Cancer Therapy

- The mitochondrial Bcl-2 protein family as drug targets for inducing cell death

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## 1. Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIF</td>
<td>Apoptosis - inducing factor</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
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<td>Bcl2</td>
<td>B-cell lymphoma 2</td>
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<td>CD</td>
<td>Circular dichroism spectroscopy</td>
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<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma viruses</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
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<tr>
<td>PC</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
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| POPC         | 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine \textbf{POPE}  
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine \textbf{PSA}  
Prostate specific antigen |
| TMCL         | 1,1', 2,2'-tetra-myristoylcardiolipin |
2. Abstract

Apoptosis is an evolutionary conserved cell death program which tightly regulates tissue homeostasis and removal of harmful cells in higher organisms. Failures in its regulation trigger severe pathological disorders, including abnormal development, neurodegenerative disorders and cancer formation. Besides an extrinsic pathway, an intrinsic (mitochondrial) pathway exists where mitochondria are actively involved in initiating cell death upon sensing intracellular stress. During this process the mitochondrial outer membrane (MOM) has to undergo permeabilization, resulting in the release of cytochrome c and further apoptotic factors into the cytosol. This permeabilization process is tightly regulated by the Bcl-2 protein. In many types of cancer is the gene B cell chronic leukemia / lymphoma 2 (Bcl-2) encoding the protein Bcl-2 involved. The increased expression of Bcl--member 2 family can develop into different types of malignancies and lead to metastatic cancer by inhibition of apoptosis. It can also lead to resistance to cancer therapy. The hope is that the direct targeting of Bcl-2 family members could provide better cancer treatment.

The aim of this study was to study the basic properties and interactions of mitochondrial membranes with the Bcl-2 protein family targeting drug Gossypol, by differential scanning calorimetry (DSC), nuclear magnetic resonance spectroscopy (NMR) and circular dichroism spectroscopy (CD). The substance Gossypol was chosen due to its demonstrated promising effect on the treatment of prostate cancer, of the most common cancers in Sweden. In the treatment of prostate cancer the potential targets of Gossypol are the Bcl-2 family members, where the strategy is to inhibit the anti--apoptotic members and also promote pro-apoptic members of the Bcl2 family. By this strategy the induction of apoptosis would also be triggered in cancer cells (who normally evade natural or drug induced cell death), which would finally stop the further growth and spreading of tumor cells.

Gossypol itself is a derivative originating from the genus Gossypium. By examining MOM mimicking lipid mixtures prior and upon the presence of gossypol, it was revealed by DSC and NMR methods that found that gossypol interacts severely with these mitochondrial membranes;; the place were in vivo also the antiapoptotic Bcl-2 protein (main culprit in cancer resistance to treatment) resides. In addition, CD studies revealed that Gossypol could interact with another member of the Bcl-2 family, the apoptotic Bax protein, which has a high sequence homology with the Bcl-2 protein itself.

Keywords: Gossypol, prostate cancer, Bcl-2 protein family, apoptosis, differential scanning calorimetry and nuclear magnetic resonance spectroscopy
3. Introduction

3.1 Cancer

The phenomenon of existence is the life as unique evidence. The result is the set of biological processes that occur in each organism, therefore in every cell. The birth, growth and death of each cell are determined by the cell cycle (in each phase in our life). However, if any misregulation occurs in this cell--associated metabolic process, and the cells cannot compensate for that, these cells can become cancer cells [1].

In Latin the word cancer means crab, a name also used in Sweden previously. In breast cancer, for example the image that appears looks like a crab and thence it is named after its appearance. Cancer of itself is a generic term that covers 200 different types of diseases that can occur in 60 different organs (caused by disorders of the cell cycle). Cancer can start at different places in the body, but in general cancer always starts from an ordinary cell, that changes and begins to spiral out of control [1].

Cancer is an invasive disease (picture 1.), which passes through several phases. A normal cell has a mutation that preserves the genetic material that is the first step and it is called initiation. The next phase is progression; there the "broken" cell participates in another mutation. The greatest risk is the latter mutation that repeatedly triggers a fast proliferation of high speed and removes nutrients from the environment to the extent that it becomes a cancer. When cancer cells begin to migrate with the help of the blood/lymph system, then this process is called metastasis, because where the cancer cells stop moving they can start to generate new cancer cells [2].

A lump of cancer cells is called the tumor and there are two types of tumors. It can be either benign tumor or malignant tumor. We know that the cells in the body form the tissues, such as muscles and bones. These in turn provide organs. Benign tumors grow around the organs and tissues but do not form metastases and are not malignant [1,2].

Different cancers have different origins and different cancers dominate in different countries. In Sweden there are around 50,000 new cancer cases each year. The largest two groups are breast cancer (7000 cases) and prostate cancer (10,000 cases) [1,2].
3.1.1 Prostate Cancer

Prostate cancer (PC) is one of the most common cancers in the male population and mainly occurs in later years. The prostate, itself is important for the functioning of the reproductive and urinary system of man. The prostate is a gland located around the urethra and produces fluid for sperms. The fluid helps sperms to move. Through the blood and lymph to other tissues and organs, PC spreads similar to other cancer types. Unlike some cancers, this type of cancer is not as "fast", but a slow-growing form. For comparison with other forms if detected in time, it can be treated efficiently [1,2].

A couple of studies have shown that there may be some connections between genetic inheritances, virus infections (herpes virus) and PC occurrence [1,3,4]. After detection of high PSA values, men often get the diagnosis of prostate cancer. However, PSA is a prostate specific antigen, which does not directly indicate the presence of a tumor, it just shows the possibility of cancer. A prostate biopsy (tissue sample) is recommended when high PSA levels are found together with an abnormal prostate enlargement, or if risk factors exist [3,4].
Difficulties in urinating, inability to maintain a continuous stream, frequent urination, burning and pain during urination blood in urine is the typical symptoms, which must be, sought care for. Before treatment is proposed, it is necessary to determine the stage of prostate cancer. As the factors necessary for the assessment are Pre-Treatment PSA, and the biopsy Gleason score: indicating the proportion of the quantity and aggressiveness of cancer and T stage: includes the size of the interaction of tumor-lymph node-metastasis. These factors allow to determine whether the cancer is confined to the prostate gland and thus potentially manageable. These factors allows also see if here is a risk of recurrence of cancer after surgery or radiation therapy. Risk measures include: low risks - more than 85% chances of no recurrence of cancer, medium - 50-70% chances of a no recurrence of cancer and high about 33% chances that there is no cancer in the next 5 years [4].

In addition to blood tests, there are other approaches such as ultrasound scans and bone imaging. Histopathological studies of the biopsies are classified using a Gleason scale of 1-10. At scores of 6 and higher it will indicate a faster growing tumor and below 6 slow- growing tumors. Ultrasound examination is performed rectally, where sound waves shows in a computer what is happening in the body. The picture shown is just an extra help to determine the diagnosis. There are different types of treatments such as: external and internal radiation, prostate surgically removed completely, hormone treatment, cytostatics and more. Ideally, several drugs would be exist, who could slow down or stop metastasis [4,5].

### 3.2 Apoptosis

In a publication by Alberts et al. [6], it is stated that, cells of an organism comprise an efficiently coordinated association. The cells keep dividing but finally they undergo suicide, a process activated by a "death program". In a paper published by Wylie, Currie and Kerr in 1972, they used the term apoptosis to describe the process of a "morphologically distinct form of cell death", although certain aspects of this term had already been suggested, previously [6].

Apoptosis is a normal part of cell development defined by "morphological characteristics and energy-dependent biochemical mechanisms" [7]. Death by apoptosis is not limited to cells, which are scheduled to die during development, but is essential in many aspects of live processes. Apoptosis is an essential part of numerous mechanisms participating in "normal cell turnover, proper development and functioning of the immune system, hormone--dependent atrophy, embryonic development and chemical-induced cell death" [7].

A cell can commit suicide by two major mechanisms. One is generated by death activators (extrinsic pathway), which bind to the receptors on the cell's surface and another result of signals originating from within the cell (intrinsic pathway). The death receptors, which are affiliated with tumor necrosis factor alpha (TNF-α), are a component of the sequence of events in externally stimulated apoptosis. FasL/FasR and TNF-α /TNFRI models ideally exemplify them. The most significant externally initiated apoptosis mechanism uses transmembrane receptor--mediated interactions [8,9].

Besides this extrinsic pathway [9], the intrinsic (mitochondrial) pathway acts using intracellular stress. During this process the MOM has to undergo permeabilization, resulting in the release of cytochrome c and further apoptotic factors into the cytosol.
This permeabilization process is tightly regulated by the Bcl-2 protein family. After permeabilization, cytochrome c gets released causing final cell death.

How is apoptosis different to other ways for cells to die? When cells are damaged, contents leak out, leading to inflammation of surrounding tissues. Cells that are induced to commit suicide, on the other hand, shrink; they develop bubble-like blobs on their surface; the chromatin in their nucleus degrades; their mitochondria break down, releasing cytochrome c; they break into small, membrane-wrapped fragments [9,10].

There are two reasons why we want a cell to commit suicide. Programmed cell death is necessary for proper embryonic development. Through apoptosis occurs formation of fingers and toes of the fetus requiring removal of the tissue between them. Shedding of the inner lining of the uterus at the start of menstruation also occurs by apoptosis. Formation of the proper connections between neurons in the brain stay connected, that surplus cell’s pray eliminated, this is happening again by apoptosis. In addition, cells with damaged DNA also undergo apoptosis. Since cancer cells escape apoptosis it is important to find treatment, which induces apoptosis specifically in cancer cells for a therapy to be successful [10,11].

### 3.3 Mitochondria

Mitochondria are cell organelles, of variable size and shape. They are present in nearly all eukaryotic organisms. They take up a large part of the cytoplasm of all eukaryotic cells and they are essential for the evolution of animals. They can possess widely various shapes but mostly they are filamentous. They consist of the outer membrane (MOM), inner membrane (IMM), intermembrane space, outer chamber (IMS) and matrix (internal chambers).

*Picture 2: How a Mitochondrion looks like. (Adapted;; Molecular Expression) [11].*

Mitochondria are the power plants of cells. It is at the center of the cell’s metabolism and they produce the energy needed for the cells via ATP synthesis by oxidative phosphorylation and the work of the tricarboxylic acid cycle. Mitochondria are often located in the vicinity of extensively consuming ATP locations [10-13]. Mitochondria have the ability to increase the number in the cell by simple division. Mitochondrial DNA (mtDNA), which is contrast to the nuclear DNA, processes the shape of a closed
circle, called a ring of DNA. The mitochondrial genome is very similar to the genome of the bacteria of the genus Rickettsia, which is an internal parasites that reproduce only inside eukaryotic cells. In humans, the gene encoding the 13 mitochondrial proteins, is involved in the transport chain and oxidative phosphorylation. The outer and inner mitochondrial membranes consist of phospholipids bilayer and proteins. It is the location for intramembranous enzymes involved in phosphorylation of nucleotides [13].

The MOM separates the mitochondrion from the cytosol, covering the entire surface of organelles and its composition is similar to the plasma membrane of eukaryotes. Porins -- transmembrane proteins are constituent's binder channels through which the water--soluble molecules and ions diffuse. It also contains the enzymes involved in the conversion of fatty acids who become metabolized in the mitochondrial matrix synthesis to membrane phospholipids. IMS of the organelle is separating matrix from IMS; is thinner than MOM, and impermeable. The inner cone is formed the cristae, in contrast to the exterior and they are in two forms lamellar and tubular type. Extension of several inner surface membrane inner cones has the task of increasing the possibility of production of ATP-a. The MOM surface is about 5 times smaller than the IMM and internal cone has more protein than any of the cell membrane and also have proteins with multiple functions. The proteins perform redox reactions in the oxidative phosphorylation and transport proteins that regulate passage of metabolite in or from matrix etc. They contain a large quantity of cardiolipin -- phospholipid molecules with 4 chains of lipid acids, making it impermeable for most ions, protons and smaller molecules [12].

IMS (intermembrane space) is the space between the MOM and IMM and except for transport protein, IMM contains protein complexes consisting of the electron transport chain and as well as complexes involved in the synthesis of ATP or ATP synthesis. IMM encloses the mitochondrial proteins and contains enzymes that participate in the catabolism of pyruvate and beta fatty acid oxidation, Krebs cycle enzymes and the enzymes for the expression of the mitochondrial genome, mtDNA, RNA, ribosomes, and of the matrix granules [12,13].

Clearly, mitochondria are very important to store energy in the form of ATP. And they are heavily involved in the metabolism of glucose and fatty acids in the Krebs cycle by oxidative phosphorylation. In the synthesis of steroid hormones, storage of ions of Ca, and apoptosis mitochondria play also an important role. Especially in the context of apoptosis, they are the key regulators cell life and death. In this process of cell death synthesis of ATP in the mitochondria of cells drops, a process accompanied by mitochondrial form pores and thereby triggers a disturbance in the cytoplasm where the cell is killed [10-13].

### 3.4 Phospholipids

The membrane enclosing the mitochondria is built up of by large number of various lipids. A group of phospholipids along with cholesterol build these cell membranes. Phospholipids are composed of glycerol, two fatty acids and phosphate containing headgroup e.g. choline (as seen in Figure 2.)

Their head is polar and hydrophilic and the tail is the opposite, non-polar and hydrophobic. These lipids, they create a semi-permeable membrane and hydrophilic, non-polar substances can go through the membranes. The membrane may expire by strong organic solvents and by change of pH value [12, 14].
During the study following lipids were used (Figure 2): 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol-amine (POPE) and 1,1', 2,2'-tetramyristoylcardiolipin (TMCL). Here we examined the phase behaviour of mitochondrial membrane mimicking lipid mixtures (PC/PE/CL) prior and upon the presence of drug candidate to reveal if these candidates interact with the membrane [10, 15].

**DMPC**

![DMPC diagram]

*Phospholipid: 1,2-dimyristoyl-sn-glycer--3-phosphocholine*

**POPC**

![POPC diagram]

*Phospholipid: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine*

**POPE**

![POPE diagram]

*Phospholipid: 1-palmitoyl-2-oleoyl-sn-glycero-3phosphoethanolamine*
3.5 The Bcl-2 protein family

B-cell lymphoma 2 (Bcl-2) family is a small protein family, a group of proto-oncogenes encoding growth factors, which in turn prolongs the survival of hematopoietic cells. In the U.S. 85% of the known types of lymphoma contributes malignant B-lymphocytes. The causes that the B-cell lymphoma cells separate Bcl-2. Bcl-2 gene from chromosome 14 is the side of the Ig heavy chain locus on chromosome 18, leading to an excess of Bcl-2 in B cells. There are resorts to block / induce apoptosis in case overpressure with Myc prevents the oncogenic activity of Myc. As a result of differential characters and splicing are two main (α and β) forms of the products [16-17].

Pro-apoptotic and anti-apoptotic proteins are the main counter player in the Bcl-2 family. The family itself is divided in three subfamilies: Bax subfamily (pro-apoptotic): Bax, Bak and Bok, Bcl-2 subfamily (pro-survival): Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1 and BH3 subfamily (pro-apoptotic): Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL. Bax (Korsmeyer group purified a protein) can associates with and modulates the activity of Bcl-2. Bax by itself can also cause apoptosis in a Bcl-2-independent manner. Another Bcl-2-interacting and death inducing--protein, Bid, which only has one Bcl-2 homology domain (BH3) has already identified. Subsequently, more Bid--like death--inducing proteins were identified, all of which has only one BH3 domain. This protein family was called Bcl-2 subfamily, only BH3. A gene, named bcl-x, which can be alternatively spliced to generate optionally two proteins that have opposite functions in apoptosis has been identified by Thompson's group [17]. The short form (Bcl-xs) cause cell death and the long form (Bcl-xL) inhibits apoptosis [16-18].

The Bcl-2 family plays an important role in prostate cancer. Malignant development can be observed by looking at a failure of apoptosis function. Apoptosis induction in malignancies by apoptosis--related Bcl-2 family proteins is important for the prediction of patient's response to chemo--or radio--therapy as well as of survival rates. One promising treatment of malignancies is chemosensitivity testing and individuell chemotherapy based on the patient's specific response ability. Dysfunktional apoptosis
leads to a dramatic increase in PC, instead of the normal startup phase of the cell cycle. In some studies it has been shown that the expression of anti-apoptotic members of the Bcl--2 family in close association with developing the normal cells in to the cancer cells. In normal prostate cells expressed Bel-2 antiapoptotic members are confined to resistant basal cells (androgen) in the prostate glandular epithelium. But PC cells leads to a rapidly increasing levels of Bcl2, BclxL and MCL, antiapoptotic members of the Bcl-2 family. Antiapoptotic protein increase leads to an attenuation of apoptosis. This in turn can lead to prostate tumor progression may begin [19]. For therapeutic purpose it would be ideal to boost the action of proapoptotic proteins in the tumor cells while blocking the cell protecting function of the anti--apoptotic Bcl--2 proteins [19,20,21].

4. Aim of this thesis

There is the large problem in cancer treatment that tumor cells can become immune to treatment induced apoptosis by an increased production of anti-apoptotic proteins, most prominent the mitochondrial membrane protein Bcl-2 itself. The aim of this work is to understand the mechanism behind the cytoprotective function of Bel-2 by focussing on the investigation of a potential interaction of this protein with a suitable drug candidate. The main aim was to find and characteriz a suitable drug candidate with focus on its affinity to Bcl-2 protein family and the location of action, namely the mitochondrial outer membrane system. This work should then form the basis for further development into a useful drug that selectively attacks the mitochondria, located Bcl--2 protein in tumor cells.

5. Method

Literature searches were made through the search engines Google, PubMed, Umeå University’s library and Ph.D. theses from Umea University. Following keywords were used: Cancer, prostate cancer, Bcl-2 protein, Bcl-2 protein blocks PC, Bcl-proteins blocked by drugs... The searches yielded many results. From those, few articles were chosen. After long periods of literature searches, it emerged that there is a drug candidate that could fit into our main aim, namely Gossypol, whose interactions with mitochondrial membranes and their proteins were studied by following three methods: 1. Nuclear magnetic resonance, 2. Differential scanning calorimetry and 3. Circular diachronic spectroscopy [21-23].

5.1 Gossypol

Gossypol (see fig3) is derived from the genus Gossypium, the cotton plant and is a natural phenol. There is a yellow pigment and has been tested that male oral contraception and is also estimated to have anti-malarial properties. In the present study, Gossypol was studied in the presence and absence of the protein Bax and in the presence of mitochondrial membrane mimicking lipid vesicles. It would have been desirable to have Bcl-2 is also included in the study, but this was not available in the laboratory [21].
Figure 2
The Chemical structure of Gossypol, drawn by Arine Bagdasarian

Among many researchers gossypol became an interesting compound in cancer drug research. Gossypol is a bioactive agent, which has been studied in three different and independent laboratories where e.g. Suresh R. Volate and William L. Farrar, National Cancer Institute [22] could show that some cancer cells were affected by the drug. In this study it was concluded that up to 70 mg per day can safely be administered. Increasing evidence in clinical trials has shown that gossypol can inhibit cancer cell growth and stopping metastasis, the main aim for many researchers and for us as well (in order to prove). The goal of their study was to study the chemotherapeutic effects of Gossypol on prostate cancer cell lines, and they chose a little different approach to go on with. In our study, we will concentrate ourselves to look on Gossypol has any effect on the phospholipids in the presence and absence of the protein Bax. The first step was to see if it affects the phase behaviour of mitochondrial membranes on its own without any presence of Bcl-2 family members. It is the first step to see if Gossypol may be sufficient good as new drug candidate for the PC treatment. Common to our and their study is that we wish to have a substance that can be effective against PC [22].
5.2 Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) is a technique that by exploiting the magnetic properties of atomic nuclei determines the structural details and physical properties of molecules. In addition, NMR gives detailed information such as the dynamics, interactions between certain parts of molecules, molecule size and the chemical environment of molecules. NMR spectroscopy is used here to analyse the interaction of drug candidates with its target membranes [23,24].

Normally ¹H NMR spectroscopy is used for small compounds, while multi–dimensional multi–nuclear (often ¹³C, ¹⁵N) NMR is often used to explore biomacromolecules. Each drug candidate should be monitored by NMR for it to be certain that the sample is pure and not decomposed. Without NMR, it would be impossible to determine new medicines and their structures at the site of their action (often target protein). Without NMR it would be hopeless to screen new compounds at the active site of proteins, and especially to obtain a three–dimensional picture of the molecule during its action (e.g. as inhibitor or activator). Similarly, NMR can e.g. also be used to study Gossypol interacting with target membranes or target proteins such as Bcl-2 or Bax [24].

5.3 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is a technique which was developed in 1962 by ES Watson and MJ O’Neil. In 1964 Priyalov and DR. Monaselidze developed a differential scanning calorimeter, which also could be used in biochemical research [25]. This technique measures the difference in the amount of heat required to change the temperature of a sample compared to a reference undergoing the same change in temperature. The resulting thermograms are the heat capacities as a function of temperature. Deviations from zero on the graphs indicate that heat is required (endotherm) to drive a phase–transition process at the given temperature, or vice versa that heat is given away (exotherm) by performing the process [25].

Melting or freezing of any compound is either endothermic (+), respectively exothermic (–) and it is not associated with any chemical change in a system, but only its phase state. Thus, the ¹H and the Gibbs Free Energy ¹Go are fixedly connected in conjunction with absolute temperature and entropy. The equations used in DSC are the thermodynamic values of Gibbs free energy equations. For larger compounds, the enormous length and intermolecular attractions play a major role in the physical properties of the macromolecule. Before melting, the polymer undergoes a glass transition (Tg) [25].

DSC analysis of analysis provides information about melting or better, unfolding of the biopolymer accruing at a specific temperature. This is a method to determine if enzymes are very heat sensitive. Importantly, DSC provides unique information about the melting behaviour of phospholipids forming membranes (e.g. melting of the lipid fatty acid chains) and effects which external membrane effectors such as drugs have on its melting behaviour.

In Figure 4 you can see that there is nothing special, but we simply load membrane samples in the machine and the variations measured in the temperature as the "melting point" or to conditions that the same mass of a sample.
5.4 Circular dichroism spectroscopy (CD)

CD is a method that exploits the optical rotational properties of chiral molecules. By studying the secondary structures of proteins, the CD - spectroscopy provide a lot of information about the shapes and chirality of a molecule. Even a helix of poly-glycine, the only achiral amino acid that is still a chiral macromolecule because it forms a helix and displays helical chirality [10]. Almost all the DNA in our bodies is a right-handed helix. Z-DNA hybrids are unusual and have some left-handed helices in them. A consequence, that circular dichroism is exhibited by most biological molecules, because of the dextrorotary (most sugars are that) and levorotary (all amino acids except glycine and cysteine molecules) they contain. Noteworthy as well is that a secondary structure will also impart a distinct CD to its respective molecules. The beta-sheet, alpha helix, double helix of nucleic acids and differentially wound regions of proteins has therefore CD spectral hallmark characteristic of their structures. The CD spectroscopy is used to study the secondary structure of the Bax protein [10].

5.5 Material and Methods

The lab started with making lipid stock mixtures of: POPC / POPE / TMCL lipids, which corresponded to 38.2 mg POPC 30.22 mg POPE and 31.56 mg of TMCL, a total
of 100 mg. After weighing, the three lipids were mixed in a round bottom flask of 100 ml and dissolved in ca. 20 ml of CHCl₃/MeOH mixtures. Evaporation took place without heating because these lipids are heat sensitive. The lipids were not water soluble, but we want to have homogeneous solutions, therefore, was used CHCl₃/MeOH was used as an organic solvent prior drying. This homogeneous solution was evaporated and a white lipid film remained. Upon resuspension in pure water, the suspension was shock frozen using liquid nitrogen and then freeze-dried, thereby generating a white lipid mixture powder for further use. In the beginning it was 100 mg total weight and the next day we got out 93.82 mg, that means, it was nearly 7 mg lost, by processing and getting the solution homogeneous. To run the DSC, it was needed to figure out how much of the lipid is needed to 4 mL of buffer solution to have 3 millimolar (mM). According to the calculation we received approximately 10.3 mg of lipid needed to mix with 4 mL of buffer solution to 3 mM. Exactly, it was 10.18 mg. DSC buffer contents: 25 mM (NaPi), sodium phosphate, 100 mM (KCl) KCl, 1 mM (EDTA) at pH = 7.4. The sample with 10.18 mg lipid stock and 3.95 mL DSC buffer was freeze-thawed (liquid–nitrogen and 40 C warm water bath) to provide homogenous multi-lamellar vesicles. Importantly we also had to use gentle sonication and vortexing. Freezing in nitrogen bath, heating, sonication and vortexing was done every time at every occasion where new trial would run in the DSC. The DSC machine was prepared using a cleaning procedure prior loading, and the machine would always be cleaned after each test run. The cleaned by 5% SDS solution and milli-Q water.

The DSC thermograms were obtained by scanning from 5°C to a maximum temperature of 50°C with a scan rate of 60°C/h. These settings were held throughout the laboratory trials. Before the lipid mixture was ready for analysis, 800 µL and 200 µL of the DSC buffers were mixed, poured into preparations tube where there was a small magnet and degassing was done in 10 minutes. The lipid mixture was then injected into the DSC sample cell and the pure buffer as reference in the reference cell. The machine did then three scans: 1. Quick up scanning 2. fast down scanning and 3. Slowly up scanning.

Gossypol mixture was made together with a lipid mixture to provide a 20:1 molar raio of lipids to gossypol with a volume of 1 ml and a 2 mM lipid concentration. Samples were produced by two different methods: First, the lipid and gossypol were dissolved together and freeze-dried overnight and got a new powder or other option is to use existing lipid suspension and adding a small volume of gossypol dissolved in DMSO (Dimethyl sulfoxide, a solvent). Option two was chosen to save time. Gossypol was dissolved in a small volume of 41.5 µL, of DMSO. Sonication and vortexing of the suspension a few times was a necessary step to get a homogeneous mixture. After incubating the suspension in the refrigerator over an hour, followed by freeze-thawing 1 mL of the suspension was degassed and loaded into the DSC machine. For the second batch, Gossypol without DMSO was used, but with the same model membranes and the same 1:20 Gossypol to lipid molar ratio. Gossypol weight was 0.35 mg and lipid powder weight was 11.51 mg. Complete suspension is obtained by extracting 11.51 mg lipid with HCCL₃ 1 MeOH 2:1 v/v. Later, it was mixed with 0.35 mg of Gossypol. Using a flow of gas nitrogen MeOH was removed from the sample and a small oily substance obtained. Under vacuum any remaining MeOH was removed by freez-drying upon resuspension of the mixture with pure water. The next day when it was weighed, it was 12.43 mg. It was more than what it was from the beginning, which resulted that there was water left in the suspension. Afterwards used 1.5 mL buffer to 12.43 mg of Gossypol / lipid suspension. The same method was used to getting DSC analysis graphs.

For NMR analysis a hydrated lipid mixture pellet without and with gossypol was needed. The lipid/gossypol 20:1 mixture was prepared as described above, however the hydration with deuterated water was kept at a 60:1 molar ratio of D2O/lipids. The
amount of total would be 30 mg. Gossypol was used at a "20:1 molar ratio," of lipids too drug and that this corresponds to a weight of 2.95 and the rest of lipid stock mixture. In order to later compare the difference is a mixture of gossypol / DMSO, in which 30 mg is the total and with the same conditions. For further analyzes were mixtures with 1:60 lipid + gossypol: D2O with 24.6 µL D 2 O, and 1.60 lipid / D 2 O (25:35 mL D 2 O) with a total weight of 20 mg lipid. The remaining suspensions were then, to packed into small MAS NMR rotors ready for use at the 500 MHz MAS NMR machine.

The Bax protein was checked by SDS-PAGE before the **CD-spectroscopy experiments** were carried out: containing gel plate plus the running buffer, where was loaded two Eppendorf with one 4 microliters Bax and 4 microliters staining buffer = Laemmli sample buffer and in the other 7.5 microliters staining buffer and 7; 5 microliters of protein Bax. New DSC samples were run at 4 mL DSC buffer with the following conditions: 1 mL of DMPC, DMPC / gossypol DMSO 100:1;; 50:1 and 20:1. Because of wrong calculation was made a run at 10:1, 5:1 and 2:1. Samples that were more "concentrated" and showed no clear graphs. Change the translation was made and the right was run again in the DSC.
6. Results and Discussion

6.1 PCPECL mixtures (lipid vesicles)

As seen in thermogram in Figure 4, the POPC/POPE/TMCL lipid membranes are undergoing a phase transition from a gel- to a liquid-crystalline phase at 21.7 °C.

![Graph showing phase transition](image)

*Figure 4: 3 mM lipid mixture (POPC/POPE/TMCL) in DSC--buffer*

In Figure 5 the thermogram is shown for the same lipid mixture but in the presence of Gossypol. In this graph we can see clear shift in the melting point compared with Figure 4. Here is the melting point of 23.87 degrees Celsius. The phase transition is also broader than the one seen in Figure 4 for Gossypol-free membranes.
Figure 5: 3 mM lipid (POPC/POPE/TMCL) mixed to 1:20 +/- Gossypol:Lipid (gossypol powder; 1:20), suspended in DSC-buffer.

The thermogram shown in Figure 6 is a mixture of 3 mM lipid vesicles to 1:20 +/- gossypol: lipid where gossypol is dissolved in DMSO solution and suspended in DSC buffer. DMSO concentration was 1% v/v. The +/- Gossypol notation means that the substance is a racemic mixture.

The graph in figure 5; shows the difference compared with Figure 4, but no difference compared with figure 5. Therefore, DMSO does not induce much interference compared to the DMSO-free preparation of gossypol-lipid systems.
Figur 6: 3 mM lipid (POPC/POPE/TMCL) mixed to 1:20 +/- Gossypol:lipid (using Gossypol dissolved in DMSO), suspended in DSC-buffer. Final DMSO-concentration = 1% (v/v)

A clear change can be seen in Figure 7, where various thermograms were superimposed thermogram A shift in the melting point and a broader slope are the two significant changes to be seen. These observations indicate that the presence of Gossypol induces changes in the membrane organization. In contrast, not much difference appears between gossypol lipid mixtures prepared without and with DMSO.
Figure 7: Superimposed thermogram with 3 mM POPC: POPE: TMCL 42:36:21, and 20:1 lipid:Gossypol

6.2 DSC: Gossypol titrated to DMPC

Thermograms of DMPC vesicles upon addition of Gossypol are seen in Figure 8 where DMPC/gossypol was measured at three different molar ratios, namely 100:1, 50:1 and 20:1, buffer.
Figure 8: Superimposed thermogram with: * Pure DMPC, * lipid: Gossypol 100:1 molar ratio, * lipid:Gossypol 50:1 and * lipid:Gossypol 20:1 molar ratio.

The graph shows thermograms identical to the one in Figure 8, but all thermograms have been scaled so that the intensity at the same temperature as the pure DMPC which is equal to one in this case.

Samples of DMPC/Gossypol at 20:1 molar ratios shows a melting point already at about 21°C, a subsequent melting. DMPC/Gossypol in ratios of 20:1 shows two peaks, which is difficult to interpret what happened, but it is equal to pure DMPC melting point.
Figure 9: The thermogram as in 5), but all thermograms has been scaled so that the "intensity at the same temperature as the pure DMPC" is = 1.

As in figure 8 and 9 and in Figure 10, are the same, but have mentioned from the beginning it was wrong ratios calculated. So there were circumstances 10:1, 5:1 and 2:1 of the stored thermal with pure DMPC, the lipids: gossypol.

The peaks are nicely aligned, but we cannot draw any conclusions that there are results that we desire. DMPC has no major impact. At fewer lipids such as 100:1 ratios, it happens more changes because Gossypol affects negatively the loaded vesicles.

Figure 6-9 is thermogram where 3 mM DMPC has been run with different concentrations of Gossypol (titration of Gossypol). The list below represents 5) and 6) experiments that drove; intention was to get the concentrations from 100:1 to 20:1, but it was wrong by a factor of 10. In figure 10, the concentration was too low and melted before DMPC melted.
Figure 11: The thermogram as in 4), but all thermogram has been scaled so that the "intensity at the same temperature as the pure DMPC" is = 1.

6.3 Effect of DMSO

DMPC 3mM, DMPC and Gossypol 3mM, titrated with ratios of 2:1 DMPC gossypol, and 3 mM DMPC + DMSP involved have not shown significant changes in the graphs in Figure 12.

The results show that DMSO induces an attenuation of the DMPC peak intensity, and the top becomes increasingly broad. But the effect of DMSO cannot explain the effects that are seen upon the titration of increasing amounts of Gossypol.

One addition of Gossypol leading to 2:1 lipid:Gossypol molar ratio corresponds to the largest an additive of DMSO is any sample. In all other experiments, the concentration of DMSO was scaled down, so we can say with certainty that the DMSO effect can never be larger than what we see in this case. Therefore, we can trust that titration experiments not particularly influenced by DMSO, but what we see is a clean Gossypol effect (Fig. 12).

DMSO is in the presence also in the aqueous solution, so it may be possible that some of the Gossypol dissolves in the water. This leads to a reduction of the Gossypol fraction remaining in the membrane. This may be possible because Gossypol has a stronger effect of expansion of the signal than the DMSO.
Figure 12: Superimposed thermogram with: 1) 3 mM DMPC, 2) 3 mM DMPC and Gossypol titrated to Granny the ratio 2:1 DMPC: Gossypol and 3) 3 mM DMPC + addition of DMSO corresponding to the volume that you set up in paragraph 2)

The effect of adding DMSO should reasonably induce a reduction in intensity in the corresponding thermograms, at least. It is therefore surprising that our thermogram with POPC / POPE / TMCL mixtures, shows a rather small signal when we have not added DMSO than when we have added DMSO and this complicates the interpretation.

6.4 CD running

We studied two Bax protein-containing samples, with either Gossypol being present or without it. The corresponding CD spectra are displayed in Figure 13 (without Gossypol) and figure 14 (with Gossypol), respectively. After the scanning temperature was raised up to 95 °C, an additional CD profile was measured (red curve in both Figures). They may not look like the same, but in figure 14, which also has Gossypol present, one can see the shape of the high temperature curve, which we would also have expected in Figure 13. Intensity trends look similar. After a down scan of the temperature (seen in Figure 15 and 16), similar trends are seen. That shows that maybe we would get a result identical in both samples if we were to repeat the experiment.

CD experiments showed the same results with and without Gossypol, at up scanning to 90° C and down scanning to 20° C.

This observation can be interpreted that the Gossypol optionally prettier graphics, but Gossypol charts can be interpreted as insufficient readable. One hypothesis we wanted
to test if the Bax may alter its stability and hence its secondary structure. But Bax did not convert into another secondary structure, which we would have seen.

Figure 13: CD spectra of Bax protein at 20° C and 95° C and 20° C after heat denaturation

Figure 14: CD running with 2:1 Gossypol:Bax under 20° C after heat denaturation. CD running with 2:1 Gossypol:Bax at 20° C and 95° C.
At wavelength 222 nm the CD signal as a function of temperature, with a scanning temperature of 20 degrees Celsius per hour (Figure 16). After the CD spectra had been collected, data were treated using a data processing program Origin 8.5, where the function SG smooth was applied to the data to filter out some noise and make the data more easily understandable. At lower wavelengths, showing less signal is observed and much of the secondary structure is visible. At the curve = 0 slope, that means no secondary structure is shown. From 95 °C down to 20 °C, it is less signals shown. Bax 20 degrees Celsius equivalent - 30 CD profile. Proteins are in their common secondary structure, no changes appear. At 95 degrees Celcius the entire curve ends up near the top (Fig. 13), then it appears less Bax, a part of the secondary structure has been lost.
Figure 16: Temperature dependent CD experiments at 222nm describe the temperature scans here of Bax.

Figure 17: CD running by Smoothed Y1 at same temperature; 20–95 °C of Bax and 20-95° C for Bax upon addition of Gossypol.
6.5 NMR--data

NMR provides information on Bax induced disturbances across the membrane. The 31P NMR shows detailed insight into the lipid interactions with protein. In figure 18 on can see 31P NMR spectra of pure lipid vesicles that produce broad NMR signals. These signals are separated by different isotropic chemical offsets. All spectra are referenced to DMPC signal at -0.9 ppm AT 308K, which means that the spectra are offset laterally (low field).

The NMR spectrum of the Gossypol containing lipid sample, shows most pronounced phosphor--lipid signals at: -0.97 ppm, -0.41 ppm, -0.19 ppm and 0.00 ppm respectively. In the pure lipids mixtures the signals have following chemical shift values: for POPC (-1.1 ppm), POPE (-0.57 ppm), and TMCL (-0.42 ppm).

![Figure 18: A Top with three 31P spectra (watched the signal from phosphorus in lipids divisions) From the top to the bottom: 1.DMPC at 308 K, 2. POPC/POPE/TMCL at 308 and 3. POPC/POPE/TMCL + Gossypol at 308 K](image)

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6.6 General Discussion

In a study written by Brian Leber1, Fei Geng, Justin Kale1 and David W. Andrews, [26] had as main goal to screen drugs that target Bcl-2 family members, with the main purpose to produce a "new" strategy to combat cancer. This was also the major motivation in our study presented here, to find drug candidates that could induce cell death by affecting the mitochondrial Bcl-2 protein family. Bcl-2 family has two members, Bax and Bak, who play an important role in the regulatory phase, where they are involved in the disruption of the outer mitochondrial membrane system. The result is a mitochondrial release component and binds to and activates APAF1 execution phase the caspase protein. Another group of Bcl-2 proteins (Bcl-xL, Bcl-w and MCL-1) prevents the activation of Bax and Bak and in turn prevented apoptosis [26].

That study has also focused on ABT-263, obatoclax and BH3 peptides Bcl-2 antagonists. Since Bcl-2 family proteins have been implicated in autophagy (a caspase independent process), results showed that accumulation of autophagic vesicles in the cells causes degradation of the ER, etc., According to the authors, there are also some cases, where autophagy can be cytoprotective, and it is therefore important inhibiting joint autophagy and facilitating apoptosis [26].

In their studies [26], they focussed on the developments of small molecules that change the function by directly binding to Bcl-2 proteins. ABT-263 and obatoclax was their drug candidates, which they used in practice, which have shown that these agents kill cancer cells where traditional chemotherapy does not work anymore. They also write that clinical studies show that one can apply doses, which patients can cope with [26].

In their study they had both Bax and Bak involved, but in this study because of time, we investigated only Bax how it affects lipid membranes. Interesting for this study was Gossypol and no such obatolax etc. Since Gossypol was the candidate selected, which could practically be ordered for our lab. But we had thought about making use of obatoclax also to later compare with Gossypol. Unfortunately obatoclax found, could not be ordered and used at the University, since the form that was only available as a radioactive component, and such substances cannot be used in a "normal" laboratory environment.

Gossypol, in turn, is a small molecule that inhibits Bcl-2/Bcl-xL/Mcl-1 who is in phase II trials as an adjuvant therapy for prostate cancer, writes Yang Meng, Wenhua Tang, Yao Dai, et al. [27]. Their goal was to analyze Gossypol molecular mechanism of action and how much capacity it has in treating prostate cancer. They acquired the evidence that the mitochondrial pathway of apoptosis in prostate cancer can occur apoptosis and inhibition of Gossypol. In turn by fluorescence resonance energy transfer assay it could be shown that Gossypol can modulate interactions of Bcl-X with Bax. Studies (Western blot and real-time PCR studies) have shown that by adding Gossypol, they may contribute to cell death to some extent. But there is a dose-dependent increase of proapoptotic BH3 - proteins that helps the cell death to occur. Their conclusion was that Gossypol through inhibition of the antiapoptotic protein Bcl- xL engaged in anti-tumor activity. In vitro and in vivo studies Gossypol was shown to increase in the activation of the protein and in turn, gives hope that Gossypol can be used for the treatment of prostate cancer. This was an advanced generating study, which is not true in comparison to that basic biophysical study we have done, but it supports our choice of candidate drug, Gossypol to have it as a new drug to treat prostate cancer [27].
There is also another study that has investigated the antitumor activity of Gossypol in prostate cancer. There Liang Xu, Yang Dajun, Shaomeng Wang, et al. [28] have examined the antitumor activity of Gossypol. It can give us a hypothesis that Gossypol may provide better cancer therapy and help patients to get a better effect on radiation, and helps to inhibit metastasis rewarding prostate cancer. The radiation induced apoptosis; they have tried to make tumor cells more sensitive to radiological treatment. They had DMSO solution as stock solution, while we had three lipid mixtures in our study. We had the objective to get the answer to our issues, while they had the in vitro experiments where Gossypol administered in cancer cells. We used the most common analytical methods (DSC), while they had a radiation clonogenic assay. In this study, took the cells in the well plates and exposed by 1 to 5 micromol / l Gossypol for irradiated adjustment one hour using 300 kV x-rays. After a few days of exposure, the plates were stained with crystal violet and counted the cancer colonies. Where by means of a reference sample could be if there were cells that had survived by Gossypol attack. Cell survival is measured with a linear - quadratic model, where the calculation was done as the ratio of the average inactive dose of the control divided by the average of inactive dose of processed cells. Their conclusion was that Gossypol enhances ORDER the induction and suppresses the growth of prostate cancer [28].

In general, these studies are not accurate compared to our study, and it is not any of the other studies either, as was mentioned. Because these studies are well-prepared and well-executed studies. We have performed a study, but there was not much time and not so many choices. We could not, for example, test the cancer cells, which would have been required for further studies. But cancer cell lines were not available in our lab (it was also not the main purpose of this project). Never the less we managed to come up with answers to the questions we wanted answered.

The purpose of this study was to learn the mechanism of cell-protecting mitochondrial membrane protein Bcl-2. We had access to Bax in our experiments, (it's a family of Bcl-2), and it was clear that a mechanism has been used where a drug candidate can be attached to a membrane protein to be able to execute its apoptosis inducing action. The hypothesis that Gossypol, could be such a candidate, can be said to be right. It has the ability to represent its effect on a membrane protein and its membrane, which is seen most clearly in our NMR experiments using mitochondrial model membrane system. The other biophysical approaches did not show such a clear result as our NMR experiments did. In the NMR spectra (Figure 18) one can clearly see, how the addition of Gossypol changes the organization of the lipid membrane system. The answer to the question, which of the drug candidates that may be conceivable, is clearly Gossypol, and through contining research can be administered and in the other studies obtained answers that it can bind and affect Bcl-2 protein family members: important for any future strategies to battle prostate tumors. Gossypol may be conceivable, as studies have also demonstrated at practical use, but require further clinical studies for this, which unfortunately cannot be done at a university.

Bax, an apoptotic member of the Bcl-2 protein family, promotes apoptosis upon activation by translocating to the MOM. There it stimulates a pore forming process which enables the release of apoptotic factors (such as cytochrome C) from the mitochondrial interiour. Normally, cytochrome C is attached to the phospholipid cardiolipin at the inner mitochondrial membrane but upon Bax activation and pore formulation, this complex dissociates and cytochrome C becomes released. Onset of this process can also be caused by intracellular oxidative stress (such as cancer drugs can induce), which generates oxidized phospholipids. These species can modulate membrane properties and the Bcl-2 proteins in a way which e.g. activates and attracts Bax to the mitochondrial membrane, a prerequisite for further apoptotic action [28]. The study was made at Umeå University was focused on cooperation between oxidized
phospholipids and BcX protein (one related to Bax). They also viewed the interaction with lipid vesicles, which could be seen in this study with. Unlike our study, they looked at a phospholipid PazePC that has an aldehyde group at the end of a truncated fatty acid chain. Our study had three different lipid mixtures. However the results did not allow us to identify a single lipid component solely responsible for the interaction with the Bax protein. The Umeå group used in their study, the same measurement technique as our study, namely DSC, NMR and CD spectroscopy. They have managed to connect PazePC with Bax protein, which showed more stable Bax protein. This study compared to their study, we can draw the conclusion that our obtained sktructural changes of Bax protein and these changes are due to changes in temperature. In our case, we had Gossypol instead of there PazePC used by them and a clear linkages between Bax together with Gossypol has failed to prove (more tangible results are needed), but a hypothesis, it emerged that they have an interconnection. The NMR measurement could observe some changes in it and their study phospholipids have undergone. The study they have conducted have proved that PazePC promotes the integration of Bax into the membrane and Bax associates to form a pore that allows the contents of mitochondria can come out and make sure that the cell dies. Such clear results could not be demonstrated in our study [29].

7. Conclusions and outlook

There is a large problem in cancer treatment that tumor cells can become immune to treatment induced apoptosis by an increased production of anti–apoptotic proteins, most prominent the mitochondrial membrane protein Bcl-2 itself. After understanding of the mechanism behind the cell protective function of Bcl-2 protein and by the study of the interaction of this protein with selected drug candidate Gossypol, we can draw a conclusion (based on our study). The aim was to understand the mechanism, and it was successful. We found a suitable candidate – drug Gossypol, which can be useful for cancer treatments.

The three analysis methods were selected in the study (DSC, NMR and CD), there were high hopes that DSC analyzes would provide better information, which could be interpreted and that Gossypol clearer, some this work on lipid membranes. However, the hypothesis that was initially used. Gossypol can become a new drug for prostate manipulation, and also for other cancers that are linked to the Bcl-2 protein family. But to count gossypol an active ingredient in a drug, it requires several clinical trials and several other experiments.

8. Acknowlegement

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