline solution (no treatment). Leukemia is a white blood cell cancer, so the annexin A5 should be able to kill cancer cells in the blood after a daily injection and increase the survival time. Breast cancer cells will be injected into mice, and the new drug will be compared to free chlorambucil and saline solution. The results between those three groups will be compared statistically.

Materials and Methods

Materials

pET-30 Ek/LIC vector was from EMD Chemicals (Billerica, MA). Bovine serum albumin (BSA), Alamar Blue reagent, Triton X-100, EDTA, dimethyl sulfoxide (DMSO), selenomethionine, isopropyl 2-D thiogalactopyranoside (IPTG), and Tris-acetate-EDTA buffer, N- *p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethylsulfonyl fluoride were from Sigma-Aldrich (St Louis, MO). HRV-3C protease was from Thermo Fisher Scientific (Waltham, MA). Sodium phosphate and sodium dodecyl sulfate (SDS) were from Mallinckrodt Chemicals (Phillipsburg, NJ). The 2 and 100 kDa dialysis membranes were from Spectrum Laboratories (Rancho Dominguez, CA). Murine breast cancer cells 4T1 (ATCC® CRL2539™) and EMT6 (ATCC® CRL2755™), leukemia cells L1210 (ATCC® CCL219™), lymphoma cells P388D1 [P388 D1] (ATCC® CCL-46™), RPMI-1640 medium, Waymouth's MB 752/1 Medium, L-glutamine 200 mM, Dulbecco's Modified Eagle's Medium were from ATCC (Manassas, VA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Antibiotics, penicillin and streptomycin, were from Invitrogen (Grand Island, NY). His-trap columns were from GE healthcare Chicago, IL). Chlorambucil was from TCI America (Portland, OR). HPLC grade ethanol was from Acros Organics (Waltham, MA). FITC, Alexa-488, Deep Red Plasma Membrane stain, DAPI, propidium iodide, flow cytometry staining buffer, fixation/permeabilization buffer, permeabilization buffer, Slide-A-Lyzer dialysis cassettes (3.5 kDa) were from Thermo Fisher Scientific (Waltham, MA). Tryptone, yeast extract, and kanamycin monosulfate were obtained from Alfa Aesar (Haverville, MA). Sodium hydroxide, potassium chloride, and sodium chloride were from VWR inc

(Radnor, PA). HRV-C3 protease was from Sino biologics (Wayne, PA). Bradford reagent were from BioRad (Hercules, CA).

Methods

Recombinant Bacteria

The plasmid contains encoding annexin A5, pET-30 EK/LIC/ANXA5, was previously constructed in this lab by PhD student Naveen Palwai[99]. *E. coli* BL21(DE3) had been transformed to contain this plasmid.

Protein Production

In the *E. coli* BL21(DE3) cells used to produce annexin A5, the T7 RNA polymerase is controlled by a checkpoint, Lac regulatory construct, only active in the presence of IPTG (isopropyl β-D-1-thiogalactopyranoside). The *E. coli*-BL21(DE3) transformed cells are cultured in a 10 mL lysogeny broth (LB) media containing 35 µg/mL of kanamycin for 12 hours before being added to 1 L of LB media. Bacteria has to be shaken and kept at a temperature around 37°C. The culture is made first in a small batch to increase the speed, and a first growth of bacteria will start the growth phase and use the LB media as a nutrient. The liter is dispatched in 4-1 L flasks to increase the contact with the air, and the culture is incubated again at 37°C with shaking. The media will become foggy, and after a few hours, the concentration of bacteria is determined by spectroscopic turbidimetry. The optical density should be measured and when it reaches a minimum of 0.5, the solution of bacteria is ready for the next step, the induction. annexin A5 will be expressed only if the bacteria containing the plasmid is in a medium containing isopropyl β-D-thiogalactopyranoside (IPTG) to induce the expression of T7RNAP which in turn will induce the expression of annexin A5 in the cytoplasm of the cell. Only a concentration of 0.4 mM is enough to induce protein expression. The solution incubates

at 30°C with shaking for 5 h to increase the time of contact of bacteria and IPTG and increase the protein production. Then the cells are harvested by centrifugation at 1000 x g for 10 minutes into 4 Falcon tubes at 4°C to slow down the bacteria metabolism and avoid the lysis of the protein by proteinases. Bacteria are resuspended into a sonication buffer containing N- *p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (a protease inhibitor), phenylmethylsulfonyl fluoride (PMSF) (a serine protease inhibitor), and 2-mercaptoethanol to prevent protein oxidation. Ethanol is added to dilute insoluble components like TPCK and PMSF, and sodium phosphate dibasic is present to keep annexin A5 in its active form. Bacteria resuspended into the sonication buffer are sonicated to destroy the bacteria walls and release intracellular proteins. At this step the solution contains different proteins and bacteria walls. A centrifugation at 12 000 x g for 30 min will separate all the soluble proteins from the insolubles. The solution after the centrifugation contains only different intracellular components of bacteria including annexin A5. Imidazole and NaCl are added to the solution to reduce non-specific protein binding.

The purification is done by a chromatography; the column contains nickel beads (Ni-NTA resin) where the histidine can bind. Three solutions are necessary for three steps in a purification by metal affinity chromatography:

The first buffer contains sodium phosphate dibasic and sodium chloride, two components necessary to mimic the physiological condition and keep protein soluble. The phosphate buffer inhibits the kinase, keeping the pH around 7.4 and avoiding interferences with the protein. NaCl increases the ionic strength of the phosphate buffer, removing components bound to the column. Non-specific ionic and hydrophobic interaction binding between proteins is reduced to increase the selectivity of the column

for His-tag proteins. A small concentration of imidazole is necessary to clean the Ni-NTA resin in the column from contaminants. It will compete with the other proteins to bind to the nickel in immobilized metal affinity chromatography.

When the soluble protein fraction is eluted in the column, the His-Tag will bind to the Ni^{2+}-NTA while the flow of the second buffer will elute the rest of the solution containing proteins.

The second buffer also contains sodium phosphate dibasic, sodium chloride, imidazole, and Triton X-114. Triton reduces on a higher level non-specific hydrophobic interaction without interfering with the binding of the tagged protein. However, the Triton in high concentration interferes with the Bradford assay by a mechanism not elucidated yet. This technique will be used to determine the final concentration of the protein, so we have to remove this molecule from the column. Before the elution of the targeted protein, the column is washed with the first wash buffer to remove the presence of Triton X-114.

The elution buffer contains also phosphate dibasic, sodium chloride but imidazole in a higher quantity to compete with His-Tag-annexin A5 bonded to the Ni^{2+}-NTA resin. However, a protein contained in *E. coli* can contaminate our solution; SlyD is a common contaminant, a 25 kDa peptidyl-prolyl isomerase[100,101].

The column will be regenerated by using 25 mL of each following solution: 1 M potassium chloride, 1 M sodium hydroxide, DI water and 1 M ethanol. Potassium chloride blocks non-specific interactions between protein and matrix, sodium hydroxide removes the lipids trapped in the column, and water is to adjust the pH and prepare the column for the ethanol/water solution, ideally to store the column.

The protein eluted is in a solution highly concentrated in imidazole and salt. A 3 hour dialysis in a sodium phosphate buffer at physiological pH is done to replace the salty solution with a buffer suitable for the cleavage of C-terminal His-tag. A Bradford protein assay determines the concentration of annexin A5. Bradford reagent (250 μL) with 5 μL of the protein will be used. The absorbance at 595 nm is read, and a standard curve gives the protein concentration.

To cleave the C-terminal His-tag, HRV3C protease is used to cleave at the artificial HRV cleavage site between the His-tag and the protein. A quantity of 10 U of enzyme per mg of annexin A5 has to be added to the solution containing the right buffer, necessary for the enzyme to be active. The first wash buffer has to be added to the column to prepare the Ni^{2+}-NTA. After 16 hours, this solution containing enzymes and annexin A5 will be fed through the column. Free protein will be eluted directly while the His-tag will remain in the column. The uncleaved protein and free His-tag are eluted with the elution buffer containing a high concentration of imidazole.

The free protein has to be dialyzed again with a buffer containing sodium phosphate dibasic and sodium chloride to mimic the physiological environment and have a protein ready to use.

An SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) will verify the quality of the production. Theoretically the annexin A5 has a weight of 36 kDa.

Conjugation Between annexin A5 and chlorambucil

For this step we decide to synthetize 10 mg of the conjugate annexin A5-chlorambucil. The chlorambucil is a drug that is not soluble in water. To be able to use the drug into water and bring the molecule in contact to the protein, we have to change

the condition of the environment. By decreasing the pH, the chlorambucil is becoming soluble. A drop of HCl 12 M is added for each 0.1 milligram of chlorambucil. In a beaker, 10 drops of HCl 12 M and 1 mg of chlorambucil are added while stirring. Then 1 mL of sterile phosphate buffer at pH 7.4 is added drop by drop and stirred. The solution should stay acidic to prevent the chlorambucil from crystallizing but not too acidic to be able to add NHS to the molecule. For 0.1 mg of chlorambucil, 7 mg of sulfo NHS and 10 mg of EDC are added. For 10 mg of annexin A5 and 1 mg of the alkylating agent, we have to add 70 mg of sulfo NHS and 100 mg of EDC. The solution is stirred for 5 min and 2 µL of 2-mercaptoethanol are added.

The solution of annexin A5 has to have a concentration around 1 mg/ml. If the concentration is more than 2 mg/mL, the protein can precipitate during the conjugation. A pH meter and a solution of sodium hydroxide must continuously maintain the pH around 7.4 ± 0.3. The chlorambucil-NHS is added drop by drop to the solution and stirred gently for 12 hours.

The solution should be centrifuged at 12,000 g for 10 min to remove insoluble chlorambucil non-conjugate from the soluble conjugate. The conjugate solution is dialyzed into 2 L of sterile phosphate buffer for 3 hours at 4°C to have a pure drug into a pH 7.4 buffer, ready to use. Then the solution can be filtrated to 0.2 µm filter under vacuum to remove any contaminants, and the concentration of annexin A5-chlorambucil (ANXA5-CHL) can be measured by Bradford Protein Assay at 595 nm, and the quantity of chlorambucil can be measured by fluorescence after photoactivation at 358 nm. The solution has to be stocked into -80°C liquid nitrogen, by the flash freezing technique, and be kept 1 month or used in the next 12 hours.

Quantity of chlorambucil per annexin A5

A standard curve is established by adding different concentrations of chlorambucil into phosphate buffer with 50% dimethyl sulfoxide (DMSO) to increase the solubility of the chlorambucil. The different concentrations are 0.2 µg/µL, 20 and 200 µg/µL into a 1 mL centrifuge tube. Those tubes are lit by UV light for 30 minutes and, left 20 min to cool down, and the fluorescence is read at 434 nm. The emission fluorescence is plotted against the concentration of chlorambucil.

To determine the concentration of chlorambucil conjugated to our protein, 500 µL of our conjugate is added to a microcentrifuge tube with 50 % DMSO. The DMSO is an organic solvent and can precipitate, denature, crystallize proteins, but the quantity of chlorambucil stays the same after the DMSO is added to the microcentrifuge tube. After 30 min under UV light and 20 min to cool, the fluorescence is read with the multiplate reader at 434 nm. The results are directly compared to the standard curve to determine the concentration of chlorambucil in our conjugate solution and determine how many molecules of chlorambucil are on each protein of annexin A5.

In vitro experiments: Breast cancer cell line and culture conditions

Two breast cancer cell lines were chosen to study the cytotoxicity of the chlorambucil. The first one, EMT6 (ATCC® CRL2755™), is a common cell line to study breast cancer taken from mice and is metastatic within days after implantation. They are epithelial cells from breast tissue with a mammary carcinoma and growing in adherent conditions. EMT6 metastasizes after being implanted orthotopically. The second one, 4T1 (ATCC® CRL2539™), is a mammary carcinoma breast cancer cell line that is an animal model of an animal stage IV human breast cancer. They are also adherent cells. 4T1 mammary carcinoma are highly tumorigenic and invasive, and they can

spontaneously metastasize in the early stage of the primary tumor in the mammary gland to multiple distant sites: lymph nodes, blood, liver, lung, brain and bones[102–104].

The EMT6 (ATCC® CRL2755™) cells were grown in 85% Waymouth's MB 752/1 medium with 2 mM L-glutamine and 15 % fetal bovine serum. The cells were grown at 37°C with 5% CO_2. The cryopreservation medium is the complete culture medium with 5% DMSO.

The murine breast cancer cells 4T1 (ATCC® CRL2539™) were grown in RPMI-1640 medium enriched with 10 % FBS and penicillin/streptomycin antibiotics (100 $U.ml^{-1}$ and 100 $\mu g.ml^{-1}$, respectively). The cells were grown at 37°C with 5% CO_2. The cryopreservation medium is the complete culture medium with 5% DMSO.

In vitro experiments: Leukemia cell line and culture conditions

L1210 (ATCC® CCL219™) is a lymphocytic leukemia cell line grown in suspension. They are they are skin cells from DBA subline 212.

Leukemia cell line L1210 cells (ATCC® CCL219™) were grown in Dulbecco's Modified Eagle's Medium enriched with 10% horse serum. The cells were grown at 37°C with 5% CO_2. The cryopreservation medium is the complete culture medium with 5% DMSO.

Lymphoma cell line P388D1 cells [P388 D1] (ATCC® CCL46™) are monocytes, macrophages growing in suspension.

The P388D1 cells [P388 D1] (ATCC® CCL46™) were grown in Dulbecco's Modified Eagle's Medium enriched with 10% horse serum. The cells were grown at 37°C with 5% CO_2. The cryopreservation medium is the complete culture medium with 5% DMSO.

The L1210 and P388 cell lines are murine models highly used to evaluate anticancer activity and develop new drugs. Some advantages are that they grow rapidly, homogeneously and are easily reproducible[105].

In vitro experiments: Cytotoxicity studies on cancer cell lines

The Alamar Blue assay was used to determine the cytotoxicity of the chlorambucil compared to Annexin A5-Chlorambucil on each cell line over 20 hours and 4 more hours for the Alamar Blue assay in 96 well plates.

In vitro fluorescence visualization

4T1-Td cells (ATCC® CRL2539™) cells were grown until 70% confluence on cover slips. Annexin A5 (1.5 mg/mL) was tagged with FITC following protocol of J. Nueves et Al.[106] and incubated with the cells for 2 hours, followed with PBS washing of any unbound protein. Cells were fixed in 4% paraformaldehyde, and images were taken on a Nikon Fluorescence microscope. The same protocol was used for the L1210 (ATCC® CCL219™).

In vivo experiments: 4T1 breast cancer cell line

All procedures complied with a protocol approved by Institutional Animal Care and Use Committee (IACUC) of the University of Oklahoma. BALB/c female mice 6 weeks of age, weighing 18 – 20 g, were used. Mice were on a standard chow diet. Mice were injected with 5×10^4 4T1 (ATCC® CRL2539™) cells in mammary fat pad number four. Cells were suspended in 50 µL PBS. Mouse body weight was monitored every 3-4 days. Mice bearing tumors were randomized into groups (5 per group) prior to initiation of treatment when tumors reached 100 mm^3. ANXA5-CHL fusion protein (200 µL at 0.5 mg CHL/kg body weight) was administered over 21 days daily and started 5 days after the injection of 4T1 (ATCC® CRL2539™) cells. Mice were euthanized once ascite

development occurred or animals seemed distressed, and tumor, blood, and organs were collected.

Tumor volume was calculated with the modified ellipsoid formula volume = (1/2) x (length x width^2) using caliper measurements of the longest dimension and perpendicular width.

In vivo experiments: Leukemia cell line

All procedures complied with a protocol approved by Institutional Animal Care and Use Committee (IACUC) of the University of Oklahoma. DBA female mice 6 weeks of age, weighing 18 – 20 g were used. Mice were on a standard chow diet. Mice were injected with 5 x 10^5 L1210 cells (ATCC® CCL219™) by intraperitoneal injections. Cells were suspended in 50 µL PBS. Mouse body weight was monitored every 3-4 days. Mice were randomized into groups (5 per group) prior to initiation of the treatment 4 days after the inoculation. ANXA5-CHL fusion protein (200 µL at 0.5 mg CHL/kg body weight) was administered over 21 days daily and started 48 hours after the injection of L1210 (ATCC® CCL219™) cells. Mice were euthanized once animals seemed distressed, weak or swelling, and tumor, blood, and organs were collected. Information about the studies using mice is summarized in Table 1.

In vivo mice number

Table 1: Summary of mice used during in vivo studies

Type of cancer	*Breast* 4T1 (ATCC® CRL-2539™)	*Leukemia* L1210 (ATCC® CCL219™)
Model	BALB/c	DBA
Number of mice per group	5	5
Age (weeks)	6	6

Statistical analysis

Data was analyzed with Excel 2019, Graphpad Prism 8^{TM} software, and FIJI. Statistical significance of cytotoxicity results was assessed using a one-way ANOVA and Tukey-Kramer multiple comparisons test. Statistical significance of survival curves was determined by the Gehan-Breslow-Wilcoxon test and Mantel-Haenszel log-rank test. Multiple comparisons were done by using the Bonferroni threshold with a number of samples n = 5. Errors are represented graphically as standard error, or SE.

Results

Results of the SDS-PAGE

Chlorambucil, with a molecular weight of 304,212 Da, has been conjugated to the ANXA5 protein, and the SDS-PAGE gel is shown in Figure 8. The EDC/NHS protocol conjugates the carboxylic function of the chlorambucil to the amine functions on the annexin A5. By estimating the average weight of the ANXA5-CHL at 39 kDa, approximately 10 molecules of chlorambucil are fixed by amide bonds on the protein. However, another technique is used to determine the concentration of chlorambucil present after conjugation.

Results of the determination of the concentration of chlorambucil

To find the concentration of chlorambucil in our conjugate solution, we used a fluorescent microscopy after photoactivation. Following the assay procedure described above, a standard curve is first made with three different concentrations of chlorambucil, respectively 0.2, 2 and 20 mg/mL of chlorambucil. After excitation at 358 nm, the fluorescence is read at 434 nm and the standard curve is made, and we found a linear relationship between the fluorescence (y) and the concentration (x) shown in Figure 9.

$$y = 172.2\ln(x) + 725.31.$$

Then, we read the fluorescence of our conjugate after photoactivation and used the standard curve to determine the concentration of the alkylating agent in our conjugate sample solution. Our annexin A5 (36 kDa) has a concentration of 0.1 mg/mL, or 2.7 µM, and we read a concentration of chlorambucil at 9 mg/mL, or 27 µM. For one molecule of annexin A5, we have 10 molecules of chlorambucil.

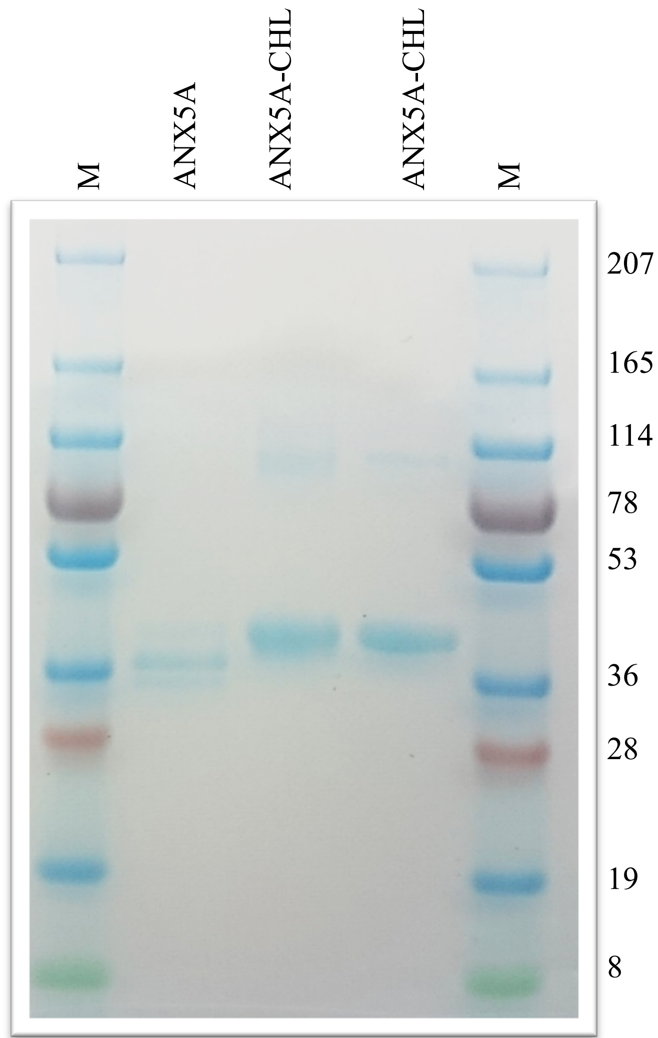

Figure 8: SDS-PAGE gel electrophoresis of annexin A5 (left) and two different batches of the conjugate (middle & right) with two protein marker ladders (M) with the indicated molecular masses in kDa. The ANXA5 has a molecular weight around 36 kDa confirmed by the bibliography. The conjugate has a molecular weight around 38 to 40 kDa, indicating that the conjugate is heavier than the ANX5A.

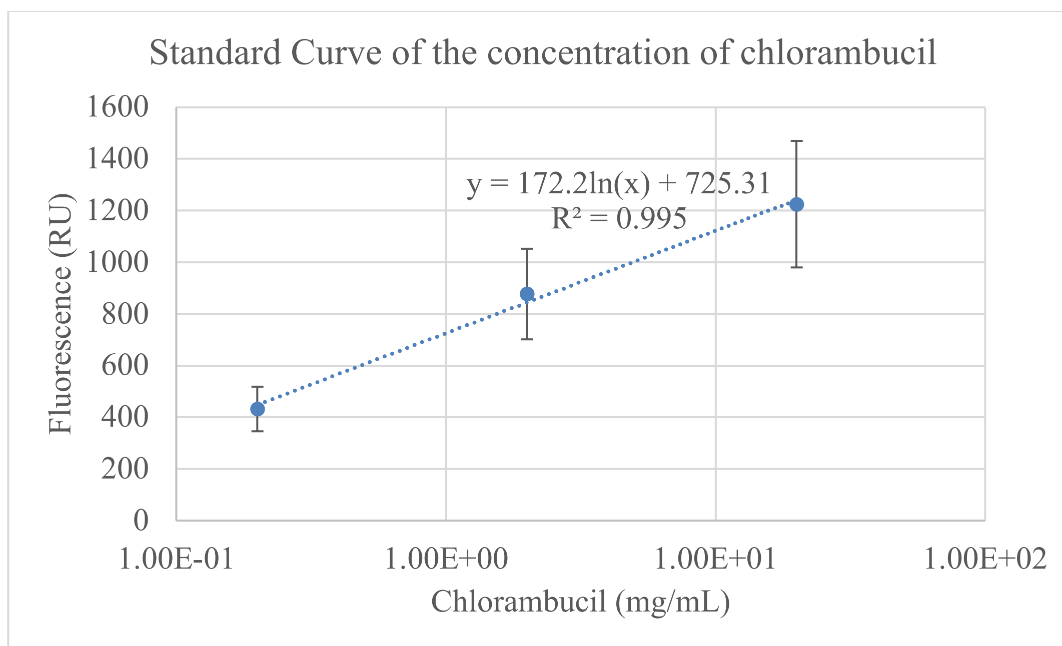

Figure 9: Standard curve of the determination of the quantity of chlorambucil in a solution by spectroactivation.
The fluorescence is proportional to chlorambucil concentration (equation is $y = 172.2\ln(x) + 725.31$). We can use this equation to determine the concentration of chlorambucil in our annexin A5-chlorambucil solution. Because the free chlorambucil is not present in the solution due to the previous dialysis, the concentration of chlorambucil in this solution is only the chlorambucil bound to the protein. Samples depicted as mean ± SE (n = 3).

In vitro results of cytotoxicity assays

Cytotoxicity studies indicate a significant cytotoxic effect of the ANXA5-CHL molecule on breast cancer cell lines EMT6 and 4T1, leukemia cells L1210, and lymphoma cells P388. The results of the cytotoxicity experiments, with a duration of 16 hours, is shown for the four cell lines in Figures 10, 11, 12, 13 and 14. The conjugate treatment was particularly effective for each cell line. For the leukemia cell line L1210 and lymphoma P388, cytotoxicity studies show that a free chlorambucil concentration of 50 µM is not enough to kill 30% of the cells[107].

Results of in vitro cytotoxicity assay on EMT6 breast cancer cell line

The cytotoxicity of the conjugate for the EMT6 cells is 100-fold better than the free drug as we can see in Figure 10. The conjugate starts to kill cancer cells above 0.1 µM with a very good toxicity, killing 75% of the cells between 0.1 and 0.5 µM. The free chlorambucil is less effective, starting to be active at 10 µM and requiring a concentration of 200 µM to kill 80% of the cells. The LD50's for EMT6 with the conjugate and the free chlorambucil are, 0.3 µM and 100 µM, respectively. The conjugate is more effective in killing EMT6 cancer cells than the free chlorambucil.

Results of in vitro cytotoxicity assay on 4T1 mammary cancer cell line

The cytotoxicity of the conjugate on the 4T1 cells (Figure 11) is 10-fold better than the free drug. The conjugate starts to kill cancer cells above 0.2 µM and nearly completely kill them with 10 µM. The LD50 is 3 µM for the conjugate and 180 µM for the chlorambucil. The free chlorambucil starts to kill cells above 1 µM; however, the cytotoxicity is less efficient and can barely kill 60% of the cells with 300 µM. These cells represent a stage IV triple negative breast cancer, in which cells are less sensitive to

chemotherapeutic drugs[108–111]. The conjugate cytotoxicity is less effective than for EMT6 cells but still stays more effective than the free chlorambucil.

Results of in vitro cytotoxicity assay on L1210 leukemia cell line

The cytotoxic efficacy of the new conjugate, ANXA5-CHL, was evaluated for L1210 leukemia cells as seen in Figure 12. The free chlorambucil takes around 60 minutes to bind to the DNA of the L1210 cells [112]. Significant cell death is seen after one day of ANXA5-CHL when only 1 μM is necessary to start to kill cancer cells, while the free chlorambucil needs a concentration 10 times higher. Only 4 μM of the conjugate kills 90% of the cancer cells, and 200 μM of free chlorambucil is necessary for the same result; their LD50's are, respectively, 1.3 μM and 10 μM. The conjugate is 10-fold more effective.

Results of in vitro cytotoxicity assay on L1210 resistant leukemia cell line

Resistant cells against chlorambucil should need, in theory, more chlorambucil to be killed. The LD50 for the conjugate is 7.5 μM and for the chlorambucil 145 μM (Figure 13). The viability curves for the resistant cells were shifted approximately 10 times higher in concentration compared to non-resistant cells (Figure 12).

Results of in vitro cytotoxicity assay on P388 lymphoma cancer cell line

The effect on the conjugate on the viability of the lymphoma cell line P388 was compared to that of the free drug (Figure 14). The conjugate is effective at 1 μM, and its toxicity for cancer cells is higher than the free chlorambucil with more than 90% of the cells dead with 3 μM. The free chlorambucil is less toxic; it starts to kill cells above 8 μM but needs a concentration 14 times higher to be able to kill 75% of the cells. These results are similar to the leukemia cell line L1210 (Figure 12), and this was predictable since these two cell lines evolve in the same way in the body. In effect, those two cell

lines are lymphoblasts and involves lymphocytes. The LD50 for the P388 cell line is 1.5 μM for the conjugate and 80 μM for the chlorambucil. The *in vitro* cytotoxic effects of the free chlorambucil on P388 cells agree with those found in the literature[65].

Results of in vitro cytotoxicity assays

The conjugate shows clearly better results than the free chlorambucil on cancer cell lines. The cancer cells can be from a solid tumor, as a breast cancer tumor, or from a non-adherent cancer type, leukemia. This effect could potentially result from active transport across the cell membrane due to the annexin A5. The increased cytotoxicity could be due to a better penetration of the drug induced by the active endocytosis of the ANXA5-CHL into the cells. Chlorambucil alone is clinically limited to leukemia patients too weak to support a strong chemotherapy. The increased cytotoxicity and the targeted system using the conjugate potentially could give a better treatment for patients. The use of the conjugate at the same dose as the free drug would result in fewer side effect.

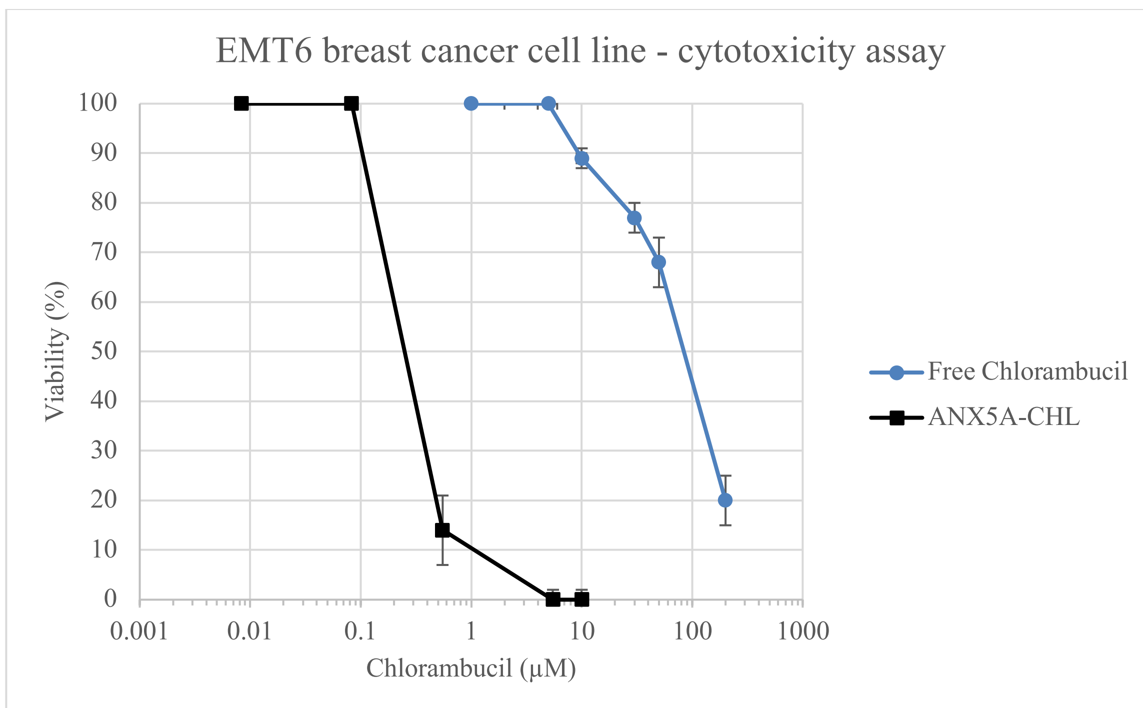

Figure 10: Cytotoxicity assay on a 96 plate-wells of EMT6 breast cancer cell line and the conjugate annexin A5-chlorambucil (ANXA5-CHL) and free chlorambucil.

The effect of free chlorambucil is compared to the effect of the conjugate. The two groups were treated at t = 0, and the results were read at t = 24 hours of the study. Each well was at a confluency of 70% at t = 0 with 180 µL of media and 10 µL of chlorambucil or conjugate added at different concentrations. Viability was determined by the Alamar Blue assay at t = 24 hours by fluorescence measurement at 590 nm after an excitation wavelength of 550 nm. The viability curves are significantly different ($p \leq 0.005$). Data depicted as mean ± SE (n = 8).

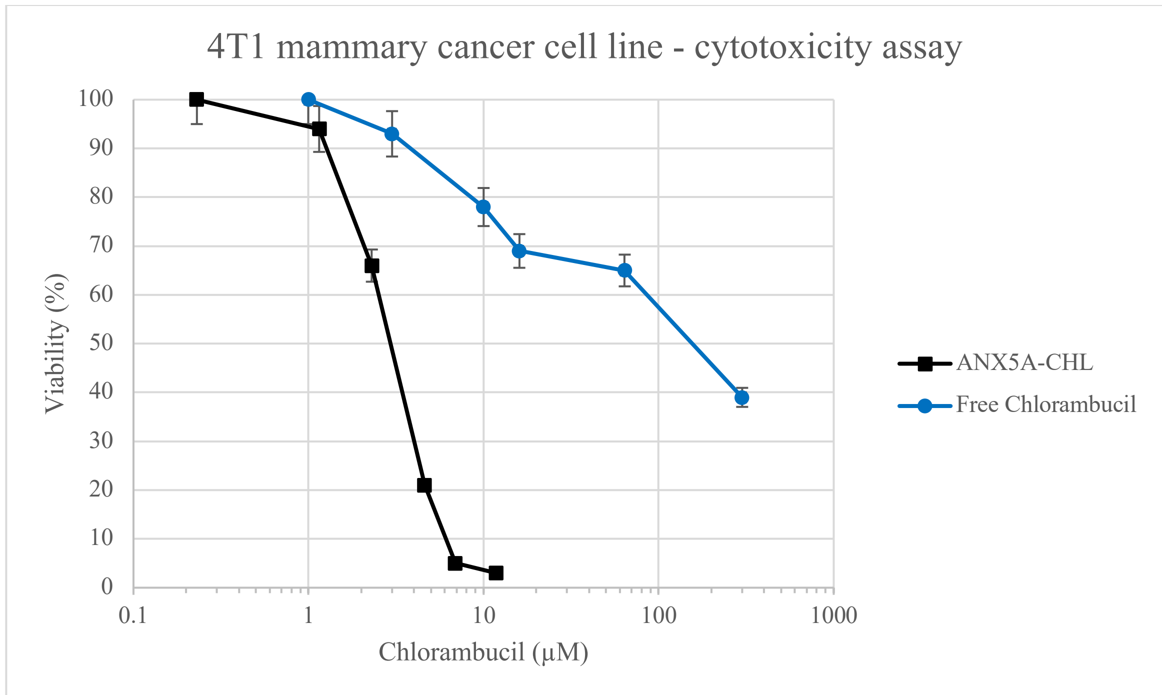

Figure 11: Cytotoxicity assay on a 96 plate-wells of 4T1 breast cancer cell line and the conjugate annexin A5-chlorambucil (ANXA5-CHL) and free chlorambucil. The effect of free chlorambucil is compared to the effect of the conjugate. The two groups were treated at t = 0, and the results were read at t = 24 hours of the study. Each well was at a confluency of 70% at t = 0 with 180 µL of media, and 10 µL of chlorambucil or conjugate added at different concentrations. Viability was determined by the Alamar Blue assay at t = 24 hours by fluorescence measurement at 590 nm after an excitation wavelength of 550 nm. The viability curves are significantly different ($p \leq 0.005$). Data is presented as mean ± SE with n=12.

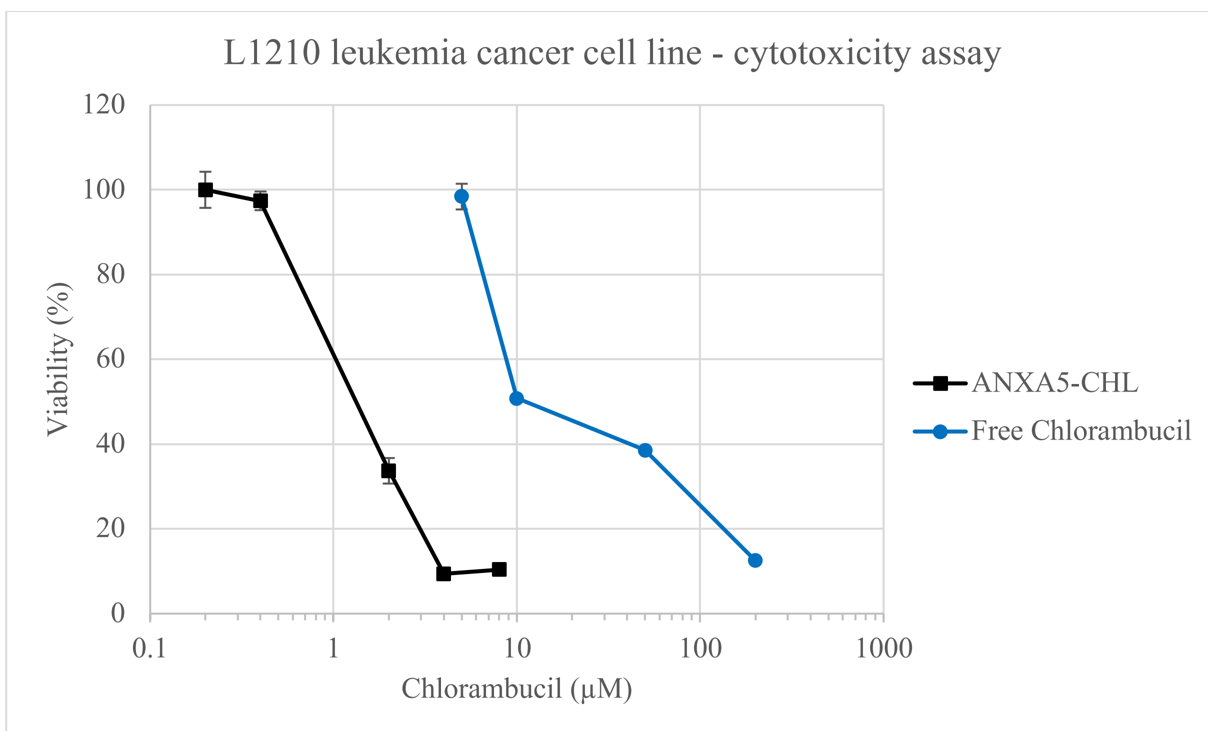

Figure 12: Cytotoxicity assay on a 96 plate-wells of L1210 leukemia cell line and the conjugate annexin A5-chlorambucil (ANXA5-CHL) and free chlorambucil.

The effect of free chlorambucil is compared to the effect of the conjugate. The two groups were treated at t = 0, and the results were read at t = 24 hours of the study. Each well had at least 25,000 non-adherent cells at t = 0 with 180 µL of media and 10 µL of chlorambucil or conjugate added at different concentrations. Viability was determined by the Alamar Blue assay at t = 24 hours by fluorescence measurement at 590 nm after an excitation wavelength of 550 nm. The viability curves are significantly different ($p \leq 0.005$). Data depicted as mean ± SE (n = 8).

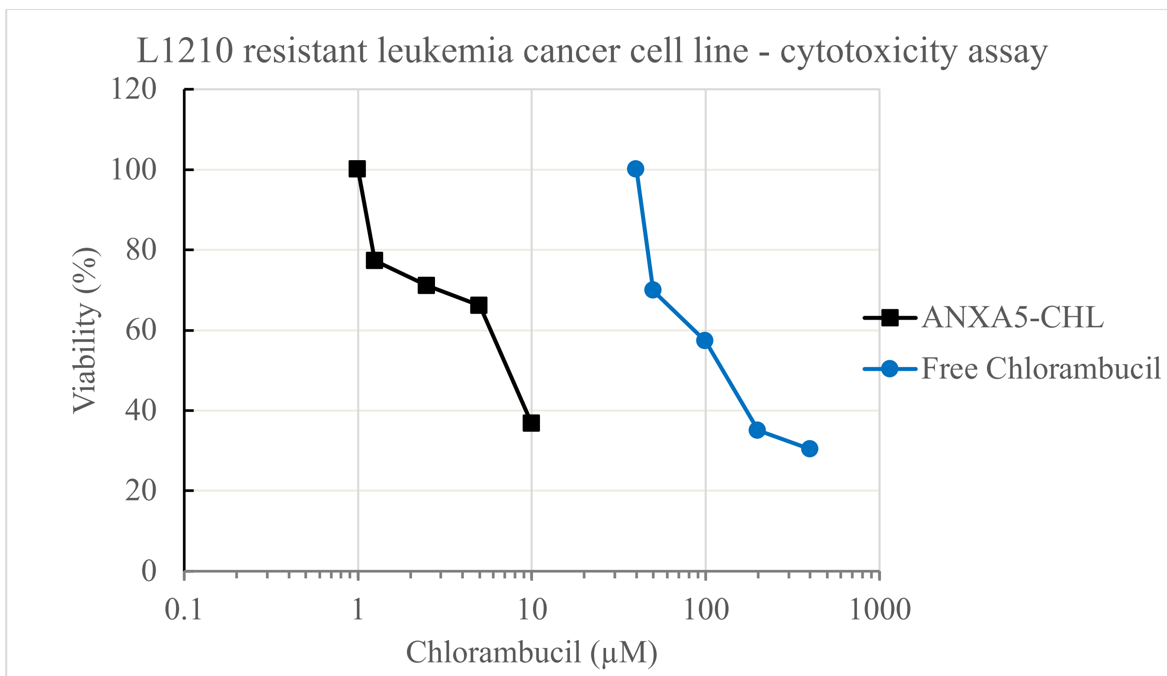

Figure 13: Cytotoxicity assay on a 96 plate-wells of L1210 resistant leukemia cell line and the conjugate annexin A5-chlorambucil (ANXA5-CHL) and free chlorambucil.

The effect of free chlorambucil is compared to the effect of the conjugate. The two groups were treated at t = 0, and the results were read at t = 24 hours of the study. Each well had at least 25,000 non-adherent cells at t = 0 with 180 µL of media already containing free chlorambucil at 10 µM and 10 µL of chlorambucil or conjugate added at different concentrations. Viability was determined by the Alamar Blue assay at t = 24 hours by fluorescence measurement at 590 nm after an excitation wavelength of 550 nm. The viability curves are significantly different ($p \leq 0.005$). Data depicted as mean ± SE ($n = 8$).

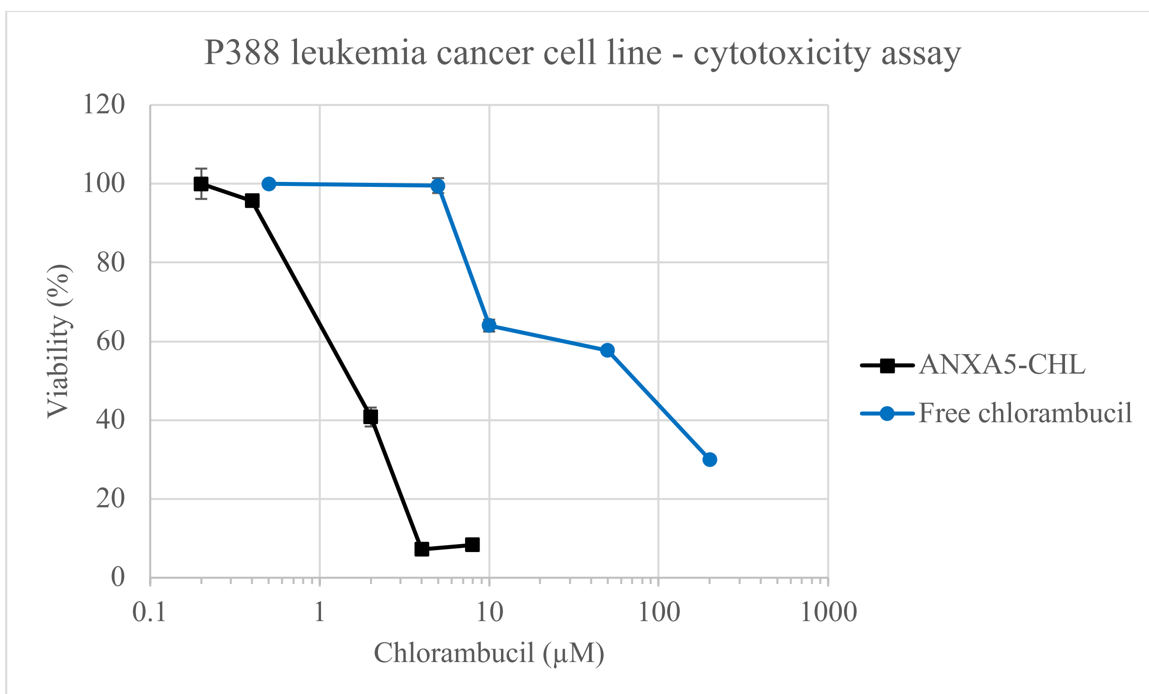

Figure 14: Cytotoxicity assay on a 96 plate-wells of P388 Lymphoma cell line and the conjugate annexin A5-chlorambucil (ANXA5-CHL) and free chlorambucil. The effect of free chlorambucil is compared to the effect of the conjugate. The two groups were treated at t = 0 and the results were read at t = 24 hours of the study. Each well had at least 25,000 non-adherent cells at t = 0 with 180 µL of media already containing free chlorambucil at 10 µM and 10 µL of chlorambucil or conjugate added at different concentrations. Viability was determined by the Alamar Blue assay at t = 24 hours by fluorescence measurement at 590 nm after an excitation wavelength of 550 nm. The viability curves are significantly different ($p \leq 0.005$). Data depicted as mean ± SE (n = 8).

Results of the in vitro visualization

The fluorescence microscope reveals that the conjugate can bind the cancer cells (Figure 15). The mammary cells are around 25 µm and appears red due to their expression of Td tomato (Figure 15 B). Binding of the conjugate, indicated by the green fluorescence, is only to the cells (Figure 15 C).

The L1210 leukemia cells were also red due to the Tomato Td modification (Figure 16 A). After treatment and washing, we can visualize more precisely the high presence of ANXA5 on the cell (Figure 16 A and B). It confirms that the conjugate can bind any cancer cells, adherent or non-adherent from, respectively, breast cancer and leukemia.

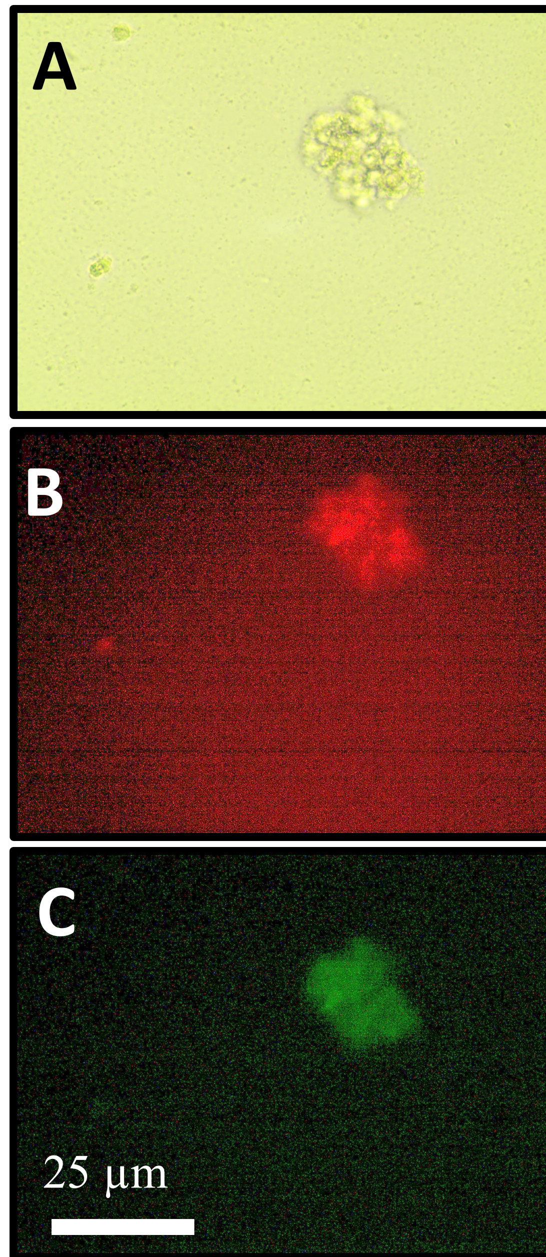

Figure 15: Pictures of 4T1 cells under fluorescence microscope showing 4T1 cells (A and B) and in vitro binding specificity ANXA5-CHL on the cell (C).

Observation under a fluorescence microscope (NIKON) of a 4T1 cell without any fluorescence (A). Fluorescent microscopy shows localization of tdTomato labeled 4T1 cancer cells (B) and FITC-labeled ANXA5-CHL (C).

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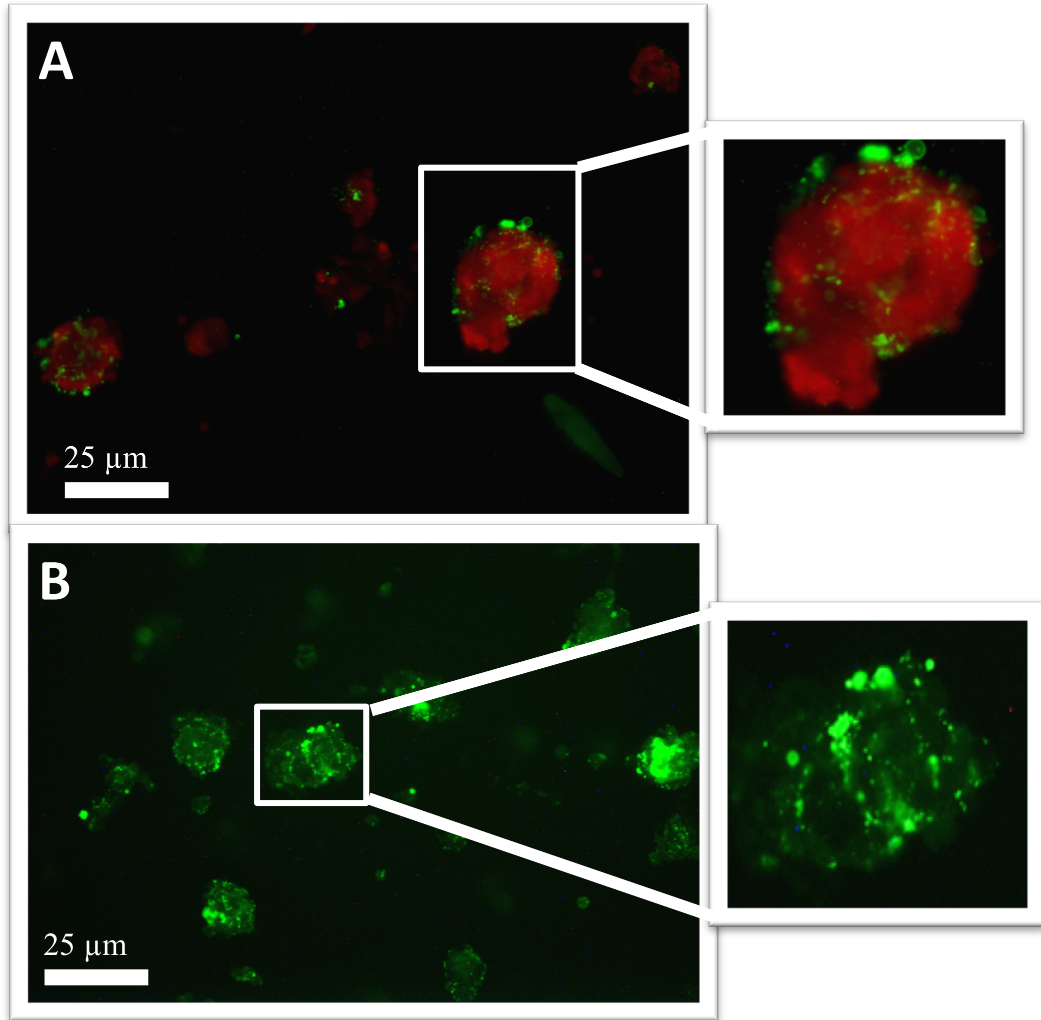

Figure 16: Picture of L1210 cell (red) and ANXA5-CHL (green) observed under a fluorescent microscope after photoactivation of the chlorambucil under UV (x100) (A) or only ANXA5-CHL (green) (B).

Observation under a fluorescence microscope (NIKON) of td Tomato labeled L1210 cells (red) (A). The annexin A5 is conjugated to a green fluorescent marker (FITC) and bind the cells (A and B).

Results of 4T1 in vivo experiments

The results for the effect of the ANXA5-CHL conjugate on the tumor growth and survival of mice with orthotopic 4T1 tumors are shown in Figure 17 and Figure 18, respectively. The ANXA5 reduced the volume increased by 77% compared to the two other groups with a slightly volume increase after day 9 for each group. At 12 days, the volume of the tumor for the control group was 180 mm^3, while the volume of the group of mice treated with the conjugate is only 60 mm^3, so a volume three times smaller.

The survival was monitored to evaluate the efficacy in vivo of the therapy. Free chlorambucil increased significantly the survival of mice with 4T1 tumors with a mean survival time of 29 days. The conjugate increased the survival and gave an average lifetime of 31 days and has a significantly increased the survival of the 4T1 mice as shown on Figure 18. The conjugate is more effective on the long-term, but we had to stop the measurement after day 50, indicating that the ANXA5-CHL increased significantly the survival time of the mice.

As we can observe on Figure 19 not negative effects or weight loss was displayed as a result of treatment with the conjugate. The Saline solution as expected decreased the average weight of the mice until they lost 10% of their body weight.

The conjugate significantly improved the survival of the mice compared to the control group and also inhibited the weight loss induce by the growing tumor because of the small increase of the tumor volume of the solid tumor.

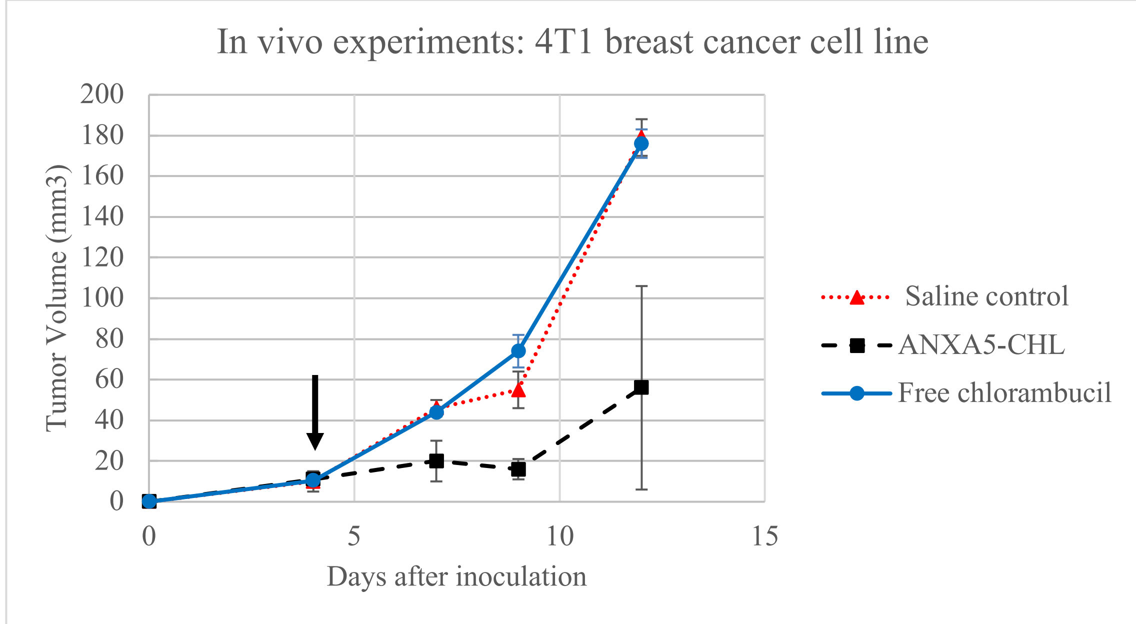

Figure 17: Antitumor effect of ANXA5-CHL and chlorambucil against 4T1 tumors on the mammary fat pad number four of BALB/c mice.
ANXA5-CHL and free chlorambucil were administered, by daily IP injection, at 0.5 mg/kg and 5 to 20 mg/kg respectively. Treatment occurred for 11 days and the start of treatment is indicated by the arrow. Data is presented as mean ± SE (n = 12).

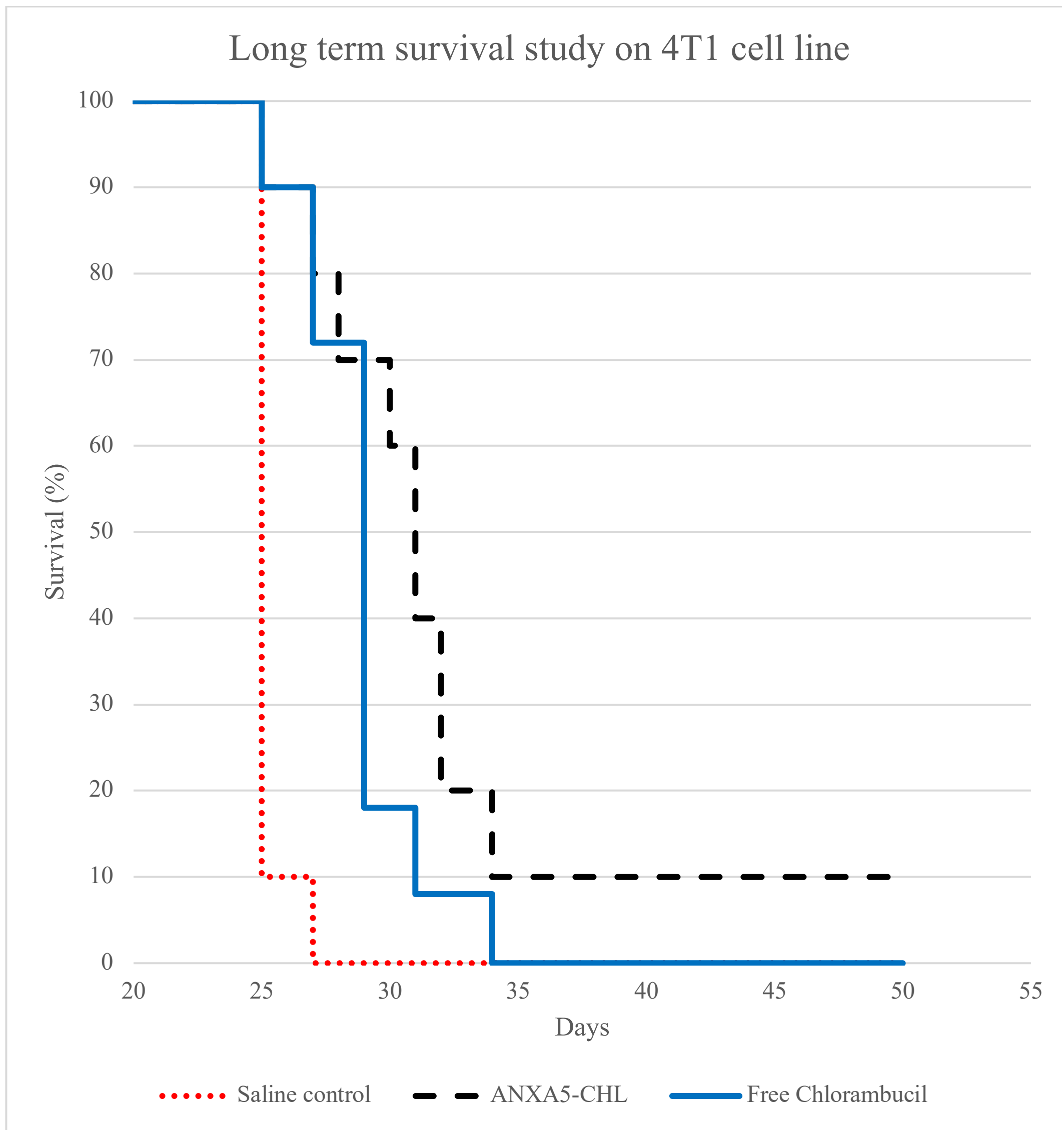

Figure 18: Long term survival study of BALB/c mice with 4T1 breast cancer cells treated with the conjugate, free chlorambucil or saline solution.
Treatment on BALB/c mice started 10 days after inoculation with 5 x 10^5 4T1 breast cancer cells. Mice were treated with ANXA5-CHL, free chlorambucil and saline solution daily for 15 days. The dose was 0.5 mg/kg of chlorambucil. Survival was monitored, and mice were euthanized if there was more than 10% abdominal swelling or mice seems distressed. Data presented as mean ± SE (n = 9).

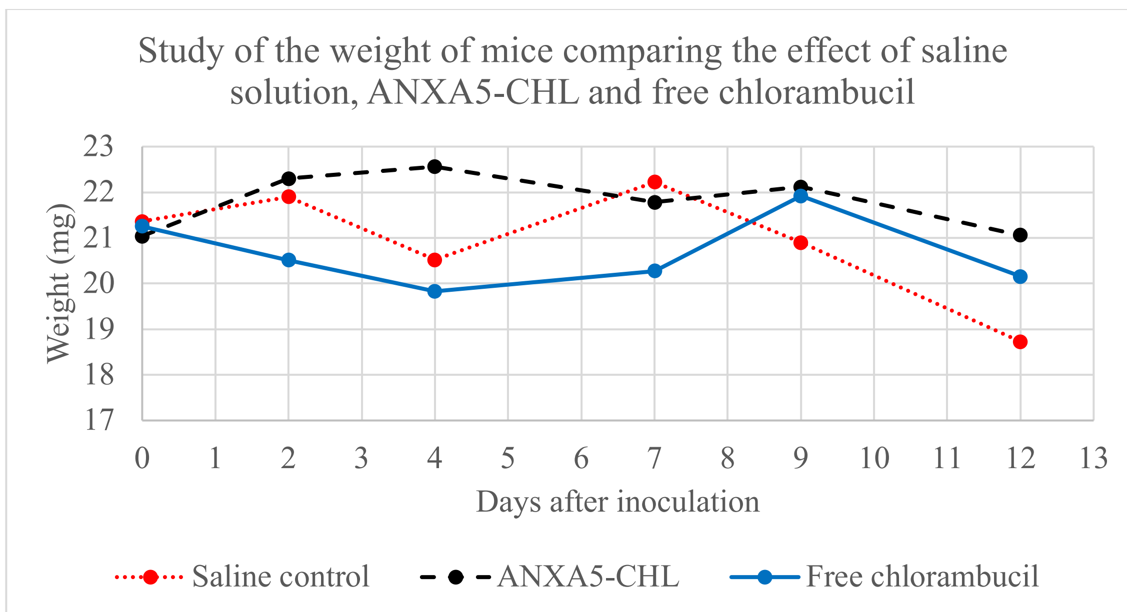

Figure 19: Minimal effect of ANXA5-CHL on 4T1 breast cancer mouse weight. Treatment started 5 days after inoculation with 5 x 10^5 4T1 mammary breast cancer cells. Tumor weight was measured every 2 days over a 12 days period. Data is presented as mean ± SE (n = 12).

Results of L1210 in vivo experiments

The survival of the mice is also increased with the conjugate, shown on Figure 20, and indicated a beneficial effect from the conjugate. The survival was monitored to evaluate the efficacy in vivo against leukemia of the proposed therapy. As seen in the Figure 20, mice treated with the ANXA5-CHL had a significant increase in survival, each result is measured by the Mantle-Cox test (p=0.0004) and the Wilcoxon test (p=0.0005). The median survival for the control group is 8.7 days and 10.4 days for the free chlorambucil, increasing the survival mice bearing L1210 leukemia cells. The conjugate has a median survival time of 10.9 days, increasing the survival of mice bearing leukemia. The ANXA5-CHL compared to free chlorambucil increased significantly the survival of the mice with L1210 leukemia cell line. Moreover, better results could be obtained by increasing the number of animals or increasing the dose of ANXA5-CHL. We can conclude that further work is needed to perfect the model.

Because chlorambucil is used to lower the number of anormal white blood cell, the expected side effect was to observe a decrease in white blood cell number. No side effects have been observed for the ANXA5-CHL treated mice.

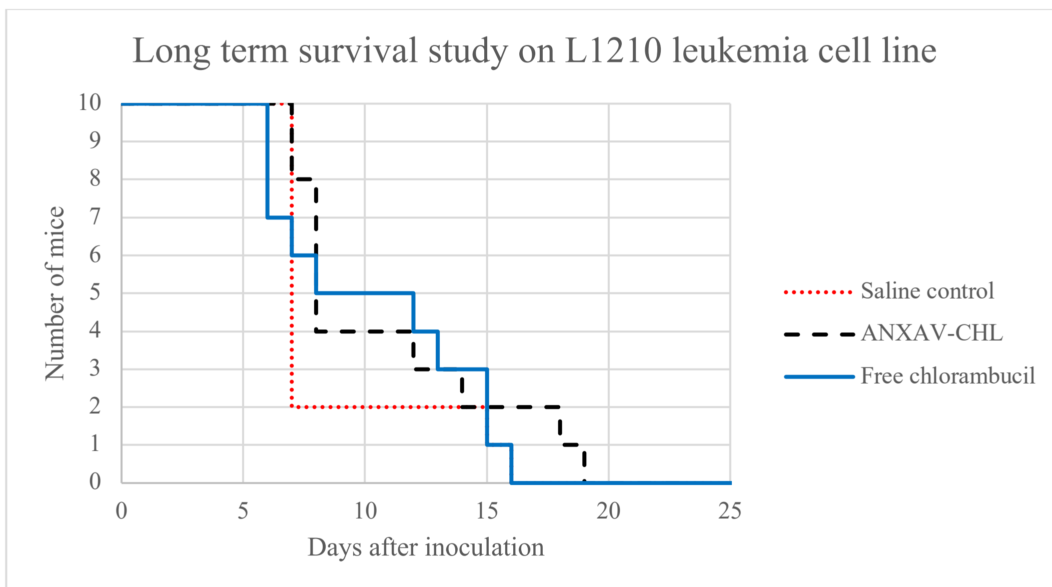

Figure 20: Long term survival study of DBA mice with L1210 leukemia treated with the conjugate, free chlorambucil, or saline solution.

Treatment on DBA mice started 5 days after inoculation with 5 x 10^5 L1210 leukemia cancer cells. Mice were treated with ANXA5-CHL, free chlorambucil, and saline solution daily for 20 days. The dose was 0.5 mg/kg of chlorambucil. Data is presented as mean ± SE (n = 12).

Discussion

Breast cancer today is one of the most important and deadly cancers, and 1 in 8 U.S women will develop invasive breast cancer over their lifetime. Different ways to treat it exist, and today the main solution are chemotherapies, radiotherapies or surgeries. Chemotherapies are effective with a survival rate around 93% but all of them are not targeted against cancer cells[11,12,15]. Leukemia is a deadly cancer with a year rate survival of 57%, but the success of the treatment depends on the age and the type of leukemia. For this type of cancer with non-adherent cells, treatments can involve chemotherapy, radiation therapy or bone narrow transplants. Targeted chemotherapies are a promising method for treating different cancers, and the target is present for both breast cancer and leukemia.

Our work proposes a new approach with a more cytotoxic treatment against cancer cells and less toxic for healthy cells. The toxicity of chemotherapies for the body, inducing side effects, comes from the death of healthy cells. Annexin A5 binds phosphatidylserine present on the surface of apoptotic cells and thereby impairs their uptake by macrophages. Annexin A5 can contribute indirectly, because of this impaired clearance, to the immune activating tumor microenvironment. Previous studies also show the anti-tumor activity of the annexin A5 in vitro and in vivo[113]. and the anti-tumor activity of a fusion protein, annexin A5-TRAIL, increasing the toxicity against cancer cells[114]. Chlorambucil is supposed to be a treatment for patients who might not be able to tolerate the side effect of strong chemotherapy[33,115]. Today with the best treatment, less than 1% of the molecule will reach the tumor, and the rest is spread in the body and can cause severe side effects. Moreover, phosphatidylserine is not only expressed on the

surface of apoptotic cells but also on the surface of macrovesicles and angiogenic endothelium, becoming a target for our conjugate.

Leukemia is the main disease treated by chlorambucil, so it is logical to try to increase the toxicity of the chlorambucil against leukemia cells. Moreover the majority of anticancer treatments in clinical use were first screened by murine leukemia[116]. Because they are cancer cells, their surfaces also present phosphatidylserine; however they float in the blood and don't form solid tumors[117]. The conjugate was able to bond those cells and kill them resulting in better cytotoxicity than with the free chlorambucil. However usually chlorambucil is used with a second component, for example a monoclonal antibody, to treat weak leukemia patients; further research could try to improve the cytotoxicity by using this second component with the conjugate. Several studies confirm the cytotoxicity of the free chlorambucil associated with Rituximab or Obinutuzumab, which are ideal to treat chronic lymphocytic leukemia by improving the survival of these fragile patients[118].

The protocol for the conjugation using EDC/NHS uses several components that could decrease the quality of the final product by denaturating the protein or hydrolyzing the chlorambucil. By using a high acidic environment to improve the dissolution of the alkylating agent in the water, we have to be more careful when we add the protein. With an isoelectric point at 4.93, ANXA5 is easily denaturated and can precipitate if the pH changes significantly. Numerous precipitations have been observed, and the quick change of pH was the problem. During the conjugation the pH is the key; EDC/NHS has to be used in a phosphate buffer to avoid the quick change of pH[119]. The chlorambucil has an half-life of 1.5 hours when it's free in an aqueous solution[69,70]. After the conjugation, our sample is instantly frozen to avoid any loss and used 10 minutes after thawing it.

In this study a completely new drug was created, conjugating a mammal protein Annexin A5 to a chemotherapeutic drug, chlorambucil. The anti-cancer efficacy was improved compared to the free chlorambucil.

Our in vitro studies are based on two different cell-lines for the breast cancer and two others for the leukemia. EMT6 is a mouse breast cancer line highly used for in vivo and vitro studies that is metastatic within days after implantation[120]. 4T1 is an aggressive triple negative breast cancer cell line, forming metastasis in the early development of the tumor. LD50's in vitro are 0.3 μM and 3 μM for EMT6 and 4T1 cells respectively. The difference in the LD50 is logical because the 4T1 cells are more invasive and resistant than the EMT6 cells. The in vivo test could be improved by increasing the dose of the conjugate and chlorambucil. We used a concentration of 0.5 mg of chlorambucil/kg, and this could be increased until 20 mg of chlorambucil/kg, above which side effects have been observed[121].Because chlorambucil is targeted to the tumor cells, it is very likely that doses of greater than 20 mg/kg could be used without causing side effects.

L1210 and P388 are highly used models for studying leukemia in vitro and in vivo[105]. However, it would be appropriate to do in vitro and in vivo tests in other leukemia cell lines to validate our findings[116].

In summary, the conjugation between a targeting protein and chlorambucil was developed, and in vitro and in vivo studies showed promising results. All of our findings suggested that this approach may have implications in cancer therapies and could be used to treat breast cancer and leukemia and decrease considerably side effects of classic chemotherapies.

Conclusion

This study has shown success in the conjugation of a protein with several chlorambucil molecules. The conjugate is able specifically to target cancer cells by binding to phosphatidylserine and by endocytosis, bringing the alkylating agent into the cells. The conjugation didn't modify the characteristic of each molecule and enhances the cytotoxicity against breast cancer and leukemia. The conjugation protocol is effective and relatively fast with a ratio of molecule conjugated of 100%. The annexin A5 with the chlorambucil has a weight increased from 36 kDa to 49 kDa as we can see on the SDS-PAGE electrophoresis. Moreover, we have around 10 molecules of chlorambucil per molecules of annexin A5, inducing a better cytotoxicity.

The ANXA5-CHL can target and kill both adherent and non-adherent cancer cells with a higher cure rate than chlorambucil alone. The conjugate needs only 0.3 μM and 1.35 μM to kill half of the EMT6 and 4T1 cells, respectively, when a concentration 100-times higher of chlorambucil will be needed to obtain the same results.

Breast cancer forms a solid tumor which can metastasize and kill patients in most cases. The EMT6 and 4T1 cell lines represent those cancer types that metastasize, and the in vivo model showed a real improvement in the survival time and in the tumor growth. The survival is improved significantly with a mean increase of 2 days for the mice with leukemia. The breast cancer treatment also shows a good improvement, the mean survival time is increased by 6.7 days when the free chlorambucil improved the mean survival time by 3.5 days. It's also significant that no treatment toxicity was observed. It would be desirable to evaluate the therapy on another type of cancer and study the pharmacokinetics of the conjugate into the body.

Future Directions

The study has shown promising results against a cancer that forms solid tumors (breast cancer) and one that is non-adherent (leukemia). Further research will be required to validate some results and increase our knowledge in this new therapy. First, the same leukemia in vivo study should be repeated by adding a CD20 targeted monoclonal antibody like Obinutuzumab. In effect, because the classic treatment is chlorambucil accompanied with an antibody, the results could be different and impact the in vivo survival difference between the conjugate and the chlorambucil and antibody. CD20 is a protein on the cell surface expressed on B cells and only weakly expressed on CLL cells[122]. An anti-CD20 antibody has shown very good improvement and is now used as treatment with chlorambucil molecule; it would act again those cells and work in synergy with the ANXA5-CHL. Further experimentation is recommended with an increase in the dosage in vivo between 5 to 20 mg of chlorambucil /kg to reduce tumor growth and increase survival[121].

Furthermore, more experiments comparing the ANXA5-CHL with only annexin A5 would give us more information of the impact of annexin A5 on solid tumor and non-adherent cancer cells. Recent articles have shown that annexin A5 can present a slightly anti-tumor effect against melanomas[113]. Other studies using different types of cancers like brain cancer should be done to study the limit of the conjugate and its toxicity in some part of the body. Moreover, a healthy cell line should be used to study the activity of the conjugate on those cells; an appropriate cell line for this experiment would be the MCF10 healthy breast cell line[123]. However, we can already expect a reduced cytotoxicity due to the lack of phosphatidylserine on the surface of the healthy cells.

The protocol used to conjugate the annexin A5 to the alkylating agent can be reused to conjugate any drug with a carboxylic function. The carboxylic function can be added chemically, and other drugs can be carried by the annexin A5. A drug more specialized against breast cancer should be studied and compared to the ANXA5-CHL. Doxorubicin and docetaxel are potential candidates for this experiment[124]. Cytotoxicity, toxicity in healthy tissues and survival rate of healthy mice should be determined and compared to the results obtained with the ANXA5-CHL conjugate on the breast cancer cell lines. The conjugate create an hypoxia in the tumor but combined with rapamycin, the hypoxia could be avoided[125]. Because this therapy also targets tumor vasculature endothelial cells, which express phosphatidylserine, this therapy should be combined with an mTOR inhibitor such as rapamycin to counter act the hypoxia created in solid tumor as a result of cutting off the blood supply to the tumor[125]. The killing of tumor vasculature endothelial cells would bond to tumor antigens being released into the bloodstream and distributed throughout the body, so this therapy could be combined with immunostimulation to treat metastatic tumors[105,126].

Abbreviation

1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride - EDC

2-Fluoro-2-deoxyglucose - FDG

Acute myeloid leukemia - AML

Acute lymphocytic leukemia - ALL

Annexin A5 – ANXA5

Adenosine triphosphate - ATP

Chronic myeloid leukemia - CML

Chronic lymphocytic leukemia - CLL

Dalton or kilo Dalton – Da or KDa

Dimethyl sulfoxide - DMSO

Deoxyribonucleic acid - DNA

Enhanced permeability and retention - EPR

Fluorescein isothiocyanate - FITC

Immunoglobulin - Ig

Isopropyl β-D-1-thiogalactopyranoside - IPTG

Median lethal dose - LD50

Luria Bertani broth - LB

Matrix metalloproteinase - MMPs

Molarity - M (unity)

N-hydroxysulfosuccinimide - sulfo-NHS

Nickel heads - Ni-NTA resin

N- *p*-tosyl-L-phenylalanine chloromethyl ketone - TPCK

Phenylmethylsulfonyl fluoride - PMSF

Phosphatidylcholine - PC

Phosphatidylethanolamine - PE

Phosphatidylserine - PS

Polymerase Chain Reaction - PCR

Ribonucleic acid - RNA

Sodium dodecyl sulfate polyacrylamide gel electrophoresis - SDS-PAGE

Tissue Factor - TF

Ultraviolet (1-400nm) - UV

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Appendix A: Chlorambucil-Annexin Conjugate

- Day before starting, autoclave the following items:
    - 1 liter LB medium
    - 4 x 1 liter Erlenmeyer flask (with aluminum foil on top)
    - 125 ml Erlenmeyer flask (with aluminum foil on top)
    - 100 ml beaker (with aluminum foil on top)
    - All size tips
    - 1.5 ml centrifuge tubes (like 100)
    - 1 liter DI water

Protein Expression

Day 1 –9:00 pm

1. Culture 5 µl of *E. coli* BL21(DE3) harboring pET303CT with the fusion gene AV in 10 ml of LB medium containing 35µg/ml kanamycin in a 125 ml Erlenmeyer flask overnight at 37°C with shaking at 200 rpm.
    - LB medium: 1 liter DI H_2O + 10 g Tryptone + 5 g Yeast Extract + 5 g NaCl.
    - Add 35 mg kanamycin to the 1 L of LB medium before taking out the 10 ml for the initial culture.
    - Incubate.

Day 2 - 9:00 am

2. Add 10 ml of the cell culture to 1 liter of fresh culture medium and kanamycin and incubate at 37°C with shaking (200 rpm). Take 1.5 mL of medium before adding the bacteria, as a blank. This cell culture was grown to mid-log phase =>OD_{600} = 0.5.
    - Take a 1.5 ml sample of just the LB medium. Label 1.5 ml tube '**LB**.'
    - Transfer 10 mL of bacteria to 1 L LB medium.
    - Transfer entire volume of medium to 4 x 1 L flasks.
    - Put in shaker at 37° C at 200 rpm.
    - After 1.5 hours of shaking, measure optical density at 600 nm (absorbance) using a clear 96 well plate and microtiter plate reader of sample vs LB medium => using 250 µl samples of each. When OD_{600nm} = 0.5, then proceed to the next step.

Day 2 –2:45 pm

3. Addisopropyl Alpha-D-thiogalactopyranoside to a final concentration of 0.4 mM to solutions in 4 x 1 L flasks and incubate at 30°C with shaking (180 rpm) for 5 h to induce protein expression.
    - Take 750 ml sample of solution before adding IPTG.
    - Add 24 mg IPTG to each flask.
    - Put back in shaker at 30°C for 5 hours.
    - IPTG stimulates the production of fusion protein.

Day 2 – 7:45 pm

4. Harvest the cells by centrifugation for 10 min at 1000 x *g*, at 4ºC.
   - Take 750 ml sample before centrifuge.
   - Centrifuge at 1000xg = 3000 rpm
   - Only 4 – 50 ml centrifuge tubes at a time, temp 4ºC, 10 mins.
   - After first centrifuge, pour out supernatant, add more culture to same 4 tubes. Bacteria will be stuck to side of tubes so inverting to pour out is not a problem.
   - Can put the 4 tubes in -20ºC freezer for overnight storage.

Day 3 – 10 am

5. Resuspend the cell pellet in 40 ml of sonication buffer.
   - Add ~10 ml to each of the 4 centrifuge tubes.
   - Vortex to resuspend cell pellets.
   - Pour contents of the 4 tubes back into the 100 ml beaker.
   - Sonication Buffer
     - 0.05 mM N- *p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)
     - 1 mM phenylmethylsulfonyl fluoride (PMSF)
     - 1% HPLC ethanol
     - 0.01% βmercaptoethanol
     - 0.02 M sodium phosphate dibasic
     - 40 mL DI
     - Dissolve TPCK and PMSF in ethanol in microcentrifuge tube, then add to beaker.
     - Make this buffer in the 100 ml beaker.
     - Correct to pH 7.4

6. Lyse the cells by sonication at 4°C for 30 sec at 4.5 watts then allow it to cool for 30 sec on ice. This cycle was repeated 4 times for a total sonication time of 2.5 min on power level 4.
   - Clean sonicator tip with ethanol before use.
   - Put beaker in tub with ice while sonicating.

7. Centrifuge the lysate obtained at 12,000 x *g* for 30 min to remove the cell debris and take the supernatant.
   - Pour beaker contents into 1 purple VWR 50 ml centrifuge tube.
   - Centrifuge at 12000xg for 30 mins
   - Take 750 µl sample after centrifuging.

Protein Purification

Day 3 – 12 pm

8. After taking supernatant sample, add Imidazole (40 mM) and NaCl (500 mM) to the lysate to reduce non-specific protein binding.
   - 40 mM imidazole
   - 500 mMNaCl

9. Equilibrate a 5 ml HisTrap chromatography column with immobilized Ni^{+2}using Wash Buffer 1.

- o Feed wash buffer thru column until the output reaches baseline, then feed lysate thru at -8.00
- o Turn on detector.
- o Turn on drop former – mode 0, rack 1, size 2, last tube 100, min 4, delay 0.
- o WASH BUFFER 1 (500 mL) (- 30 min)
  - 20 mMsodium phosphate dibasic
  - 40 mM imidazole
  - 500 mMNaCl
  - Correct this to pH 7.4

10. Feed the soluble protein fraction into the column.

11. Wash the column with 70 column volumes of Wash Buffer 2 to remove unwanted proteins and endotoxin (250 ml).

- Collect flow thru – contains unwanted proteins ’
- o WASH BUFFER 2 (300 mL) (- 1.5 hours)
  - 20 mM sodium phosphate dibasic
  - 40 mM imidazole
  - 500 mMNaCl
  - 0.1% Trition X-114
  - Correct this to pH 7.4

12. Wash the column with 20 column volumes of Wash Buffer 1 to wash the protein until the pen reaches the baseline (100 ml). (- 40 min)

- Collect flow thru – contains unwanted proteins

13. Elute the protein using elution buffer.

- Collect the elution – contains our FP
- o ELUTION BUFFER (300 mL)
  - 20 mM sodium phosphate dibasic
  - 500 mMimidazole
  - 500 mMNaCl
  - Correct this to pH 7

14. Dialyze eluted protein for 3 hours against 2 liters of dialysis buffer containing 20 mM sodium phosphate at pH 7.4 to remove NaCl and imidazole from the protein solution and make it suitable for C-terminal His-tag cleavage.

- o DIALYSIS 1 BUFFER (2 L)
  - 20 mMsodium phosphate dibasic
  - Adjust to pH 7.4
- After dialysis, put back in a 50 ml centrifuge tube.

15. Before continuing, need to regenerate the column using this procedure:

- 25 ml of 1 M KCl
- 25 ml of 1 M NaOH

- 25 ml of DI Water
- 25 ml of 1 M HPLC Grade Ethanol

16. Measure the concentration of protein (Bradford Protein assay).
    - Add 30 ml DI Water to tube with cell pellet.
17. Cleave the C-terminal His-tag by adding HRV 3C protease at 10 U/mg of protein with the recommended 10 X buffer provided. Incubate for 16 h at 4°C at 30 rpm.
    - Add HRV 3C protease. HRV 3C protease comes as 2 U/µl and we want to use it at 10 U/mg protein.

Day 4 – 10 am

18. Equilibrate the HisTrap column with Wash Buffer 1.
    - Feed thru the column until baseline is reached.

Add imidazole (40 mM) and NaCl (500 mM) to the cleaved protein solution (depends on particular volume after overnight cleavage incubation).

- 40 mM imidazole
- 500 mMNaCl

20. Feed the solution to the HisTrap column.
    - Collect first peak solution from the column is solution to dialyze. This contains our protein.

21. Feed Wash Buffer 1 into column to pull out all cleaved protein before proceeding forward.

22. Elute uncleaved protein with imidazole.

23. Dialyze purified protein for 3 hours against 2 liters of dialysis buffer.
    - DIALYSIS 2 BUFFER
        - 20 mM sodium phosphate dibasic
        - 100 mMNaCl
        - Adjust to pH 7.4

24. Regenerate the column as above.

25. Pass the sample thru a 0.2 µm cellulose-acetate filter.

26. (IF NECESSARY) Concentrate the protein using a 150 kDa, 20 ml protein concentrator (Millipore #89921).

27. Aliquot purified protein into cryovials and put in the liquid nitrogen tank prior to freeze-drying.

30. Perform an SDS-PAGE
    - Suspend 50 µl of sample + 50 µl loading buffer (95% Laemmli sample buffer + 5% β-mercaptoethanol).
    - For BI, centrifuge for resuspend in 100 µl of loading buffer.

Synthesis of ANXA5-CHL

1. Dissolve 1000 µg of chlorambucil in 10 drops of 12 M HCL
   - *Chlorambucil is insoluble at neutral pH, but dissolves readily at pHs below 5.*
   - *should not be stored for long periods of time in acidic solutions. Chlorambucil is unstable in acidic solutions*
2. Dilute the mixture in 1 mL of phosphate buffer
   - *This provides a larger working volume of chlorambucil to continue downstream production.*
3. Add 100 mg of EDC
   - *The EDC will bind the carboxylic groups of chlorambucil increasing their chemical reactivity towards primary amines.*
4. Add 70 mg of suflo-NHS
   - *Sulfo-NHS stabilizes the EDC activated carboxylic groups, increasing the efficiency of the chlorambucil-annexin reaction.*
5. Stir vigorously for 5 minutes
6. Add 2 µL of β-mercaptoethanol
   - *B-mercaptoethanol neutralizes the excess EDC and NHS preventing their interference in downstream reactions.*
7. Immediately titrate the solution to a pH of 7.4
   - *Raising the pH stabilizes the sensitive chlorambucil functional groups.*
8. Add 10 mL of a 1 mg/ml solution of annexin in phosphate buffer
   - *The annexin is kept at a low concentration to prevent precipitation and crosslinking. The dilute mixture also serves to further neutralize some of the acid from step.*
9. Stir gently for 12 hours
10. Centrifuge for 10 minutes at 12,000 G
    - *Chlorambucil is not stable in neutral pH solutions and will precipitate. The precipitate is easily removed by centrifugation.*
11. Retain the supernatant and discard the pellet
12. Dialyze the supernatant against 2 L of phosphate buffered saline for 3 hours at 4 degrees Celsius.
    - *This step removes the rest of the unbound chlorambucil as well as other upstream contaminants such as β-mercaptoethanol.*
13. Filter the dialysate using a 0.2 µm filter
14. Immediately flash freeze under liquid nitrogen and store at -80 °C until immediately before use.

Cytotoxicity Assay

Protocol:

1. Harvest 5 million cells from culture and dilute to a total of 25 mL with media.
2. Seed each well of a 96 wells plate with 180 µL of media containing 20,000 cells
3. Allow cells to rest for at least 1-2 hours.
4. Treat groups according to experimental protocol.
5. In this experiment you will add 20 µL of the Chlorambucil-DMSO per well
6. Incubate cells with chosen treatment for 20 hours.
7. Add 20 µL of Alamar Blue solution to each well
8. Incubate cells with Alamar Blue for 2-4 hours to allow dye uptake.
9. Read the absorbance immediately at 540nm.

Supplies

•5 million cells
•DMEM with 10% horse Serum and 1 % Anti/Anti (L1210 and P388) or Waymouth's medium and L-glutamine and fetal bovine serum (for 4T1 and EMT6 cells)
•DMSO
•Alamar Blue
•96 wells plate
•Chlorambucil Dissolved in DMSO

Synthesis of ANXA5-CHL

1. Dissolve 1000 µg of chlorambucil in 10 drops of 12 M HCL
   - *Chlorambucil is insoluble at neutral pH, but dissolves readily at pHs below 5.*
   - *should not be stored for long periods of time in acidic solutions. Chlorambucil is unstable in acidic solutions*
2. Dilute the mixture in 1 mL of phosphate buffer
   - *This provides a larger working volume of chlorambucil to continue downstream production.*
3. Add 100 mg of EDC
   - *The EDC will bind the carboxylic groups of chlorambucil increasing their chemical reactivity towards primary amines.*
4. Add 70 mg of suflo-NHS
   - *Sulfo-NHS stabilizes the EDC activated carboxylic groups, increasing the efficiency of the chlorambucil-annexin reaction.*
5. Stir vigorously for 5 minutes
6. Add 2 µL of β-mercaptoethanol
   - *β-mercaptoethanol neutralizes the excess EDC and NHS preventing their interference in downstream reactions.*
7. Immediately titrate the solution to a pH of 7.4
   - *Raising the pH stabilizes the sensitive chlorambucil functional groups.*
8. Add 10 mL of a 1 mg/ml solution of annexin in phosphate buffer
   - *The annexin is kept at a low concentration to prevent precipitation and crosslinking. The dilute mixture also serves to further neutralize some of the acid from step.*
9. Stir gently for 12 hours
10. Centrifuge for 10 minutes at 12,000 G
    - *Chlorambucil is not stable in neutral pH solutions and will precipitate. The precipitate is easily removed by centrifugation.*
11. Retain the supernatant and discard the pellet
12. Dialyze the supernatant against 2 L of phosphate buffered saline for 3 hours at 4 degrees Celsius.
    - *This step removes the rest of the unbound chlorambucil as well as other upstream contaminants such as -mercaptoethanol.*
13. Filter the dialysate using a 0.2 µm filter
14. Immediately flash freeze under liquid nitrogen and store at -80 °C until immediately before use.

Spectroscopy of chlorambucil

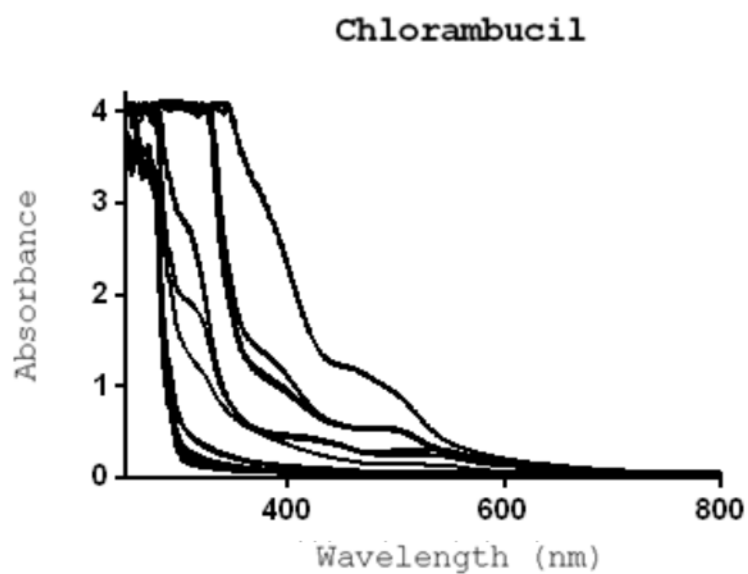

Figure 21: Chlorambucil absorbance.
Chlorambucil has strong absorbance at UV wavelengths. Pictured above are the spectra for serial dilutions of chlorambucil. ($[Chlorambucil]_{starting}$ = 10 mg/ml; serial dilutions by half). Chlorambucil was diluted in a solution with 50 % of DMSO.

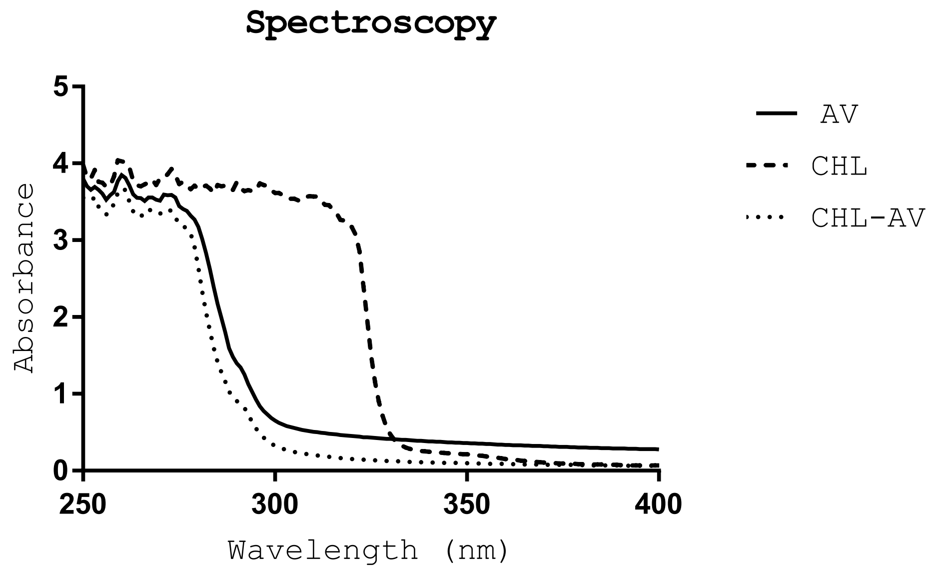

Figure 22: Absorption spectroscopic detection of CHL.
Despite strong absorbance peaks in the UV range, an unknown concentration of chlorambucil can not be determined with absorbance spectroscopy in samples containing protein. When chlorambucil is in the presence of protein, the absorption spectra of the chlorambucil – protein conjugate is dominated by that of the aromatic amino acids of the protein