THE EFFECT OF DOXORUBICIN-ALBUMIN MAGNETIC NANOPARTICLES ON PROSTATE AND LUNG CANCER CELLS

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ABSTRACT

THE EFFECT OF DOXORUBICIN-ALBUMIN MAGNETIC NANOPARTICLES ON PROSTATE AND LUNG CANCER CELLS

Chemotherapy is a major therapeutic approach for treatment of a wide range of cancers. But unfortunately, this therapeutic approach has got a lot of side effects on healthy tissues as well as cancerous cells. Although doxorubicin is one of the most potent and widely used anticancer drugs, it has some side effects on healthy tissues, as well. Therefore, most researchers have studied various doxorubicin carrier systems for targeted delivery. For this purpose, doxorubicin loaded magnetic albumin nanospheres (M-DOX-BSA-NPs) were synthesized.

The main objective of this project was basically to determine the cytotoxic, apoptotic and cell cycle effects of BSA-NPs, M-BSA-NPs, DOX, BSA-DOX-NPs and M-DOX-BSA-NPs against prostate and lung cancer cells and compare them. Beside this, it is aimed to understand how cells look like before and after giving doxorubicin and M-DOX-BSA-NPs by using optical microscopy and compare them. Another objective of this project was to determine where M-DOX-BSA-NPs reach out into the cell by using concofal microscopy; and to explore proteins' differentiations between control groups and lung and prostate cancer cells in which DOX and M-DOX-BSA-NPs were applied, by proteomic studies.

In this study, the experimental results which were also supported by literature findings, demonstrated that M-DOX-BSA-NPs were more toxic than free doxorubicin, and this complex was more effective for prostate cancer cells than lung cancer cells.

As a result of this study, it was determined that this complex was synthesized as a target chemotherapy drug delivery system. Accordingly, this complex may affect to other cancer types, and it can be carried out by new drug designers and treatments.

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ÖZET

PROSTAT VE AKCİĞER KANSER HÜCRELERİNE DOKSORUBİSİN ALBÜMİN MAGNETİK NANOPARÇACIKLARININ ETKİSİ

Kemoterapi birçok kanser türü için temel tedavi yöntemidir. Fakat maalesef, kanser hücrelerini etkilediği gibi sağlıklı hücreleri de etkilemekte ve onlara zarar vermektedir. Doksorubisin en yaygın kullanılan anti-kanser ilacı olmasına rağmen, birçok yan etki içermektedir. Bu yüzden birçok araştırmacı doksorubisin taşıma sistemi üzerinde çalışmaktadır. Bu amaç doğrultusunda, doksorubisin ile birleştirilmiş magnetik ve albümin nanoparçacıkları sentezlenmiştir.

Bu projenin esas amacı, albümin nanoparçacıkları (BSA-NPs), albümin magnetik nanoparçacıkları (M-BSA-NPs), doksorubisin (DOX), doksorubisin ile birleştirilmiş albümin nanoparçacıkları (BSA-DOX-NPs) ve doksorubisin ile birleştirilmiş magnetik albümin nanoparçacıklarının (M-DOX-BSA-NPs) prostat ve akciğer kanser hücrelerine sitotoksik, apoptotik ve hücre döngüsü etkilerini belirlemek ve karşılaştırmak; ilaçlar verilmeden önce ve verildikten sonra hücre görüntülerini almak ve bu görüntüleri kıyaslamak; yeni sentezlenen bu birleşiğin konfokal mikroskopisi kullanarak hücrede nereye gittiğini belirlemek ve bu ilaç verilmiş hücreler ile kontrol grubu arasındaki protein farklılıklarını kıyaslayabilmektir.

Bu çalışmanın deneysel sonuçlarında M-DOX-BSA-NPs birleşiğinin, diğer denenen birleşiklere oranla daha toksik olduğu ve prostat kanser hücrelerini akciğer hücrelerine oranla daha fazla etkilediği gözlemlenmiştir ve bu sonuçlar literatür bulgularıyla desteklenmiştir.

Sonuç olarak yeni sentezlenen bu birleşiğin, hedefe yönelik kemoterapi ilacı olarak etki göstermiş olduğu belirlenmiştir. Bu doğrultuda diğer kanser türleri için de tedavi edilebilirliği mümkün olabilir ve yeni ilaç dizaynı üzerine çalışan araştırmacılara yeni ufuklar açabilir.

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CHAPTER 1

INTRODUCTION

1.1. Cancer

The body is made up of many types of cells. These cells grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. When cells become old or damaged, they die and are replaced with new cells.

However, sometimes this orderly process goes wrong. The genetic material (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division. When this happens, cells do not die when they should and new cells form when the body does not need them. The extra cells may form a mass of tissue called a tumor. In Figure 1.1 is showed the difference between normal cells and cancer cells in terms of growth control mechanism (NCI, accessed 2011).

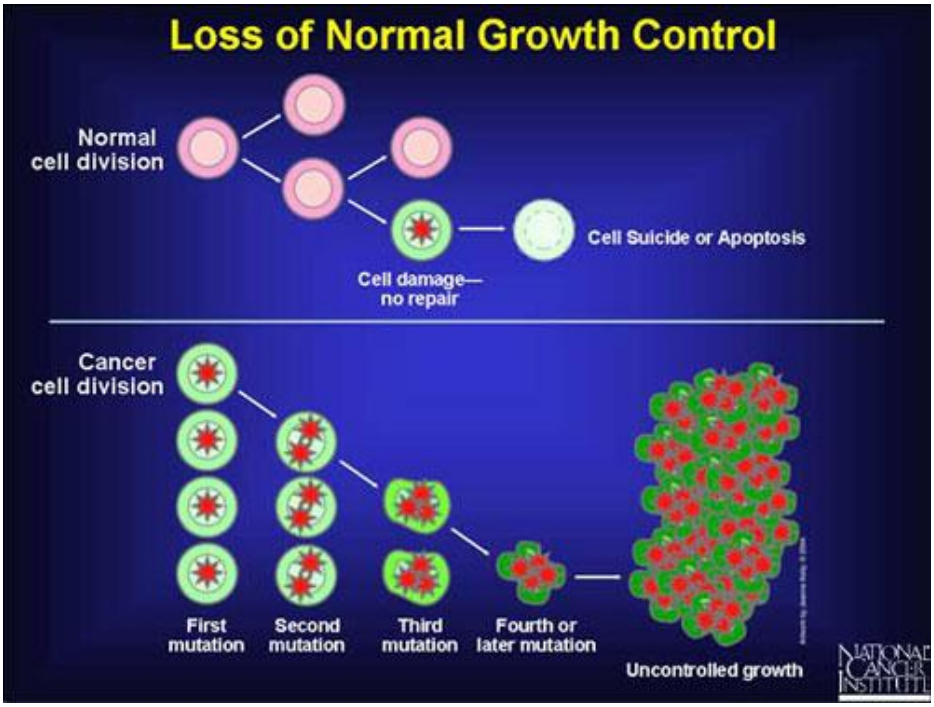

Figure 1.1. Loss of Normal Growth Control
(Source: NCI,2011).

Not all tumors are cancerous; tumors can be benign or malignant.

- Benign tumors aren't cancerous. They can often be removed, and, in most cases, they do not come back. Cells in benign tumors do not spread to other parts of the body.
- Malignant tumors are cancerous. Cells in these tumors can invade nearby tissues and spread to other parts of the body. The spread of cancer from one part of the body to another is called metastasis.

Cancer is not just one disease but many diseases. There are more than 100 different types of cancer. Most cancers are named for the organ or type of cell in which they start. For instance, cancer that begins in the lung is called lung cancer; cancer that begins in basal cells of the skin is called basal cell carcinoma.

Cancer types can be grouped into broader categories. The main categories of cancer include:

- Carcinoma: Cancer that begins in the skin or in tissues that line or cover internal organs.
- Sarcoma: Cancer that begins in bone, cartilage, fat, muscle, blood, vessels or other connective or supportive tissue.
- Leukemia: Cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.
- Lymphoma and Myeloma: Cancers that begin in the cells of the immune system.
- Central Nervous System: Cancers that begin in the tissues of the brain and spinal cord.

Cancer is a complex group of diseases with many possible causes. Firstly, genetic (biological) factors, age, obesity, declining immune system, exposure to chemicals and/or radiation, pesticides and virus are risk factors. Besides these, environment and lifestyle factors have a major impact to constitute the cancer. Relevant research demonstrates us that of all cancer-related deaths, nearly 5-10% are due to genetic factors, whereas the rest of it, nearly 90-95% are because of lifestyle and environment factors (Anand et al., 2008); (Irigaray et al., 2007).

Nowadays most of research groups are studying about cancer formation, progression and therapeutic approaches. However cancer still remains one of the leading

causes of death after the cardiovascular diseases in the world (Jemal et al., 2007). Approximately 12 million people worldwide caught this disease each year and unfortunately 7 million of these patients are dying in the same year. (Bilir, 2008); (Jemal et al., 2011b). The latest world cancer statistics report that the number of new cancer cases will increase to more than 15 million in 2020.

1.1.1. Lung Cancer

Lung Cancer is a disease characterized by uncontrolled cell growth in tissues of the lung. Most cancers that start in the lung, known as primary lung cancers, are carcinomas that derive from epithelial cell. The main types of lung cancer are small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). (Lung Carcinoma: Tumors of the Lungs) Lung cancer is a leading cause of cancer related mortality and has become a global healthy problem (Chiu et al., 2011). Also, it is responsible for 1,3 million deaths annually as of 2008 (Ferlay et al., 2010).

1.1.1.1. A549 Cell Line

A549 cells are adenocarcinomic human alveolar basal epithelial cells. There are squamous in nature and responsible for the diffusion of substances, such as water and electrolytes, across the alveoli of lungs ("A549 Cell Line: Human alveolar adenocarcinoma cell line -General Information," Retrieved 3 January 2012).

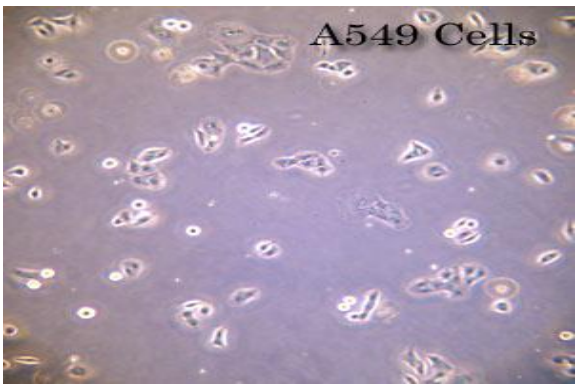

Figure 1.2. Image of A549 cell
(Source: Altogen Biosystem)

1.1.2. Prostate Cancer

Prostate cancer occurs in the prostate a gland in the male reproductive system. It tends to develop in men over the age of fifty (Siegel, Ward, Brawley, & Jemal, 2011). In addition to this, it is the most frequently diagnosed type of cancer, the second leading cause of cancer-related mortality in men in recent years and most common in the developed world with increasing rates in the developing world (Jemal et al., 2011a), (Baade, Youlden, & Krnjacki, 2009). Prostate cancer is classified as an adenocarcinoma or glandular cancer that begins when normal semen secreting prostate gland cells mutate into cancer cells. As prostate cancer is a mass of cells that can invade other parts of the body, it is considered a malignant tumor. Most commonly metastasizes to the bones, lymph nodes and may invade rectum, bladder, and lower ureters after local progression (Medecine, Archived from the orginal on 2011-04-48).

1.1.2.1. PC3 Cell Line

PC3 is human prostate cancer cell line. These cells do not respond to androgens, glucocorticoids, or epidermal or fibroblast growth factor. Besides this, PC3 has low testosterone-5-alpha reductase and acidic phosphates activity (ATCC, retrived 10 August 2012).

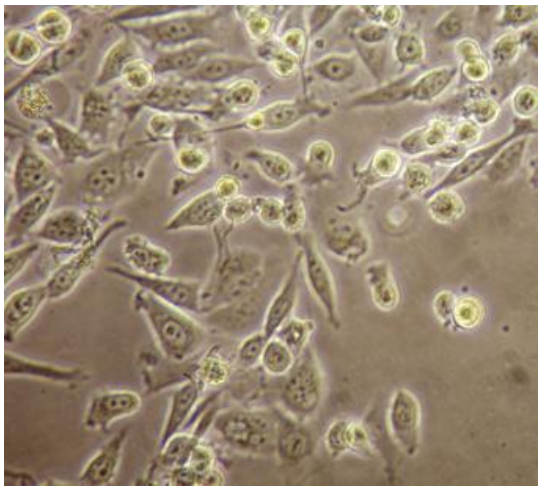

Figure 1.3. Image of PC3
(Source: addexbio.com)

1.2. Doxorubicin

Chemotherapy is a major therapeutic approach for the treatment of a wide range of cancers. Doxorubicin (anthracyclinic antibiotic) is one of the most potent and widely used anticancer drug. Figure 2.1. shows structure of doxorubicin. Its mode of action is complex and none completely understood but is believed to interact with cell DNA by intercalation and subsequent inhibition of biosynthesis (Shen, Li, Kohama, Oneill, & Bi, 2011). It is commonly used in the treatment of a wide range of cancer, including hematological malignancies, many types of carcinoma and soft tissue sarcomas.

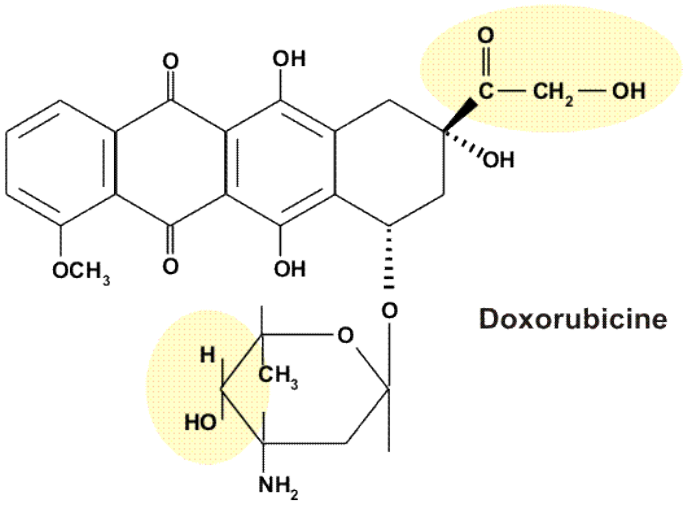

Figure 1.4. Structure of DOX
(Source: Oncoprof.net)

It is mention mechanism of action, doxorubicin interacts with DNA by intercalation and inhibition of macromolecular biosynthesis (Fornari, Randolph, Yalowich, Ritke, & Gewirtz, 1994); (Momparler, Karon, Siegel, & Avila, 1976). This inhibits the progression of the enzyme topoisomerase II which relaxes supercoils in DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replicating. The planar aromatic chromospheres portion of the molecule intercalates between two base pairs of the DNA, while the six-member daunosamine sugar sits in the minor groove and interacts with flanking base pairs immediately adjacent to the intercalation site, as evidenced by several crystal structures (Frederick et al., 1990); (Pigram, Fuller, & Hamilton, 1972).

In addition, doxorubicin is assumed to interfere with apoptosis and survival pathways during its cytotoxic action (Friesen, Herr, Krammer, & Debatin, 1996; Wang et al., 2004). Besides this, doxorubicin accumulates primarily in nuclei, but appreciable amounts are detectable in mitochondria and smaller amounts in plasma membranes, microsomes, and cytoplasm (Burns, North, Petersen, & Ingraham, 1988).

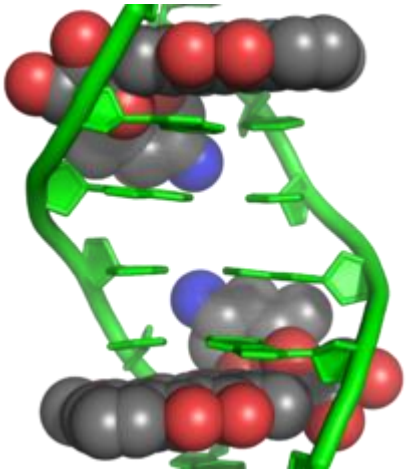

Figure 1.5. Cartoon diagram of two doxorubicin molecules intercalating DNA (Source: PDB).

Although chemotherapy is a major therapeutic approach for cancer patients, it has lots of side effects on healthy tissue as well as the cancerous cells. Unfortunately, doxorubicin has a number of undesirable side effects, too. Such as nausea, vomiting, hair loss, cardio toxicity and myelosupression that lead to a very narrow therapeutic index (Shen et al., 2011). Therefore, seeking for an effective tumor-targeted delivery system is one of the hottest topics in tumor chemotherapy (Sun et al., 2004). Because of this reason, most researchers have studied various doxorubicin carrier systems for targeted delivery.

1.3. Albumin

Albumin is the most abundant plasma protein and is synthesized in the liver where it is produced at a rate of approximately 0,7mg/h for every gram of liver (Kratz, 2008). Over the past decades, albumin has emerged as a versatile carrier for therapeutic

and diagnostic agents, rheumatoid arthritis and infectious diseases (Elsadek & Kratz, 2012). Figure 1.6 shows structure of albumin.

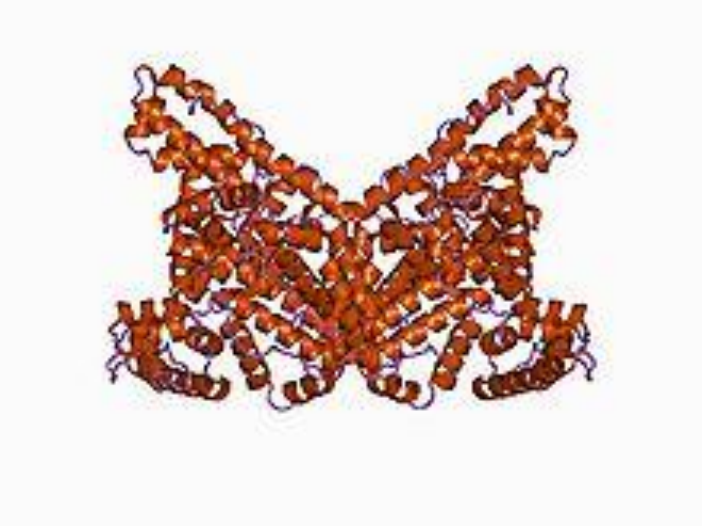

Figure 1.6. Structure of albumin
(Source: Sugio, Kashima, Mochizuki, Noda, & Kobayashi, 1999)

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Albumin is an attractive macromolecular carrier that has been shown to be biodegradable, nontoxic, metabolized in vivo to produce innocuous degradation products, non-immunogenic, easy to purify and soluble in water allowing ease of delivery by injection and thus an ideal candidate for nanoparticles preparation (Kratz et al., 1997); (Rahimnejad, Jahanshahi, & Najafpour, 2006).

Albumin has got three types.

- Ovalbumin (OVA): Ovalbumin is a highly functional food protein that is frequently used in food matrix design. OVA was chosen as a carrier for drug delivery owing to its availability and low cost, compared with other proteins. Moreover, OVA exhibits several interesting functionalities such as its ability to form gel networks and stabilization of emulsions and foams. Due to its pH and temperature sensitive properties, it has a high potential for use as a carrier for controlled drug release (Wongsasulak, Patapeejumruswong, Weiss, Supaphol, & Yoovidhya, 2010).
- Bovine Serum Albumin (BSA) : Bovine serum albumin is widely used for drug delivery because of its medical importance, abundance, low

cost, ease of purification, unusual ligand-binding properties and its wide acceptance in the pharmaceutical industry (Hu et al., 2006); (Tantra, Tompkins, & Quincey, 2010).

- Human Serum Albumin (HSA): HSA is the most abundant plasma protein with an average half-life odd 19 days. As it is extremely robust towards pH, temperature and organic solvents, it is not a standard protein. When HSA is broken down, the amino acids will provide nutrition to peripheral tissues. These properties as well as its preferential uptake in tumor and inflamed tissues, its ready availability, biodegradability and lack of toxicity make it an ideal candidate for drug delivery (Kratz, 2008); (Fasano et al., 2005).

Nanoparticulate delivery systems are investigated as a drug delivery in the pharmaceutical research. Nanocarriers not only protect a drug from degradation but also enhance drug absorption by facilitating diffusion through epithelium. Furthermore, they improve intracellular penetration and distribution. Additionally, one of the major advantages associated with the nanoparticulate systems is their ability to withstand physiological stress or improved biological stability and possibility of oral delivery which makes them more attractive as a drug delivery strategy than liposome (Kumar, 2000);.

Albumin-based nanoparticle carrier systems represent an attractive strategy, since a significant amount of drug can be incorporated into the particle matrix because of the different drug binding sites present in the albumin molecule (Patil, 2003). Due to the defined albumin primary structure and high content of charged amino acid, albumin-based nanoparticles could allow the electrostatic adsorption of positively or negatively charged molecules without the requirement of other compounds (Irache et al., 2005); (Weber, Coester, Kreuter, & Langer, 2000).

1.4. Magnetite (Fe_3O_4)

Magnetite is a ferromagnetic mineral, one of some iron oxides and a member of the spinal groups. Figure 1.7. shows structure of magnetic.

The specific tumor targeting of nanocarriers leads to better profiles of the pharmacokinetics and pharmacodynamics, controlled and sustained release of drugs, an

improved specificity, an increased internalization and intracellular delivery and, more importantly , a lower systemic toxicity (Ak G., 2012b).

Magnetic nanoparticles are being of great interest due to their unique purposes and have been actively investigated as the next generation of targeted drug delivery for more than thirty years (Chomoucka et al., 2010). In summary, for magnetic targeting first magnetic nanoparticle introduced in the body, then under the influence of external magnets, it is possible to guide nanoparticles to a particular targeted site (Arruebo, Fernandez-Pacheco, Ibarra, & Santamaria, 2007); (Danhier, Feron, & Preat, 2010).

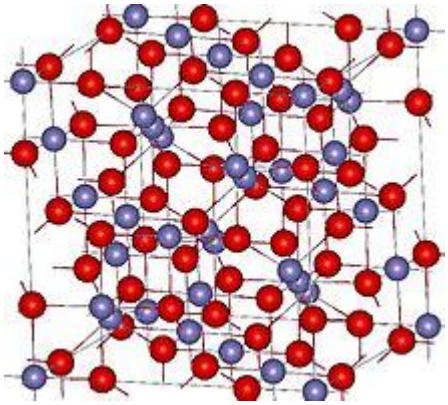


Figure 1.7. Crystal structure of magnetite
(Source: en.wikipedia.org)

1.5. Proteomics

Proteins are large biological molecules consisting of one or more chains of amino acids. Protein perform a vast array of functions within living organisms, including catalyzing metabolic reactions, replicating DNA, responding to stimuli, and transporting molecules from one location to another. Therefore, it is crucial to note that all research related to protein increase our understanding of their levels, interactions, functions, modifications, regulations, and localization in cells(Graves & Haystead, 2002).

Proteomics is the large-scale study of proteins, particularly their structures and functions (Anderson & Anderson, 1998; Blackstock & Weir, 1999). The word ‘proteomics’ is a blend of **prote**in and gen**omics**. Figure 1.8. shows biochemical interactions between genomics and proteomics.

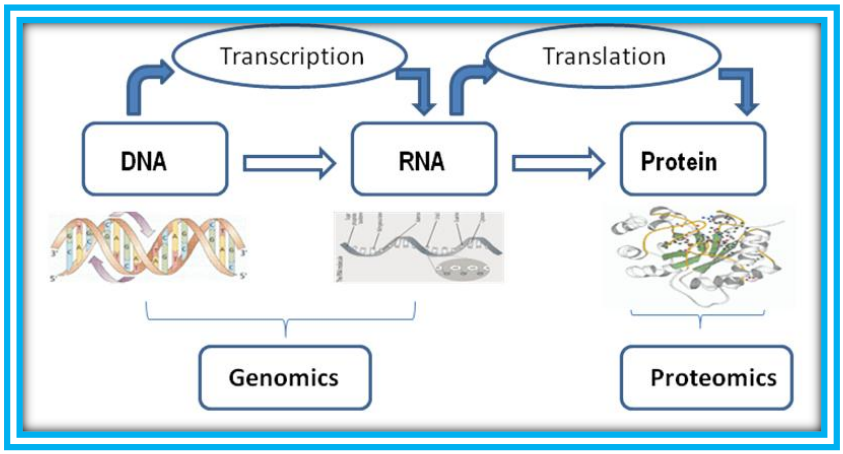


Figure 1.8. Biochemical interactions between genomics and proteomics
(Source: Turan, 2011)

Proteome is the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism or system. Proteomics is an interdisciplinary formed on the basis of the research and development of the Human Genome Project, is also an emerging scientific research and exploration of the proteome research from the overall level of intracellular protein composition, structure, and its own unique activity patterns. It is an important component of functional genomics.

While proteomics generally refers to the large-scale experimental analysis of proteins, it is often specifically used for protein purification and mass spectrometry.

1.5.1. Protein Profiling

Protein profiling is poised to provide unprecedented insight into biological events. Protein profiling can determine quantitative of protein levels and also indicate us unique expression patterns; diseased vs. healthy, treated vs. untreated, experimental vs. control; at the protein level when proteins from one cell type are compared with another. Besides these, it provides a much better understanding of an organism (Graves & Haystead, 2002).

Proteomics typically gives us a better understanding of an organism than genomics. The level of transcription of a gene gives only a rough estimate of its level of expression into a protein (Gygi, Rochon, Franza, & Aebersold, 1999). An mRNA

produced in abundance may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. Additionally, mRNA produced may go under rapid degradation that causes a reduction in translation, resulting in the production of less protein. In addition, some bodily fluids, such as serum and urine, have no source of mRNA under normal circumstances; therefore, proteomic technologies have emerged as an important addition to genomic studies (Graves & Haystead, 2002). Proteomics verifies the presence of a protein and provides a direct measure of the quantity present. An additional important reason that protein profiling is crucial is its power to analyze protein modifications. Many proteins experience post-translational modifications that profoundly affect their activities; for example some proteins are not active until they become phosphorylated. This method is that structural proteins can undergo phosphorylation during cell signaling and result in the protein becoming a target for binding to or interacting with a distinct group of proteins that recognize the phosphorylated domain. On the other hand, ubiquitination, methylation, acetylation, glycosylation, oxidation, sulfation, hydroxylation, nitrosylation, amidation are another post translation type of modification (Belle, Tanay, Bitincka, Shamir, & O'Shea, 2006). In addition to modifications, protein profiling can be also used to characterization regulatory mechanisms which are signal transduction can be turn normal cells into abnormal cells.

In conclusions, protein profiling provides determine structure and functions of an organism. Using of protein profiling in the study, we can compare with proteins of different states such as diseased vs. healthy or treated vs. untreated and understand of molecular mechanism.

1.5.2. Application of Proteomics

One of the most promising developments to come from the study of human genes and proteins has been the identification of potential new drugs for the treatment of disease. This relies on genome and proteome information to identify proteins associated with a disease, which computer software can then use as targets for new drugs. For example, if a certain protein is implicated in a disease, its 3D structure provides the information to design drugs to interfere with the action of the protein. A molecule that fits the active site of an enzyme, but cannot be released by the enzyme, will inactivate

the enzyme. This is the basis of new drug-discovery tools, which aim to find new drugs to inactivate proteins involved in disease. As genetic differences among individuals are found, researchers expect to use these techniques to develop personalized drugs that are more effective for the individual.(Vaidyanathan, 2012).

1.6. Aim of the Study

Although chemotherapy is a major therapeutic approach for cancer patients, it has lots of side effects on healthy tissue as well as the cancerous cells. Doxorubicin is a useful chemotherapy drug. However, it is so toxic not only cancer cells but also healthy cells. To increase of toxic effect of doxorubicin, it was modified with magnetic and albumin nanoparticles. For this purpose, doxorubicin loaded magnetic albumin nanospheres were synthesized by Assoc. Prof. Dr. Şenay Şanlıer Hamarat, Research Assist. Güliz Ak and Research Assist Habibe Yılmaz in Biochemical Department of Ege University. To purpose of this study to evaluate in vitro cytotoxicity effect of doxorubicin loaded magnetic albumin nanospheres against prostate and lung cancer cells.

Through this goal, the cytotoxic effects were determined by measuring the IC-50 value using MTT assay against PC3 and A549 cell lines. Apoptosis rate analysis and cell cycles were determined to effect different concentration of doxorubicin and doxorubicin loaded magnetic albumin nanospheres. Besides this, each of two cell lines were compared with the effect of doxorubicin loaded magnetic albumin nanospheres. Additionally, to take cell images, it was used optical microscopy. With this method it was determined images of PC3 and A549 as a control (no drug applied), as an applied of doxorubicin and as an applied doxorubicin loaded magnetic albumin nanospheres. Furthermore, to understand where doxorubicin loaded magnetic albumin nanospheres goes, it was used laser confocal microscopy.

Investigating protein profiles and understanding the dynamic alterations of cellular proteins are of great importance for diagnostic and therapeutic purposes in clinical settings. Therefore, proteomic studies were carried out. For this purpose, firstly protein isolation was performed from each of these cell lines untreated drugs as a control, treated doxorubicin and doxorubicin loaded magnetic albumin nanospheres, separately. Following to the extraction of total protein content, a comparison of protein

profiles in these different states was performed using mini and maxi sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS). And finally, it was determined differentially expressed protein spot (increased and/or decreased or appear and/or disappear) using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Proteomic study provides an excellent opportunity to find the differentiated proteins depending on doxorubicin loaded magnetic albumin nanospheres effect. For future perspective, differentially expressed protein spot can be identified by mass spectrometry and also it can be tried doxorubicin loaded magnetic albumin nanospheres in other cancer types.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Medias

Medias were listed in Appendix A.

2.1.2. Chemicals, Reagents and Solutions

Reagents and solutions were presented in Appendix B.

2.2. Methods

2.2.1. Cell Lines and Culture Conditions

The human prostate cancer (PC3) cell line was kindly obtained from Assoc. Prof. Dr. Kemal Sami Korkmaz in Ege Universitesi, Engineering Faculty, Department of Bioengineering and adenocarcinomic human alveolar basal epithelial cells (A549) cell line was provides by Prof. Dr. Serdar Ozcelik in Izmir Institute of Technology, Science Faculty, Department of Chemistry. The prostate cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with %5 fetal bovine serum (FBS) and 1% gentamicin sulfate, lung cancer cells were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) growth medium containing 10% fetal bovine serum (FBS) and 1% gentamicin sulfate at 37°C in 5% CO_2. Medium was refreshed every 3 days. In order to passage these cells, whole cell suspension was taken from tissue culture flask ($75cm^2$ or $150cm^2$) into a sterile falcon tube (50ml) and then centrifuged at 800 rpm for 5 minutes at room temperature. After centrifugation, the

supernatant was removed the tube and for solving, the pellet was added with 2 ml RPMI-1640 (10% FBS and 1% Gentamicin sulfate) for A549 and 2 ml DMEM (5% FBS and 1% Gentamicin sulfate) for PC3. After solving, it was transferred (1ml) into a sterile 75cm^2 or 150cm^2 filtered tissue culture flask and added 14ml for 75cm^2 flask, 24ml for 150cm^2 flask. Then it was incubated in humidified incubator 5% CO_2 at 37°C

2.2.2. Thawing the Frozen Cells

Cells (2ml) were removed from frozen storage at -80°C and quickly thawed in a water bath at 37°C so as to acquire the highest percentage of viable cells. When the ice crystals melted, the content was immediately transferred into a sterile filtered tissue culture flask (25cm^2) containing 5-6 ml of RPMI-1640 growth medium and incubated overnight at 37°C in 5% CO_2. After incubation, cells were passaged as mentioned before.

2.2.3. Freeze the Cells

Cells taken from tissue culture flask were centrifuged at 800 rpm for 5 minutes at room temperature. After centrifugation, the supernatant was carefully removed and the pellet was resuspended by the addition of RPMI-1640 or DMEM (depends on cell line) and 0.5ml dimethyl sulfoxide (DMSO). Then, gentle pipetting was applied and the cell suspension was transferred to the cryogenic vials (2 ml) by labeling. At the following step, these cryogenic vials were incubated at +4°C for 1 hour and then at -20°C for 1 hour and finally, lifted to freezing compartment -80°C for long-term storage

2.2.4. Cell Viability Assay

The assay primarily based on distinguishing dead cells from alive cells with the addition of trypan blue dye. ~900 µl of trypan blue dye was mixed ~100 µl of cells (10:1 ratio, volume/volume) in order to measure the viability of cells. When cells were treated with trypan blue dye, viable cells would be normally impermeable to it, whereas dead cells would permeate by virtue of breakdown in membrane integrity. Thus, cells

can be observed as unstained cells that are alive or blue stained cells that are dead under a microscope. By applying this assay, cells were counted using a hemocytometer in the presence of trypan blue solution, under microscope. Then the percentage of viable cells was calculated. Cell viability assay was conducted before each experiment.

2.2.5. Cell Proliferation Assay

The cytotoxicity of various concentrations of the albumin nanoparticles (BSA-NPs), magnetic albumin nanoparticles (M-BSA-NPs), doxorubicin, DOX incorporated albumin nanoparticles (DOX-BSA-NPs), doxorubicin loaded magnetic albumin nanoparticles (M-DOX-BSA-NPs) were measured using the MTT. The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye. This assay is based on the capacity of mitochondrial dehydrogenase enzymes. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells (Mosmann, 1983). A solubilization solution (usually dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed. MTT is a sensitive, quantitative and reliable colorimetric assay to measure viability, proliferation, and activation of cells. In addition, MTT assay is used to quantitative cytotoxicity.

MTT-based in vitro cytotoxicity assay was performed to investigate and compare with effects of free DOX, DOX-BSA-NPs, M-DOX-BSA-NPs, BSA-NPs and M-BSA-NP against PC3 and A549 according to the "Design of Folate-Linked Liposomal Doxorubicin to its Antitumor Effect in Mice" article (Yamada A., 2008). 95 µL of cell suspension was inoculated into 96-well micro culture plates at $1x10^4$ cells density per well in culture media. Cells were incubated for 24 h. BSA-NPs, M-BSA-NP, DOX-BSA-NPs and M-DOX-BSA-NPs were dissolved in dimethyl sulfoxide (DMSO) and dilute at appropriate concentrations with the culture medium but free DOX was dissolved in phosphate buffered saline (PBS) and filter sterilized and for diluting was used of PBS. After 24 h, 5 µL of these compounds were added and final concentrations

were 50, 25, 10, 5, 1, 0.5 and 0.1µg/ml for A549 and 10, 5, 1, 0.5, 0.1, 0.05, 0,02 µg/ml for PC3. Untreated cells were used as a control groups. After that for fixing, 1% DMSO was added all well. When these operations were finished, cells were incubated further for 48 h in CO_2 incubator at 37 °C. After the incubation, the medium was removed and cells washed with phosphate-buffered saline (PBS). %10 MTT solution (5.0mg/ml in PBS) was prepared with RPMI and DMEM for A549and PC3 cell lines, respectively. 100 µL MTT solution was added to each well and plates were incubated at 37 °C for 4 h in dark and then plates were centrifuged at 1800 rpm for 10 minutes at room temperature to avoid accidental removal of formazan crystals. After removing MTT, 100 µL DMSO was added to each well to dissolve the formazan crystals and then 96-well plates put in shaker for 15 min. Finally, the absorbance was determined using plate reader at a wavelength of 540 nm. Each composite was assayed three times in triplicate. As using "GraphPad Prism 5" software program, IC50 values (the concentration of drug that inhibits 50% of cell proliferation as compared to untreated control) of each compound was calculated. Three independent assays were repeated (n=3).

2.2.6. Apoptosis Analysis

Also called programmed cell death or cell suicide, apoptosis is an integral and necessary part of life cycle of organisms. In the human body, about a hundred thousand cells are produced every second by mitosis and a similar number of them die by apoptosis (Vaux, 1999)). The apoptotic mode of cell death can be described as a process which plays an important role in the improvement and homeostasis of multicellular organism and in the regulation and maintenance of the cell populations in tissues upon pathological and physiological conditions (Hengartner, 2000; Jacobson, Weil, & Raff, 1997; Leist & Jaattela, 2001; Meier, Finch, & Evan, 2000).

To investigate the apoptotic effects of free DOX and M-DOX-BSA-NPs against A549 and PC-3 cell lines these drugs were tested by using Annexin V- FITC Detection Kit. $1x10^5$ cells/well were seeded in a 6-well plate in 1,80 ml growth medium and incubated at 37°C in 5% CO_2 for 24h. After incubated 20 µl added free DOX and M-DOX-BSA-NPs, dissolved in PBS and DMSO, respectively. Final concentrations were 1 nM, 10 nM, 100 nM, and 1000nM and incubated at 37°C in 5% CO_2 for 48 hours. Untreated cells were used as a control groups. After incubation, the cells were taken to a

falcon tube and centrifuged at 800 rpm for 5 minutes. When centrifugation finished, the pellet was dissolved in 5 ml of PBS and centrifuged again. After that, the pellet was resuspended in 200 µL of binding buffer. 2 µL of annexin V-FITC and PI were added. The stained cells were incubated for 15 minutes at room temperature. And finally, the mixture was determined by flow cytometer (FACSCANTO, BD).

2.2.7. Cell Cycle Analysis

In order to determine the cell cycle effects of the free DOX and M-DOX-BSA-NPs against PC3 and A549 cells, these drugs were tested by propidium iodide staining. $5x10^5$ cells/well were seeded in a 6-well plate in 1,80 ml growth medium and incubated at 37°C in 5% CO_2 for 24h. After incubated 20 µl added free DOX and M-DOX-BSA-NPs, dissolved in PBS and DMSO, respectively. Final concentrations were 1 nM, 10 nM, 100 nM, and 1000nM and incubated at 37°C in 5% CO_2 for 48 hours. Untreated cells were used as a control groups. After incubation, the cells were taken to a falcon tube and centrifuged at 1200 rpm for 10 minutes. When centrifugation finished supernatant was removed and pellet was dissolved in1ml PBS and than 4ml PBS was added on ice. The cell suspension was centrifuged again. The pellet resuspended in 1ml PBS and fixed by adding 4 ml ethanol slowly on ice. All tubes were carried out low speed vortex. These tubes were incubated at -20°C at least. After incubation, cell suspension was centrifuged at 1200 rpm for 10 minutes at 4°C. The pellet was solved in 1 ml PBS and added 4 ml PBS and centrifuged again. The pellet was resuspended in 200 µl 0,1 % Triton X-100 in PBS. 20µL RNase A (200 µg/ml) was added to cell suspension and cells were incubated in 37°C in 5% CO_2 for 30 min. After 30 minutes 20 µl PI (1mg/ml) was added and incubated at room temperature for 15 min. The cell cycle distribution was determined by flow cytometer, and data were analyzed by ModFit software. It were collected at least 10,000 events for each sample.

2.2.8. Imaging of Optical Microscopy

A549 and PC3 cells were seeded in 96-well plates for image of optical microscopy. After waiting overnight, 1µg/mL free DOX and M-DOX-BSA-NPs were added and incubated 48h. And also it was prepared a control which was not given any

drug. After incubating, they were examined by using optical microscopy (OLYMPUS-CKX41).

2.2.9. Imaging of Confocal Microscopy

In order to understand where M-DOX-BSA-NPs complex go to the in cell, we used confocal microscopy. Firstly, lamella was placed in a 6-well plate and then PC3 and A549 were seeded in 2 ml growth medium and incubated for 24h. After waiting overnight, 1μg/mL M-DOX-BSA-NPs were added and incubated 2 h. After 2 h, lamella was carefully taken and fixed on lame by means of using nail polish. Finally, the lame was examined by using confocal microscopy.

2.2.10. Total Protein Extraction from PC3 cells

So as to extract the total proteins PC3 cells, firstly $5x10^6$ cells were seeded in $25m^2$ flask and incubated at 37°C in 5% CO_2 for 24h. After 24 hours, 505.7ng/ml free DOX (which is the IC-50 on PC3 cells) and 34,5ng/ml M-DOX-BSA-NPs (which is the IC-50 value of on PC3 cells) were added and incubated at 37°C in 5% CO_2 for 48h. After incubation, all the cells were lifted by using scraper, BD falcon 18 cm. Whole cell suspension was taken from tissue culture flask into a sterile falcon tube and then centrifuged at 1000 rpm for 10 minutes at room temperature, separately. After centrifugation, the supernatant was removed and the pellet resuspended in 95 μl of Lysis Buffer (containing Sodium Chloride, Tris-HCl, EDTA, %1 Triton X-100 and ultrapure water) and added 5 μl protease inhibitor before incubated on ice for 15 minutes and transferred prechilled sterile 1,5 ml ependorf tubes. The samples are homogenized by passing at least 5 times through insulin injector. After it was centrifuged at 12000 rpm for 15 minutes, it was taken supernatant into another prechilled ependorf tubes. Supernatant, which is the total protein of our samples, used for further studies (SDS, 2D-PAGE).

2.2.11. Total Protein Extraction from A549 cells

So as to extract the total proteins A549 cells, firstly $5x10^6$ cells were seeded in $25m^2$ flask and incubated at 37°C in 5% CO_2 for 24h. After 24 hours, 9134ng/ml free DOX (which is the IC-50 on PC3 cells) and 1683ng/ml M-DOX-BSA-NPs (which is the IC-50 value of on A549 cells) were added and incubated at 37°C in 5% CO_2 for 48h. Other assays are as above mention.

2.2.12. Bradford Protein Assay for Protein Determination

Protein concentrations were measured by Bradford assay by using bovine serum albumin (BSA) as the standard. The Bradford protein assay is one of the spectroscopic analytical methods that are utilized to find out the total protein concentration of a sample (Bradford, 1976). The method is also known as colorimetric assay because the addition of the sample causes color changes from brown that is cationic form to blue which is anionic form. Moreover the color of the sample becomes darker and the measured absorbance raises with the protein concentration increases. Basic principle of this method is absorption shift from 470 nm to 595 nm. When Coomassie Brilliant Blue G-250 (CBB G-250) dye binds to proteins through Van der Waals forces and hydrophobic interactions from its sulfonic groups, absorption occurs. In general, binding of dye to proteins becomes lysine, arginine and histidine residues, it also can bind tyrosine, tryptophan and phenylalanine weakly.

To obtained BSA curve, the preparation of stock BSA standard. 180µl coomassie Brilliant Blue was added 96 well-plates and added 20µl BSA solution (concentration 2, 1, 0.5, 0.1mg/ml) and incubated at room temperature for 10 minutes. For control group was added only 20 µl PBS and 180µl coomassie Brilliant Blue. For our samples, 20µl was taken and added 180µl coomassie Brilliant Blue. After that these mixture in 96 well-plates was allowed to incubate at room temperature for 10 minutes. For control group was added only 20 µl lyses buffer and 180µl coomassie Brilliant Blue. Finally, the absorbance of these plates was measured at 595 nm by plate reader.

Protein concentration of the samples was determined by using Bradford method. Besides this, it was determined by using nanodrop.

2.2.13. Mini and Maxi SDS-PAGE Analysis

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, describes a technique to separate proteins according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge) and no other physical feature. SDS is an anionic detergent applied to protein sample to linearize proteins and to impart a negative charge to linearized proteins. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

Firstly, samples were prepared. All of samples were diluted with lysis buffer and mixture sample buffer and final concentration was occurred 1 mg/ml. After that separation gel was prepared 10 ml for mini SDS, 80 ml for maxi SDS, poured carefully and incubated for 1 hour. After incubated stacking gel was added and incubated for 1 hour, again. After the samples were loaded 10 ml for mini, 20 ml for maxi (1mg/ml final concentration of proteins), added running buffer and electrophoresis was started at constant current at 32mA for 30 minutes and fallowed by 50mA for 2hour for mini but for 5-6 hour for maxi SDS. When it reached the bottom of the gel, electrophoresis was stopped. After that, the gel was stained with Coomassie Brilliant Blue and incubated overnight.

Another day, the gel was carried out distaining for 2 hours and this assay was repeated. And finally, gels were scanned and photographed by OLYMPUS-DP25 Digital camera system.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cytotoxic Effects on PC3 Cell Line

The cytotoxicity of various concentrations (10, 5, 1, 0.5, 0.1, 0.05, 0.02µg/ml) of the albumin nanoparticles (BSA-NPs), magnetic albumin nanoparticles (M-BSA-NPs), doxorubicin, DOX incorporated albumin nanoparticles (DOX-BSA-NPs), doxorubicin loaded magnetic albumin nanoparticles (M-DOX-BSA-NPs) were investigated. Using GraphPad Prism 5" software program Figure 3.1. and 3.2. were obtained. According Figure 3.1 and 3.2., neither BSA-NPs nor M-BSANPs effect on PC3 cells. Cell viability is approximately %100. These results show us that albumin and magnetic nanoparticles have not toxic effects.

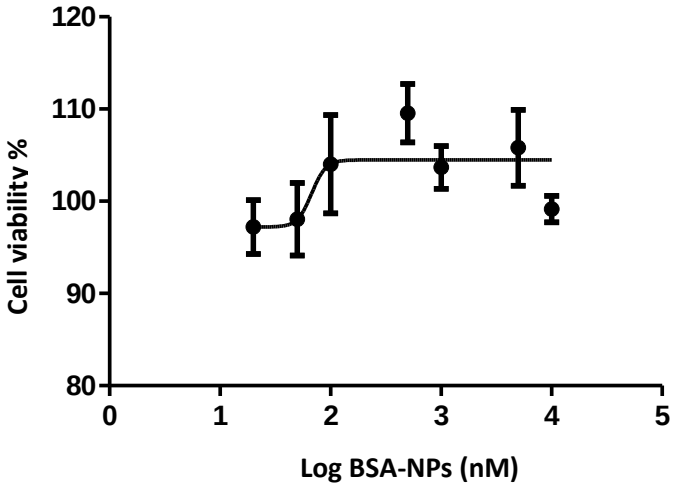


Figure 3.1. The effect of cytotoxicity of BSA-NPs against PC3 cells

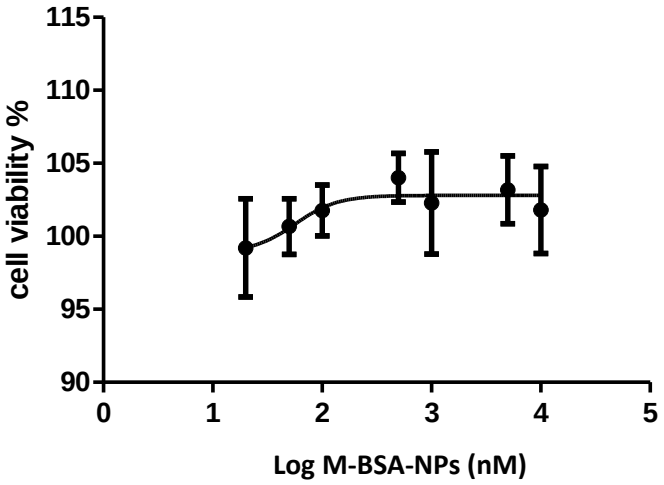


Figure 3.2. The effect of cytotoxicity of M-BSA-NPs against PC3 cells

On the other hand, when free doxorubicin, DOX-BAS-NPs and M-DOX-BSA-NPs were applied on PC3 cells, cell viability increased fast. Using GraphPad Prism 5 software program, all of these drugs was calculated IC50 values. Figure 3.3 indicate that IC50 value of free DOX is 505.7ng/ml for PC3 cells.

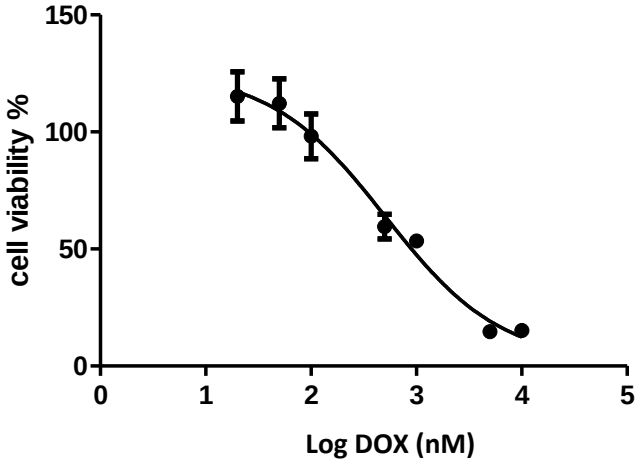


Figure 3.3. The effect of cytotoxicity of free DOX against PC3 cells.

As albumin is an attractive drug carrier, when DOX incorporated albumin nanoparticles (DOX-BSA-NPs) was applied, we expected that drug release was slowly

and thus, IC50 value was smaller than free doxorubicin. As we look at Figure 3.3, we can see that DOX-BSA-NPs is more effective than free doxorubicin. IC50 value of DOX-BSA-NPs was found 136,3ng/ml for PC3 cells

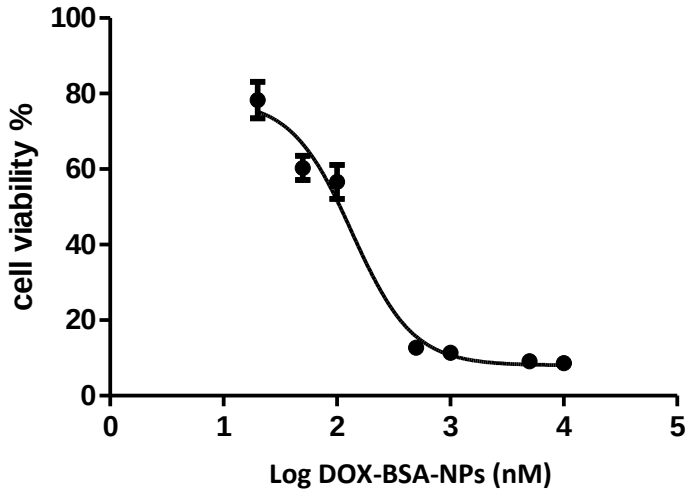


Figure 3.4. The effect of cytotoxicity of DOX-BSA-NPs against PC3 cells

It was synthesized doxorubicin loaded magnetic albumin nanoparticles (M-DOX-BSA-NPs) in order to increase side effect of doxorubicin. Therefore, it was expected most toxic and lowest IC50 value. Figure 3.5. was occurred and according to this figure, IC50 value of M-DOX-BSA-NPs was found 34.5 ng/ml for PC3 cells. The results indicate that M-DOX-BSA-NPs is the most toxic. According the results, this drug carrier system increased side effects of doxorubicin.

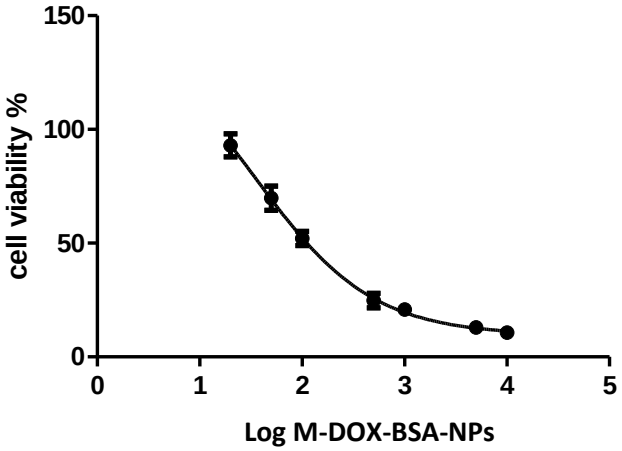


Figure 3.5. The effect of cytotoxicity of M-DOX-BSA-NPs against PC3 cells

Beside this, when it was looked at previously studies, this compound was seen more toxic against PC3 cells. For instance; "Quantum Dot−Aptamer Conjugates for Synchronous Cancer Imaging, Therapy, and Sensing of Drug Delivery Based on Bi-Fluorescence Resonance Energy Transfer" article was performed, (Bagalkot et al., 2007). According this study, IC_{50} values of quantum dot (QD) alone, quantum dot (QD)−aptamer(Apt)−doxorubicin (Dox) conjugate [QD−Apt(Dox) and free doxorubicin against PC3 cells were found 1,6µM, 1,6µM and 5µM, respectively, (Bagalkot et al., 2007). The other article was "Comparative effects of thermosensitive doxorubicin-containing liposomes and hyperthermia in human and murine tumors", (Yarmolenko et al., 2010). As regards this study, an IC50 value of thermosensitive liposome encapsulating doxorubicin (LTSL-DOX) against PC-3 cells was found 151nM, (Yarmolenko et al., 2010). The results indicated that quickly release of doxorubicin was prevented by magnetic albumin nanoparticles. So that, M-DOX-BSA-NPs was found the least IC_{50}.

3.2. Cytotoxicity Tests on A549 Cells

The cytotoxicity of various concentrations (50, 25, 10, 5, 1, 0.5 and 0.1µg/ml µg/ml) of the albumin nanoparticles (BSA-NPs), magnetic albumin nanoparticles (M-BSA-NPs), doxorubicin, DOX incorporated albumin nanoparticles (DOX-BSA-NPs),

doxorubicin loaded magnetic albumin nanoparticles (M-DOX-BSA-NPs) were investigated against A549. Using GraphPad Prism 5" software program Figure 3.6. and 3.7. were obtained. According Figure 3.6 and 3.7., neither BSA-NPs nor M-BSANPs effect on A549 cells. Cell viability is approximately %100. These results show us that albumin and magnetic nanoparticles have not toxic effects on A549 cell, like PC3 cells.

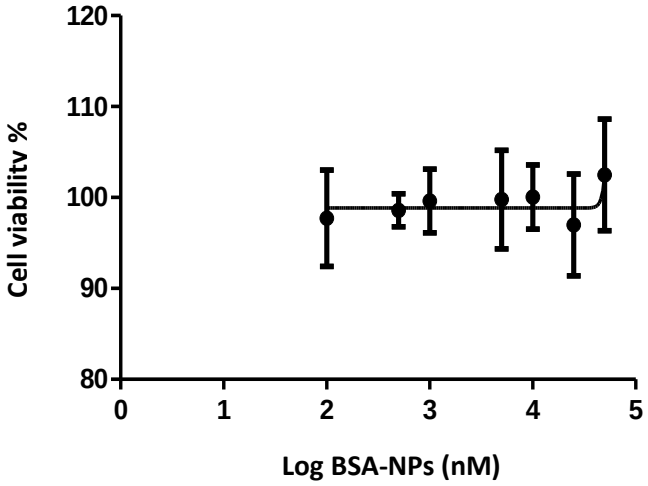


Figure 3.6. The effect of cytotoxicity of BSA-NPs against A549 cells

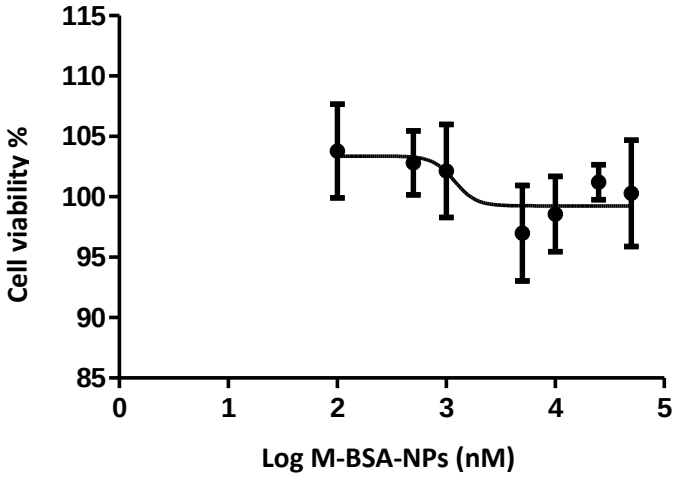


Figure 3.7. The effect of cytotoxicity of M-BSA-NPs against A549 cells

On the other hand, when free doxorubicin, DOX-BAS-NPs and M-DOX-BSA-NPs were applied on A549 cells, cell viability increased fast. Using GraphPad Prism 5" software program, all of these drugs was calculated IC50 values. Figure 3.8 indicate that IC50 value of free DOX is 9134ng/ml for A549 cells.

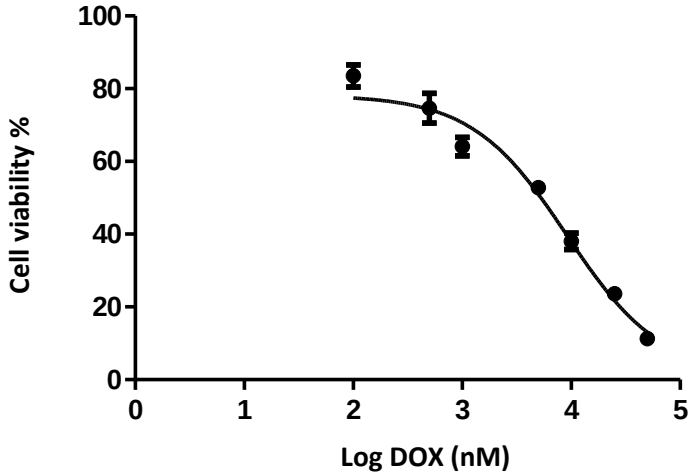


Figure 3.8. The effect of cytotoxicity of free DOX against A549 cells.

As albumin is a attractive drug carrier, when DOX incorporated albumin nanoparticles (DOX-BSA-NPs) was applied, we expected that drug release was slowly and thus, IC50 value was smaller than free doxorubicin, as described above. As we look at Figure 3.9, we can see that DOX-BSA-NPs is more effective than free doxorubicin. IC50 value of DOX-BSA-NPs was found 3238ng/ml for A549 cells.

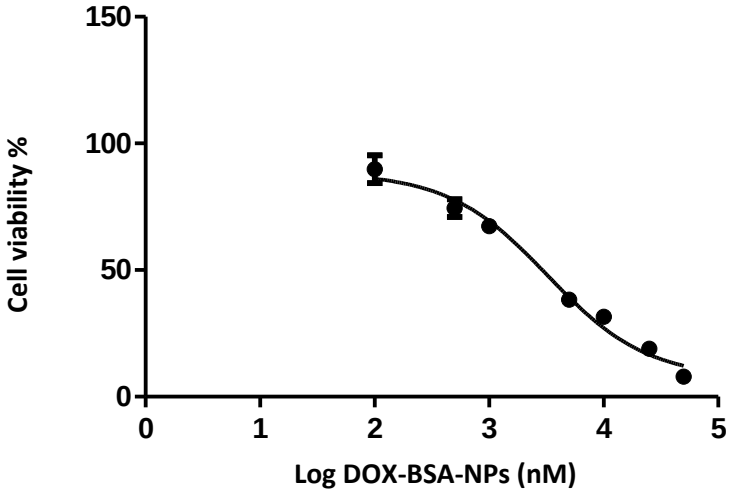


Figure 3.9. The effect of cytotoxicity of DOX-BSA-NPs against A549 cells.

It was synthesized doxorubicin loaded magnetic albumin nanoparticles (M-DOX-BSA-NPs) as a target chemotherapy drug. Therefore, it was expected most toxic and lowest IC50 value. Figure 3.10. was occurred and according to this figure, IC50 value of M-DOX-BSA-NPs was found 1683ng/ml for A549 cells. The results indicate that M-DOX-BSA-NPs are the most toxic. According these results demonstrate that the M-NPs can selectively target to cancer cells.

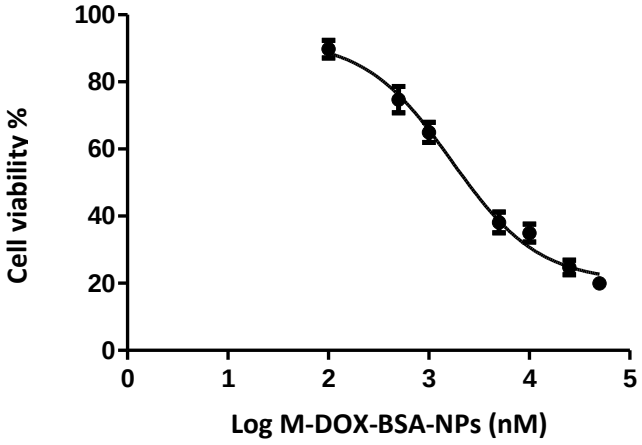


Figure 3.10. The effect of cytotoxicity of M-DOX-BSA-NPs against A549 cells.

On the other hand, previously studies supported that this compound was more toxic, too. First article was “Folate-receptor-targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin-PEG-folate conjugate” (Yoo H. S., 2004). According to this, IC_{50} values of DOX nano-aggregates and DOX/FOL nano-aggregates against A549 were found 9.0μM and 9.2 μM, respectively, (Yoo H. S., 2004). Another article was “Evaluation of folate conjugated pegylated thermosensitive magnetic nanocomposites for tumor imaging and therapy” (Rastogi S., 2011). In this study, the cytotoxicity effects of polymer nanoparticles, iron-polymer nanoparticles, folate conjugated iron-polymer nanoparticles, doxorubicin loaded folate conjugated iron-polymer nanoparticles and free doxorubicin against A549 cells was compared with each other. Toxicity was found higher for free doxorubicin. The results indicated that quickly release of doxorubicin was prevented by magnetic albumin nanoparticles. Thus M-DOX-BSA-NPs were occurred more toxic and new alternative compound as a literature, too.

Beside this, M-DOX-BSA-NPs were found more effective for PC-3 cells than A549 cells. For reason of this, lung cancer cells are unfortunately strong cancer type.

3.3. Apoptosis Rate on PC3 Cells

To investigate the apoptotic effects of free DOX and M-DOX-BSA-NPs against PC-3 cells these drugs were performed different concentrations (1, 10, 100, 1000 nM) and untreated cells as a control group by using flow cytometry based annexin V staining. Firstly it was investigated on doxorubicin. The percentage of cell phase composition was calculated and shown in Figure 3.11. According to this result, when it was applied 1000nM concentration of doxorubicin, region of Q3 (live) was no cells. Most of cells passed necrosis (necrosis is a form of cell injury that results in the premature death of cells in living tissue, (Proskuryakov, Konoplyannikov, & Gabai, 2003)) area. On the other hand, when it was applied 1 and 10nM concentration of doxorubicin, region of Q3 (live) was nearly amount of control.

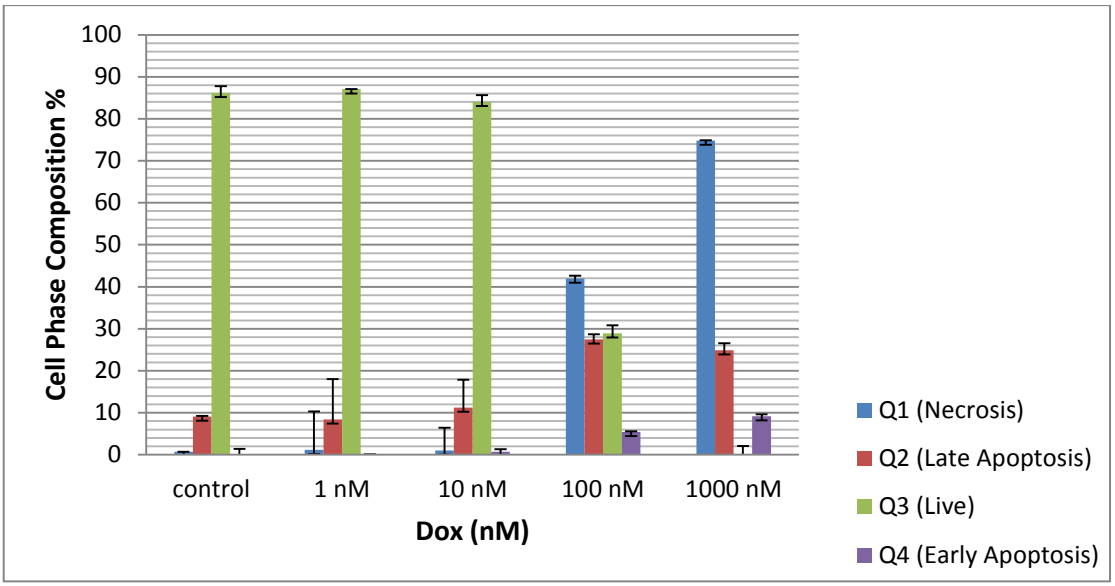


Figure 3.11. Quantification analysis of applied DOX apoptosis of PC3 cells.

Secondly, it was investigated effect of M-DOX-BSA-NPs. The percentage of cell phase composition was calculated and shown in Figure 3.12. According to this result, when it was applied 1000nM concentration of M-DOX-BSA-NPs, region of Q3 (live) was no cells. In contrast of doxorubicin most of cells passed Q2 late apoptosis (Apoptosis is a naturally occurring programmed and targeted cause of cellular death, (Proskuryakov et al., 2003)) area. On the other hand, when it was applied 1nM concentration of M-DOX-BSA-NPs, region of Q3 (live) was nearly %70 amount of cell. This results show that M-DOX-BSA-NPs is more toxic than free doxorubicin. Besides this, this experiment supports MTT assay.

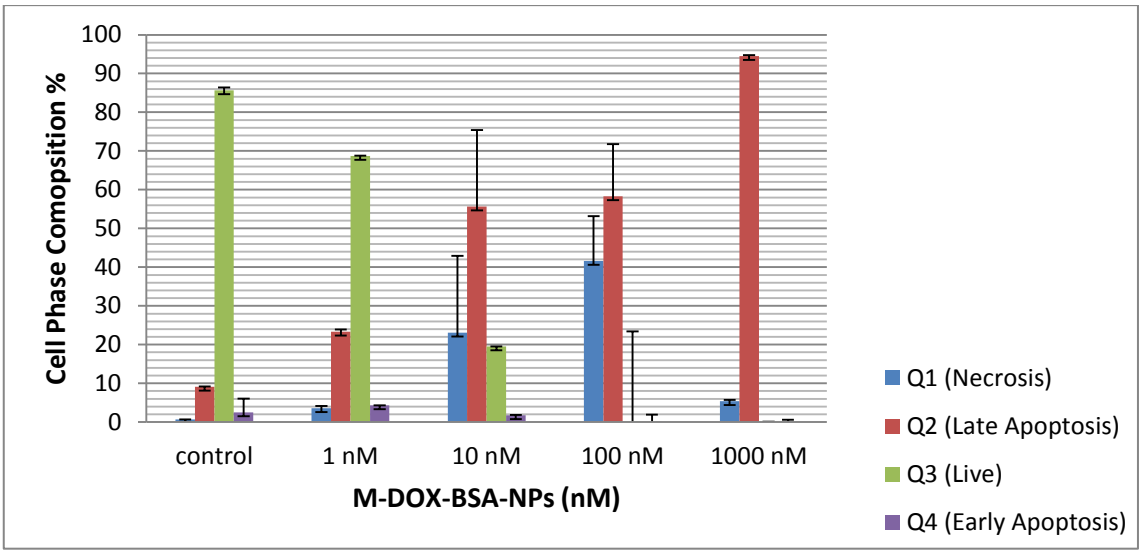


Figure 3.12. Quantification analysis of applied M-DOX-BSA-NPs apoptosis of PC3 cells.

3.4. Apoptosis Rate on A549 Cells

In order to investigated apoptotic effect of DOX and M-DOX-BSA-NPs against A549 cells, the DOX and M-DOX-BSA-NPs were tested different concentrations (1, 10, 100, 1000 nM) and untreated cells as a control group like PC3 cell as mention above.

Firstly, it was investigated on doxorubicin. The percentage of cell phase composition was calculated and shown in Figure 3.13. According to this result, when it was applied 1000nM concentration of doxorubicin, region of Q3 (live) was no cells. In contrast to PC3 cells, most of A549 cells passed Q2 late apoptosis area. On the other hand, when it was applied 1nM and 10nM concentration of doxorubicin, region of Q3 (live) was nearly amount of control like PC3 cells. However, contrast to PC3 cells, although 100 nM doxorubicin was treated, it was seen that %50 of cells were alive.

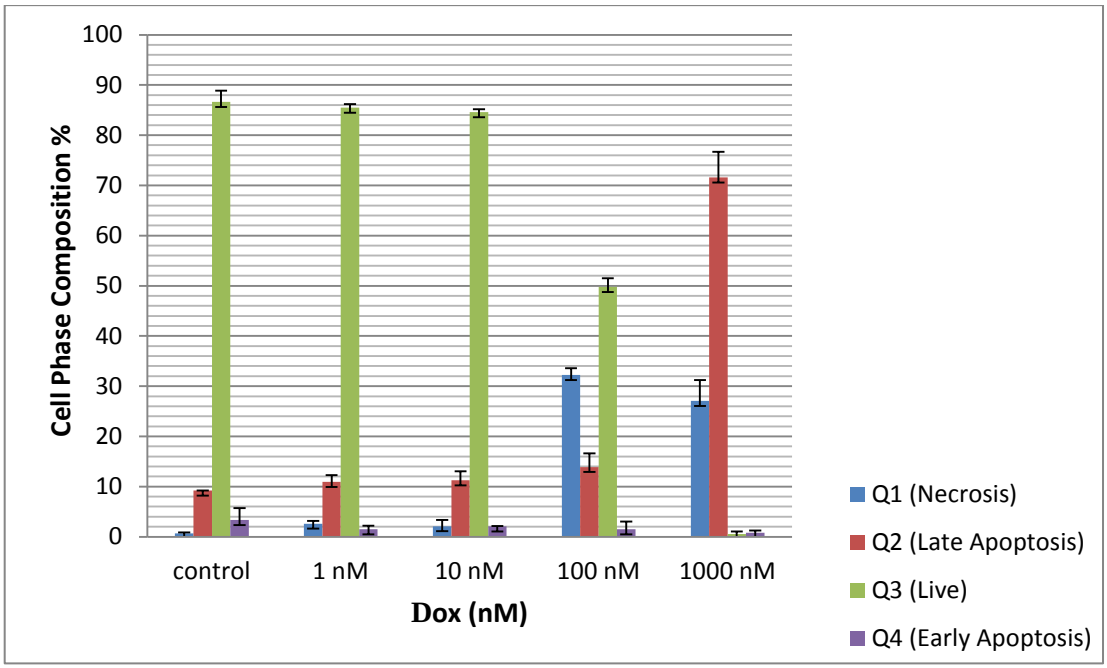


Figure 3.13. Quantification analysis of applied DOX apoptosis of A549 cells.

Secondly, it was investigated effect of M-DOX-BSA-NPs. The percentage of cell phase composition was calculated and shown in Figure 3.14. According to this result, when it was applied 100 and 1000nM concentration of M-DOX-BSA-NPs, region of Q3 (live) was no cells like PC3. Most of cells passed Q2 late apoptosis area. On the other hand, when it was applied 1nM concentration of M-DOX-BSA-NPs, region of Q3 (live) was nearly %80 amount of cell. This results show that M-DOX-BSA-NPs is more toxic than free doxorubicin and also, for PC3 cells doxorubicin and M-DOX-BSA-NPs are more effective than A549cells. These results support MTT assay.

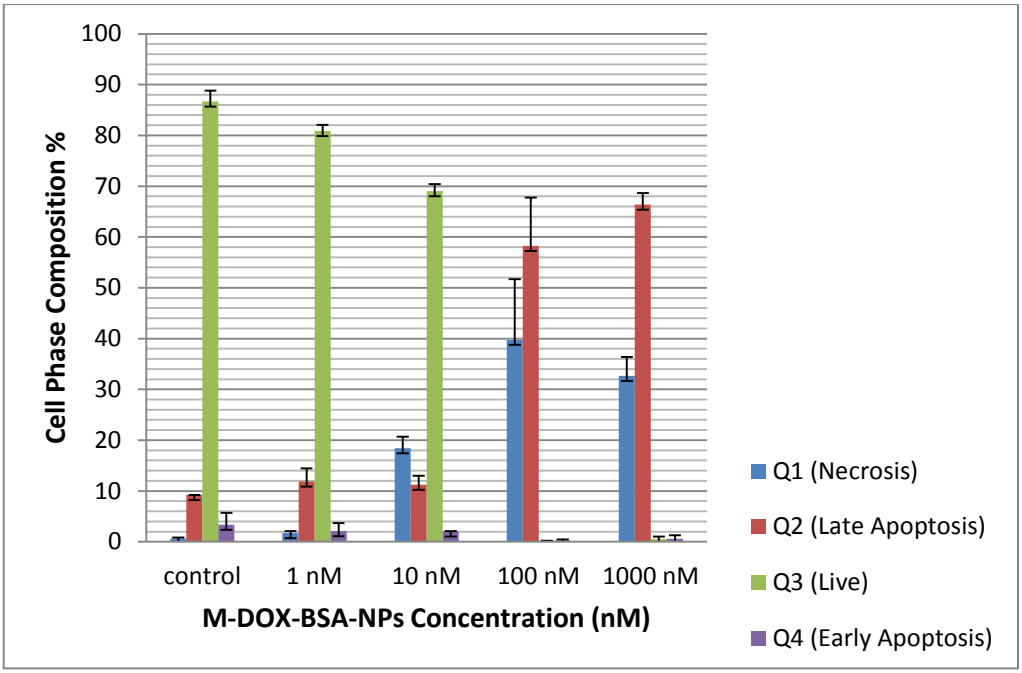


Figure 3.14. Quantification analysis of applied M-DOX-BSA-NPs apoptosis of A549 cells.

3.5. Cell Cycle Results for PC3 Cells

To investigate the cell cycle effects of free DOX and M-DOX-BSA-NPs against PC-3 cells, DOX and this compound were performed different concentrations (1, 10, 100, 1000 nM) and untreated cells as a control group by using flow cytometry based propidium iodide (PI) staining. Firstly it was investigated on doxorubicin and figure 3.15 was obtained. It was looked cell phase composition according to G2, S, and G1 phase. G1 phase is the beginning of DNA synthesis, S phase is DNA replication and G2 phase is the last phase until the cell enters mitosis. As doxorubicin affects DNA it was expected when applied high concentration of doxorubicin, G1 phase increased. According to the figure 3.15., as increased doxorubicin concentration, G1 phase increased but the highest doxorubicin concentration increased S phase. G1 phase was found nearly control group.

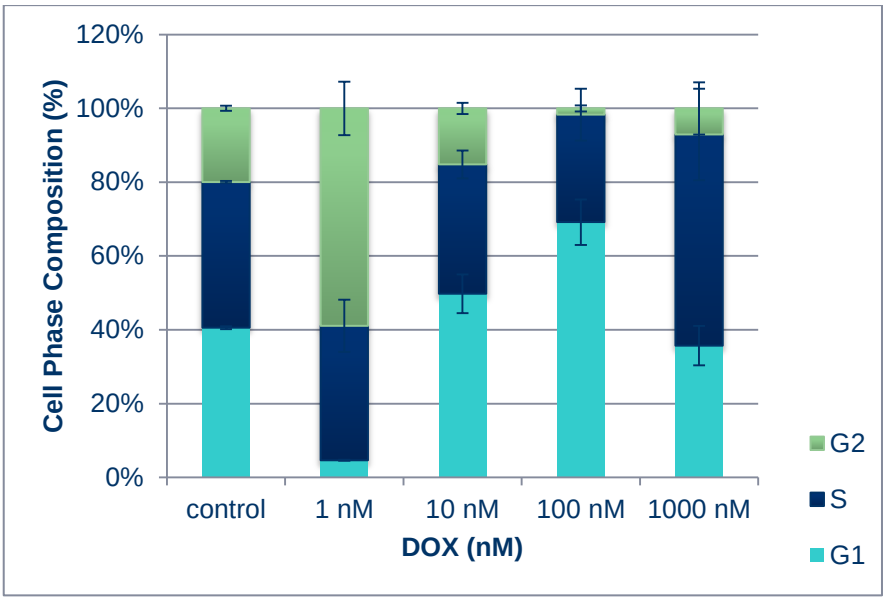


Figure 3.15. Effects of Doxorubicin cell-cycle distributions on PC-3

Secondly, it was investigated effect of M-DOX-BSA-NPs. It was occurred figure 3.16., and according to this figure, when concentration of M-DOX-BSA-NPs was increased G1 phase increased as it was expected. On the other hand when applied lower concentration of it, G2 phase increased. For reason of this, nanoparticles might affect G2 phase.

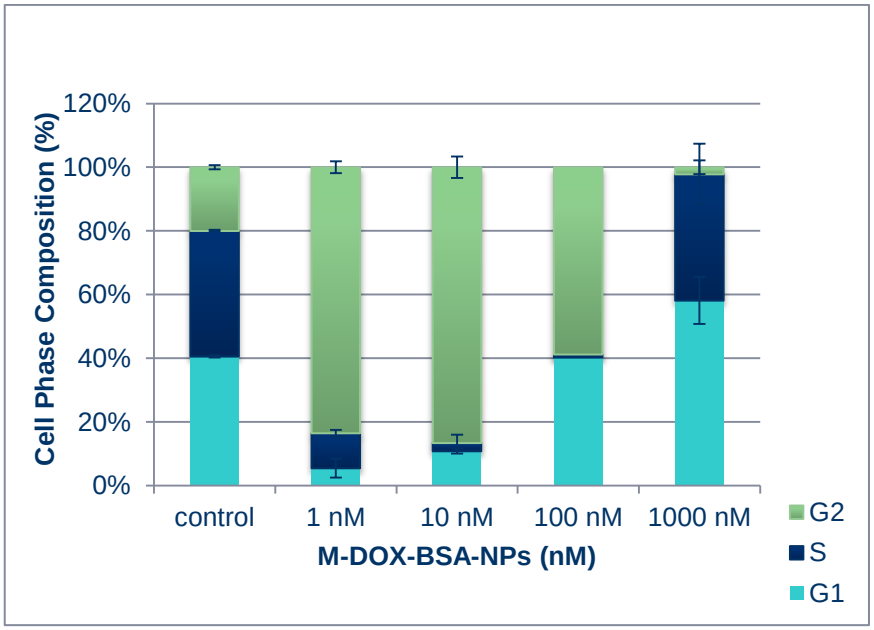


Figure 3.16. Effects of M-DOX-BSA-NPs cell-cycle distributions on PC-3

3.6. Cell Cycle Results for A549 Cells

In order to investigated cell cycle effect of DOX and M-DOX-BSA-NPs against A549 cells, the DOX and M-DOX-BSA-NPs were tested different concentrations (1, 10, 100, 1000 nM) and untreated cells as a control group like PC3 cell as mention above.

Fist of all doxorubicin was investigated and figure 3.17 was obtained. As regards the figure, G1 phase was found nearly control group at all concentration. This is because; these concentrations of doxorubicin against A549 cells were not enough for increased G1 phase.

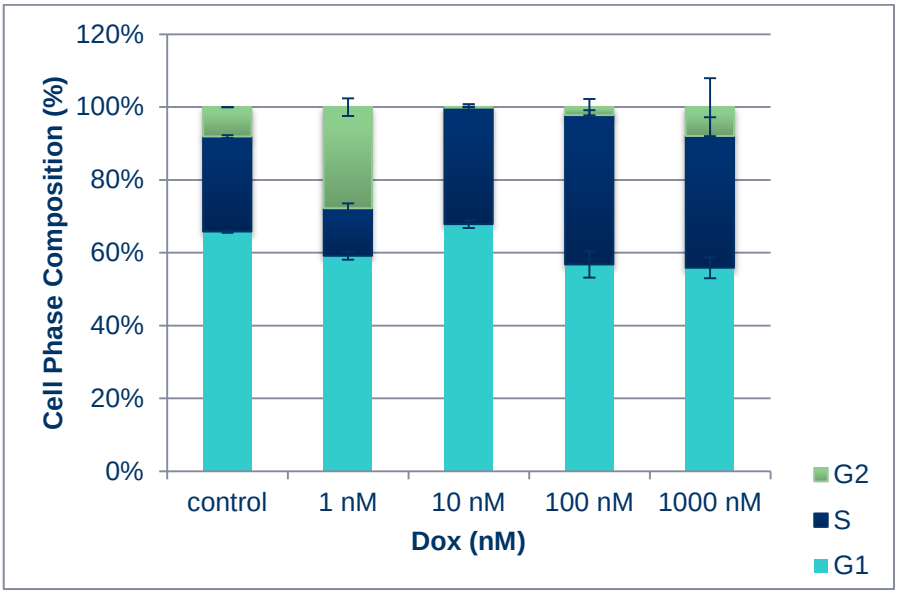


Figure 3.17. Effects of DOX cell-cycle distributions on A549

Secondly, it was investigated effect of M-DOX-BSA-NPs. The percentage of cell phase composition was calculated and shown in Figure 3.18. According to this result, as increased concentration of M-DOX-BSA-NPs, G1 phase increased, it was expected. However, when applied lower concentrations of it, G2 phase increased like PC-3 cell result. The result supported that nanoparticles affected G2 phase.

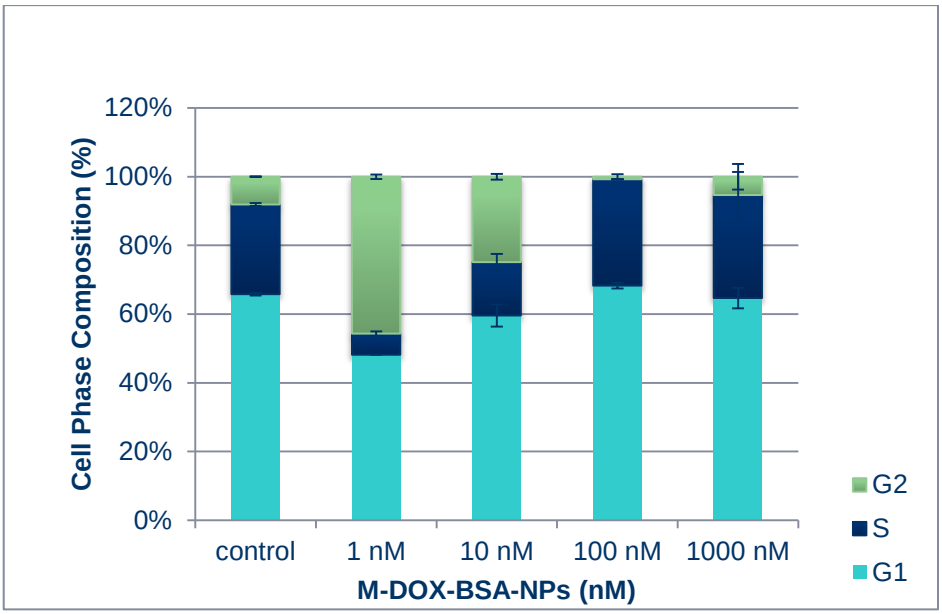


Figure 3.18. Effects of M-DOX-BSA-NPs cell-cycle distributions on A549

3.7. Imaging of Optical Microscopy

In order to understand how these cancer cells appeared before and after given free DOX and M-DOX-BSA-NPs, optical microscopy was used. According to the figure 3.19., while before given free DOX and M-DOX-BSA-NPs, the cells were alive (control groups); after applying free DOX, most of cancer cells (not only PC3 but also A549) could not be observed. Indeed, after given M-DOX-BSA-NPs, almost any cells appeared especially for PC-3cells.

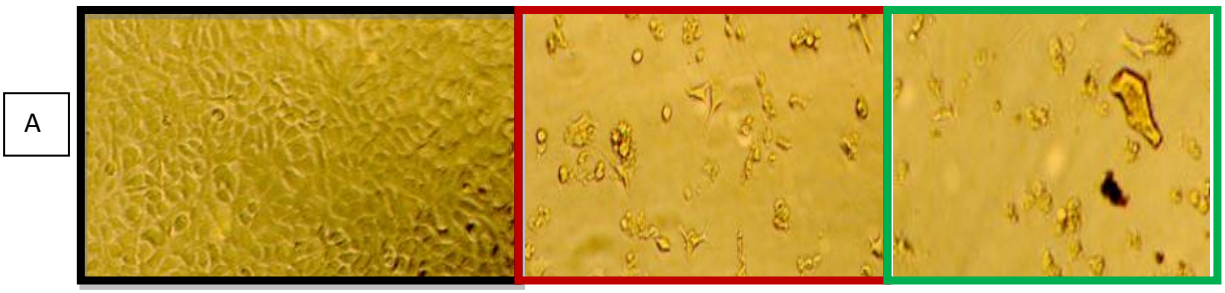
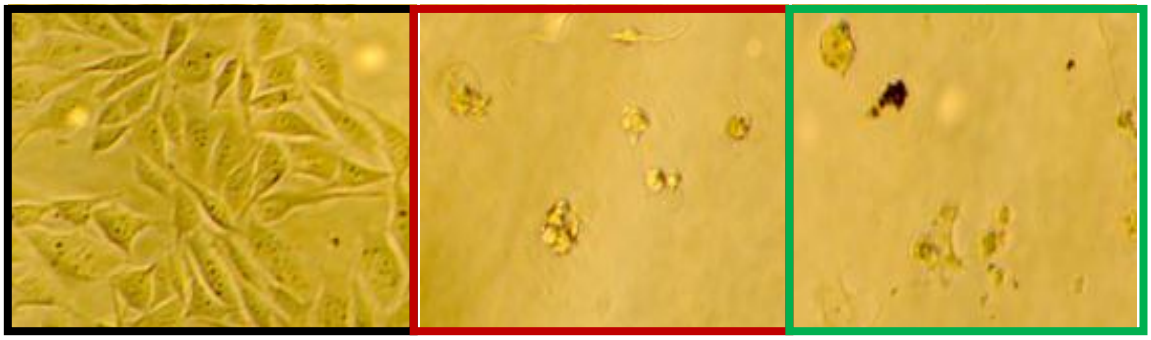


Figure 3.19. Optical microscopy images (x10) [A] A549 cells [B] PC3 cells [a] control [b]1 µg/mL free DOX [c] 1 µg/mL M-DOX-BSA-NPs.

(cont. on next page)

B



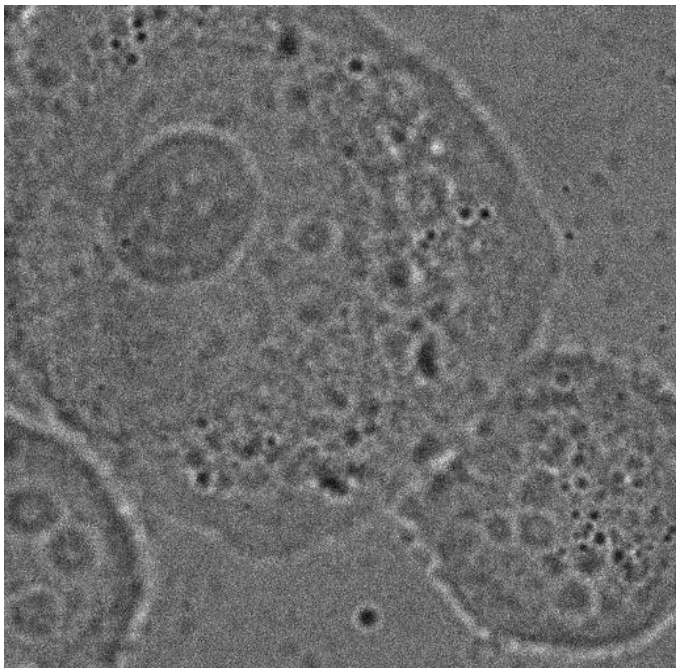
[a] [b] [c]

Figure 3.19. (cont.)

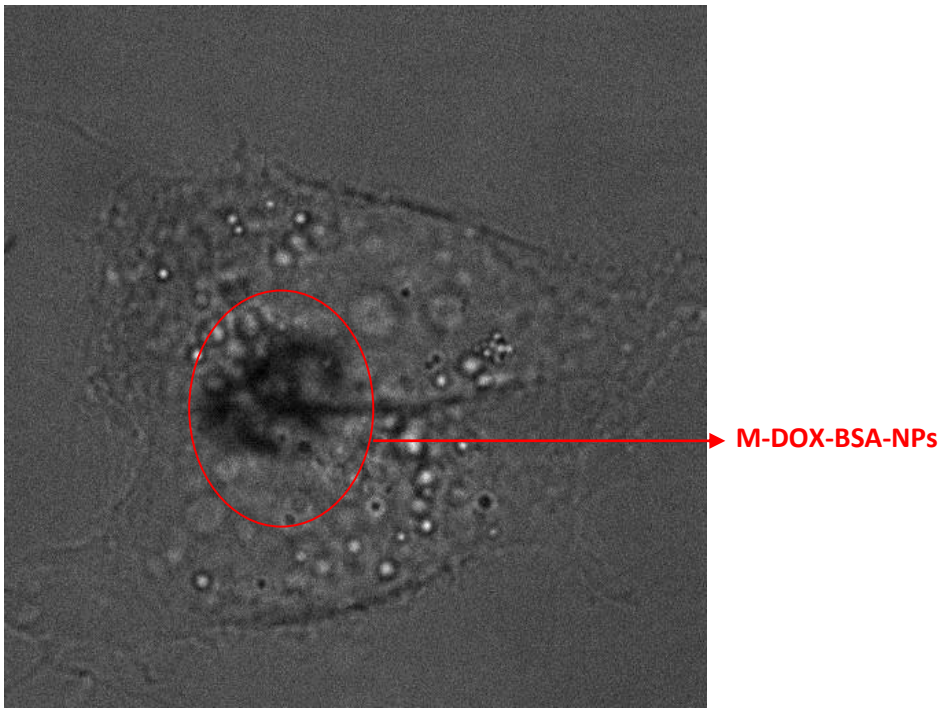
Although same concentration of DOX and M-DOX-BSA-NPs were given each of cells, it was seen few cells for A549 cells but fewer cells for PC-3 cell. It was seen again that M-DOX-BSA-NPs is more effective than free DOX for these cancer cells. This imaging result supported other results like MTT.

3.8. Imaging of Confocal Microscopy

In order to understand where M-DOX-BSA-NPs go to the in cell, it was used confocal microscopy. As doxorubicin affects DNA, it is expected that M-DOX-BSA-NPs go into the nucleus. When it was looked at figure 3.21., it was appeared that M-DOX-BSA-NPs was in the cell and reached nucleus, as it was expected.



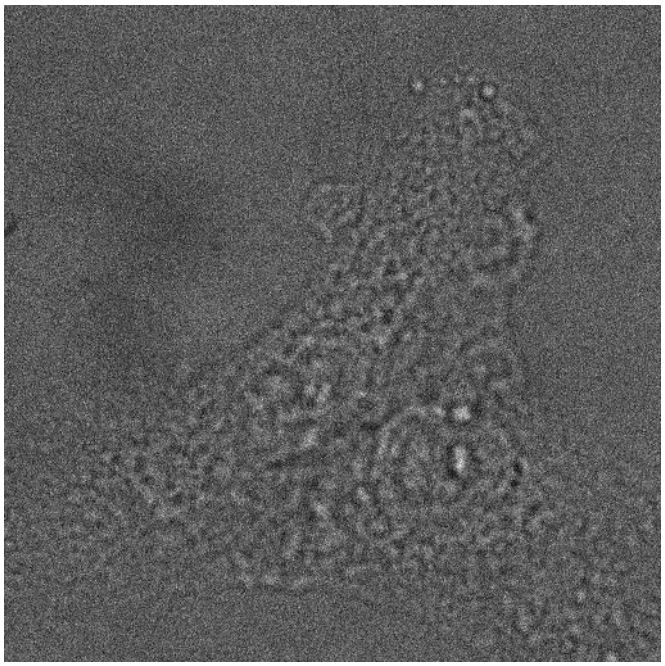
[A]



[B]

Figure 3.20. Confocal microscopy images [A] control, [B] 1 µg/mL M-DOX-BSA-NPs for PC-3 cells.

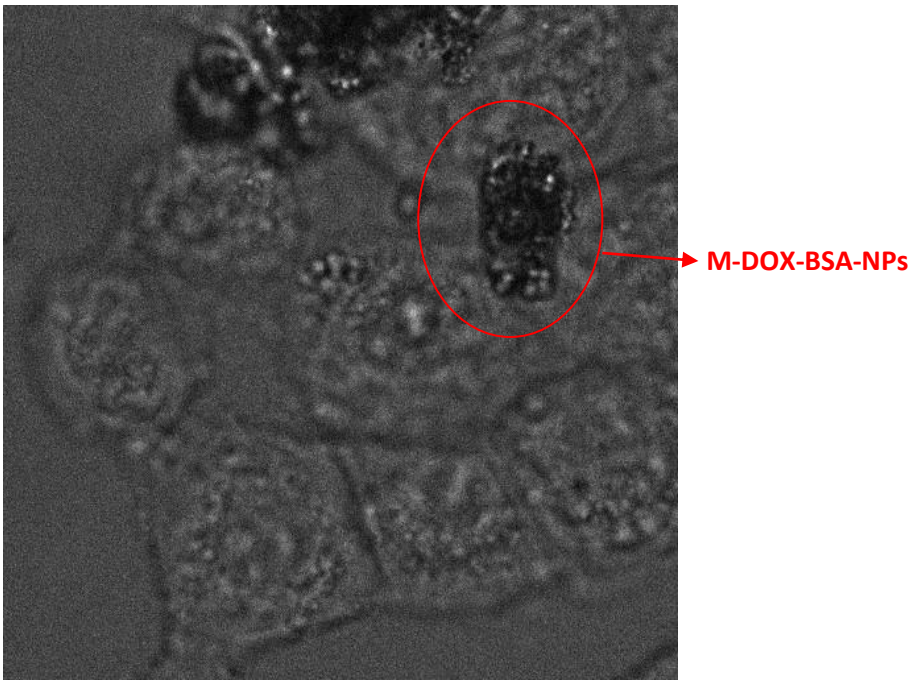
On the other hand, when it was performed for lung cancer cells, it was obtained figure 3.22. As it was looked at these images, it was seen that M-DOX-BSA-NPs could not reach to nucleus. The compound stayed in cell membrane. The reason for this, incubated time or concentration of M-DOX-BSA-NPs could not be enough for lung cancer cells. On the contrary prostate cancer cells, lung cancer cells could not go to the nucleus although it was used same concentration and incubated time. The image results indicated that this compound was more effective on prostate cancer cell. In other words, the images supported other results.



[A]

Figure 3.21. Confocav microscopy images [A] control, [B] 1 μg/mL M-DOX-BSA-NPs for A549 cells.

(Cont. on next page)

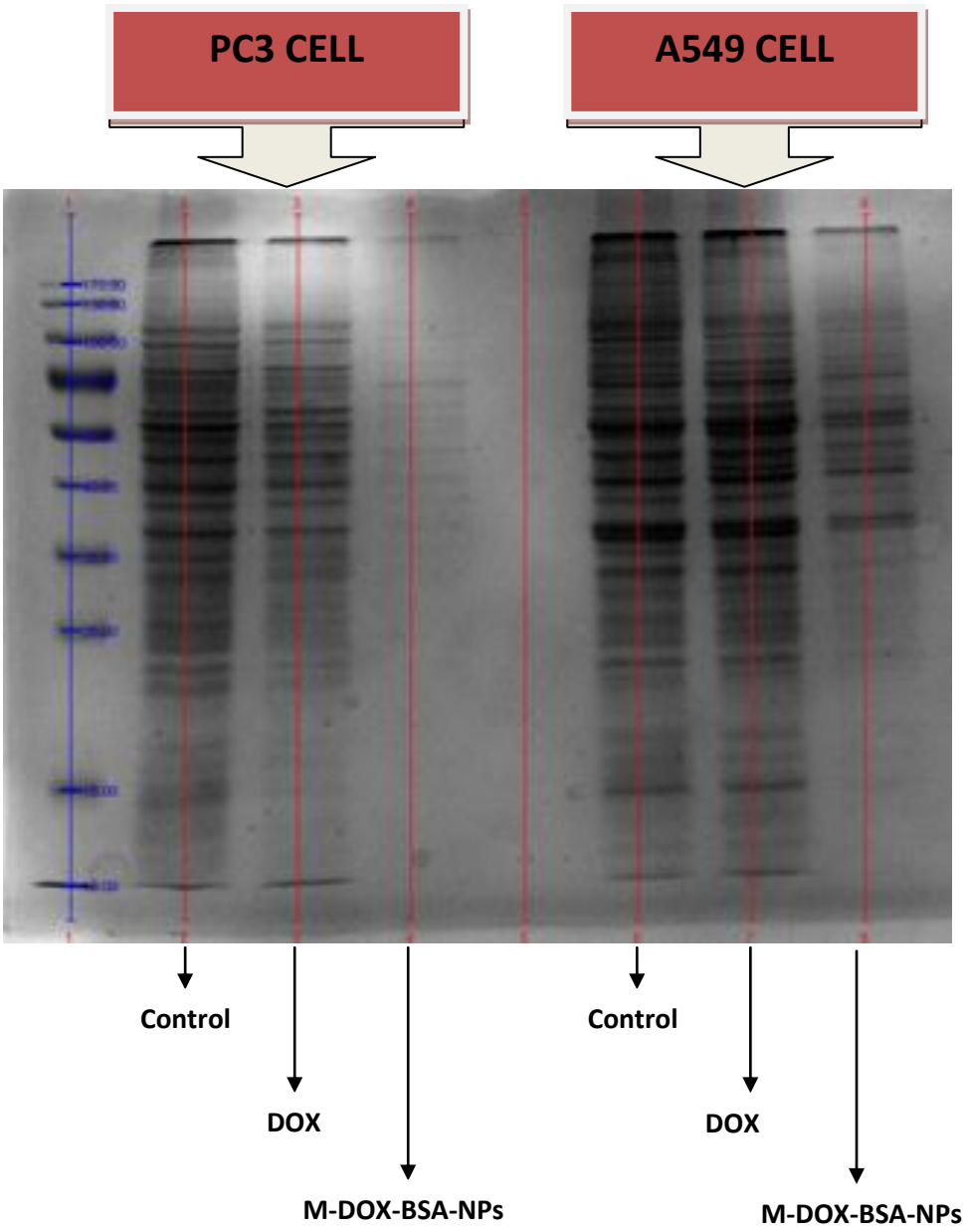


[B]

Figure 3.21. (Cont.)

3.9. Mini and Maxi SDS-PAGE Gel Electrophoresis

After determining the cytotoxic effects of albumin nanoparticles (BSA-NPs), magnetic albumin nanoparticles (M-BSA-NPs), doxorubicin, DOX incorporated albumin nanoparticles (DOX-BSA-NPs) and doxorubicin loaded magnetic albumin nanoparticles (M-DOX-BSA-NPs) on A549 and PC3 cells, apoptotic effects of doxorubicin and M-DOX-BSA-NPs on A549 and PC3 cells and cell cycle effects of doxorubicin and M-DOX-BSA-NPs on A549 and PC3 cells total protein isolation was performed from DOX, M-DOX-BS A-NPs and control group, separately. Afterwards, the isolated proteins were processed by mini and maxi Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis, respectively to identify chances in protein profiles. First of all, gels obtained from DOX, M-DOX-BS A-NPs and control group not only PC3 but also A549 cells were stained with Coomassie Brillant Blue Staining. As range of mini electrophoresis was fewer, the result was satisfactory (Figure 3.23). Therefore, it did not need another staining like silver staining.



Figüre 3.22. Mini SDS-PAGE Map

As to the map, after applied doxorubicin, band intensity decreased; after given M-DOX-BSA-NPs, band intensity almost disappeared. In addition, some band appeared before applied these compounds even so, some band lost after administered.

After carried out mini SDS-PAGE electrophoresis, maxi SDS-PAGE electrophoresis was performed and it was obtained figure 3.24. To the contrary mini electrophoresis, maxi result was not satisfactory. Rage of maxi electrophoresis was bigger than mini electrophoresis. Further, proteins run more time. For this reason, there

was no enough protein that was visualized on the gel. But still it could be seen different bond.

In order to understand protein differentiation better, it can be performed two dimensional gel electrophoresis. After carried out this study, if it can be found different spot or spots, it can be determined by using mass spectrometry. These sodium dodecyl sulfate-polyacrylamide gel electrophoresis results give us preliminary information about protein expression.

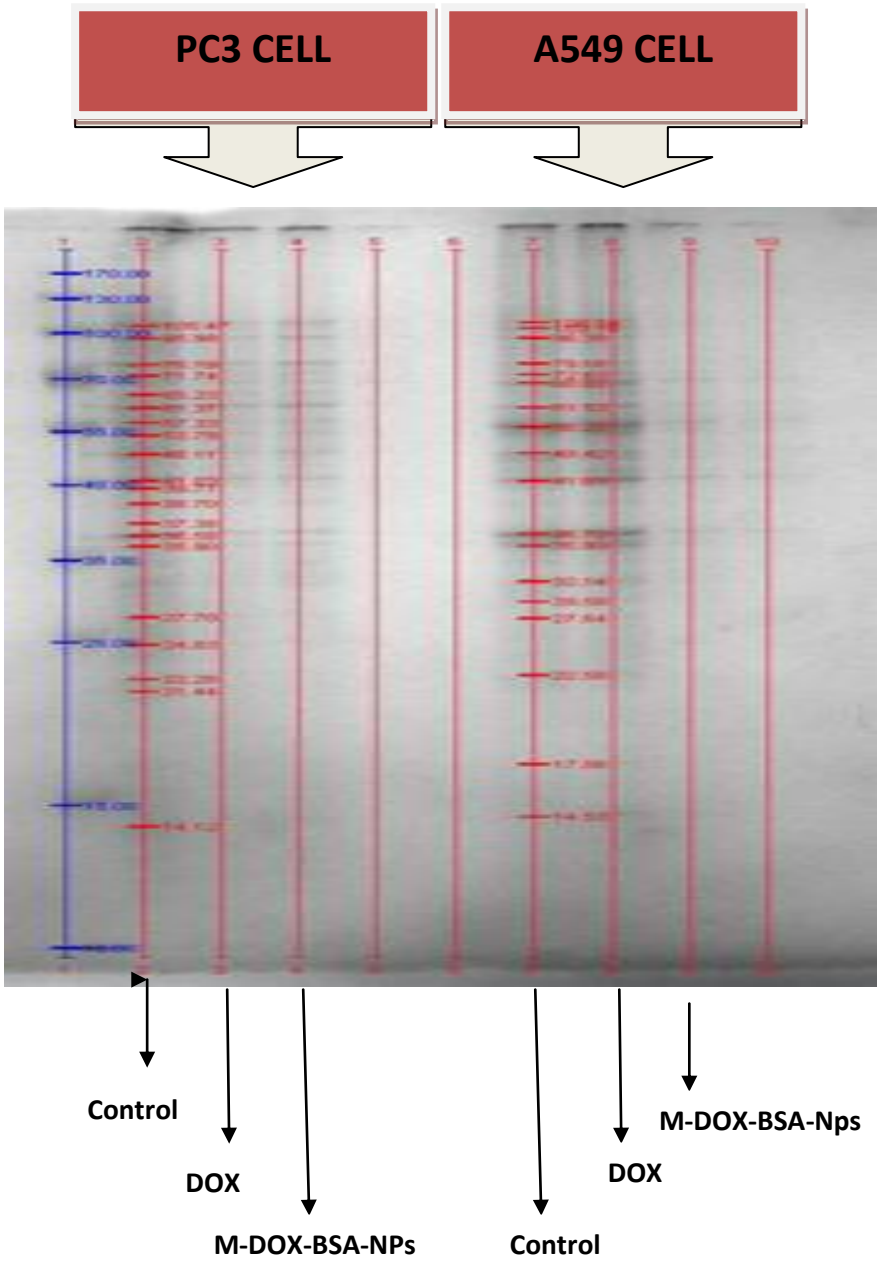


Figure 3.23. Maxi SDS-PAGE Map

CHAPTER 4

CONCLUSIONS

Unfortunately, cancer is increasing day by day. Chemotherapy is a major therapeutic approach for the treatment of a wide range of cancers. One of the most difficulties to treat a tumor is how to kill tumor cell effectively, meanwhile keep the normal cellular tissue not harmed at the best degree. However, the specificity of most anti-cancer drugs is poor. Therefore, seeking for an effective hottest topics tumor targeted delivery system is one of the hottest topics in the tumor chemotherapy (Sun et al., 2004). Doxorubicin is a powerful drug used in cancer chemotherapy. But unfortunately, it has got a lot of serious adverse effects, too. Because of this, doxorubicin loaded magnetic albumin nanospheres were consisted.

In this study, it was demonstrated anticancer potentials (both cytotoxic, apoptotic and cell cycle effects) of DOX formulation, BSA-NPs and M-BSA-NPs on PC3 and A549 cells. In addition to this, we scanned after and before given these compounds for both PC3 and A549 cells by using confocal and optical microscopy. Beside this it was investigated protein profiling when they exposed to DOX and M-DOX-BSA-NPs for each of cells.

In order to achieve our aims in the project, it investigated that M-BSA-DOX-NPs could reduce damaging side effects according to the free DOX or not. At the first experiment part, the cytotoxic effect was determined by measuring the IC50 value of free DOX, BSA-DOX-NPs, M-BSA-DOX-NPs, M-BSA-NPs and BSA-NPs and they compared with each other using MTT assay. It was calculated from cell proliferation plots and IC50 values of M-BSA-DOX-NPs were found 34,5 ng/ml for PC3 and 1683 ng/ml for A549 cells. These results indicated that M-BSA-DOX-NPs affected each cell the lowest concentration. In addition, the compound was more effective for PC3 than A549. When it was looked at literature, these results supported. For instance, as to "Quantum Dot−Aptamer Conjugates for Synchronous Cancer Imaging, Therapy, and Sensing of Drug Delivery Based on Bi-Fluorescence Resonance Energy Transfer" article, IC_{50} values of quantum dot (QD) alone, quantum dot

(QD)−aptamer(Apt)−doxorubicin (Dox) conjugate [QD−Apt(Dox) and free doxorubicin against PC3 cells were found 1,6µM, 1,6µM and 5µM, respectively, (Bagalkot et al., 2007). Also according to the "Comparative effects of thermosensitive doxorubicin-containing liposomes and hyperthermia in human and murine tumors" article, an IC50 value of thermosensitive liposome encapsulating doxorubicin (LTSL-DOX) against PC-3 cells was found 151nM (Yarmolenko et al., 2010). This result was nearly three times our compounds result for PC-3. As regards other article "Folate-receptor-targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin-PEG-folate conjugate", IC_{50} values of DOX nano-aggregates and DOX/FOL nano-aggregates against A549 were found 9.0µM and 9.2 µM, respectively, (Yoo H. S., 2004). According to the article, our compound was found eight times effectively. Beside these, in "Evaluation of folate conjugated pegylated thermosensitive magnetic nanocomposites for tumor imaging and therapy" article was investigated the cytotoxicity effects of polymer nanoparticles, iron-polymer nanoparticles, folate conjugated iron-polymer nanoparticles, doxorubicin loaded folate conjugated iron-polymer nanoparticles and free doxorubicin against A549. Free doxorubicin was found the most toxic (Rastogi S., 2011). On the other hand it was looked at our complex was found not only PC-3 cell but also A549 cell the most toxic. These results showed that as it was used magnetic albumin nanoparticles as a drug carrier, quickly release of doxorubicin was prevented. Thanks to nanoparticles, it was decreased toxicity of doxorubicin as it was expected. Then, apoptosis was evaluated by measuring the changes PC3 and A549 when M-BSA-DOX-NPs were applied. According to the results, M-BSA-DOX-NPs were more effective for PC3 than A549, as well. Besides this, when it was looked apoptosis results, the results supported MTT assay. After that cell cycle effects of free doxorubicin and M-DOX-BSA-NPs against PC-3 cells and A549 cells were investigated. On the contrary it was expected, lower concentration of M-DOX-BSA-NPs increased G2 phase. It was thought nanoparticles might increase G2 phase.

When it comes to the imaging analysis, firstly, to understand the cells look like, it was took optical imaging. For this study it was applied 1µg/ml of doxorubicin and M-DOX-BSA-NPs. As to images, after applied these compounds it was seen a few cell for A549, fewer cells for PC-3 cells, as we expected. This study supported other studies, too. Secondly, in order to understand M-DOX-BSA-NPs go to the cell, it was use confocal microscopy. Doxorubicin effects DNA of cells. Therefore, we expected when we treated M-DOX-BSA-NPs on cells, this complex went to nucleus. According to

taken confocal images, this complex went to nucleus in PC3cells. On the other hand, it could not reach to there in A549 cells. If after given this complex incubation time had extended or concentration of it has increased, maybe it could have reached there. Thus, these results showed us that this complex is more effective for prostate cancer cells than lung cancer cells.

Protein profiling provides a much better understanding of an organism, in terms of structure and function. Use of protein profiling in the study of multiple proteins, protein forms, and protein families almost always by comparing two different states (diseased vs. healthy or treated vs. untreated) is expected to expand our understanding of molecular mechanisms. For this reason, SDS-PAGE analysis was performed. It was obtained differentiated bonds.

For the further step studies, In order to understand protein differentiation better, it can be performed two dimensional gel electrophoresis. differentiated proteins can be recovered from gel and can be applied for MALDI-TOF Mass Spectrometry for protein identification and their function can be evaluated after M-BSA-DOX-NPs is treated in cells. Also, the same compound can be tried to treat other cancer types, with the help of differentially expressed proteins. Additionally, it can be improved new treatment for cancer cells.

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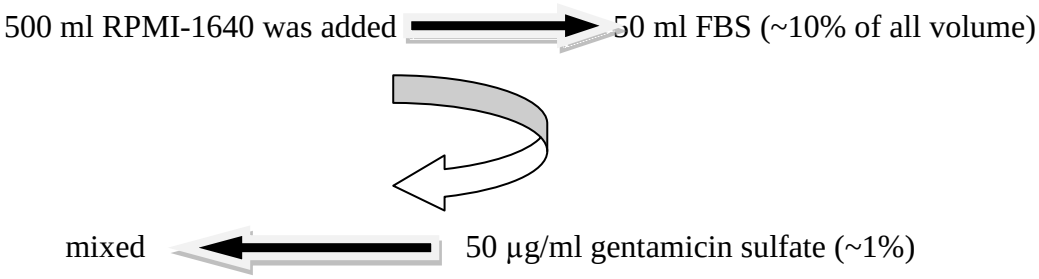
APPENDIX A

MEDIAS

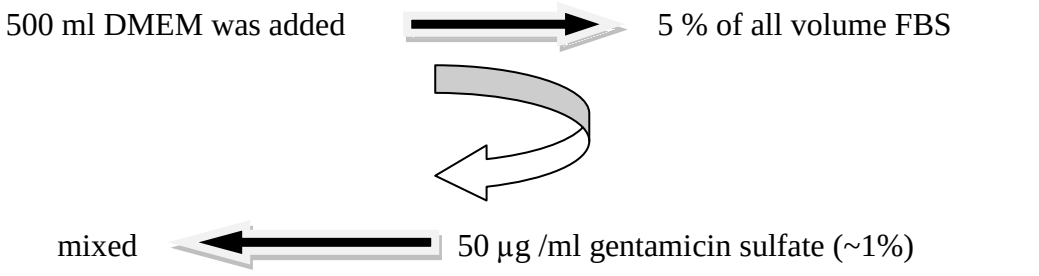
A.1. RPMI-1640 Growth Medium

Roswell Park Memorial Institute – 1640 (RPMI 1640) growth medium, fetal bovine serum (FBS) and gentamicin sulfate were obtained from Gibco, BRL.



A.1. DMEM Growth Medium

Dulbecco's modified Eagle'S medium (DMEM) growth medium, fetal bovine serum (FBS) and gentamicin sulfate were obtained from Gibco, BRL.



APPENDIX B

CHEMICALS, REAGENTS AND SOLUTIONS

B.1. Doxorubicin

Doxorubicin was supplied commercially (300mgincluding 50 mg doxorubicin). It was prepared fresh for each study. It was solved with phosphate buffered saline (PBS).

B.2. PC3 Cell Line

PC3 (human prostate cancer) cell line was provided by Assist. Prof. Kemal Sami Korkmaz in Ege Universitesi, Engineering Faculty, Department of Bioengineering.

B.3. A549 Cell Line

A549 (adenocarcinomic human alveolar basal epithelial cells) cell line was obtained from Prof. Dr. Serdar Ozcelik in Izmir Institute of Technology, Science Faculty, Department of Chemistry.

Table.1.B. Chemicals and Reagents Used in Experiments

NO	CHEMICALS	COMPANY
1	Dimethyl Sulfoxide (DMSO)	Sigma
2	Trypan Blue Dye	Sigma
3	Phosphate Buffered Saline (PBS)	Invitrogen
4	Gentamicin Sulfate	Gibco
5	Fetal Bovine Serum (FBS)	Gibco

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Table1.B. (cont.)

6	MTT Reagent (should not be exposed to light)	Sigma
7	0,5M Tris-HCl, pH 6,8	AppliChem
8	Annexin-V Apoptosis Detection Kit I	BD Pharmingen
9	Bovine Serum Albumin (BSA)	Sigma
10	Coomassie Brilliant Blue G-250 (CBB G-250) Dye	AppliChem
11	Absolute Ethanol	AppliChem
12	Phosphoric Acid	AppliChem
13	Ethylenediaminetetraacetic acid (EDTA)	Sigma
14	Protease inhibitor	Roche
15	Glycerol	AppliChem
16	Bromophenol Blue (%0.5)	AppliChem
17	CHAPS (%2)	AppliChem
18	Marcaptoethanol	AppliChem
19	SDS	AppliChem
20	phosphoric acid	AppliChem
21	Acrylamide	AppliChem
22	Bisacrylamide	AppliChem
23	1.5 M Tris – HCl pH = 8.8	AppliChem
24	Ammonium Persulfate (APS)	Sigma
25	Tetramethylethylenediamine (TEMED)	Sigma
26	Trypsin	Sigma
27	Triton X-100	Sigma
28	RNase	Thermo

B.4. MTT Reaction Solution

In order to prepare a reaction solution sufficient for a 96-well plate 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT solution was made in PBS to obtained a concentration of 5mg/ml. % 10 MTT solution was prepared with DMEM and RPMI.

B.5. Stock BSA Standard (2 mg/ml)

0,01g of BSA was dissolved in 10 ml PBS

B.6. Standard Curve for BSA

Table 2. B. Absorbance Values for BSA Standards

Concentration (mg/ml)	Absorbance 596nm	Standard Deviation
0,1	**0,1584**	**0,0574**
0,5	**0,1916**	**0,0231**
1	**0,2451**	**0,0590**
2	**0,3296**	**0,0609**

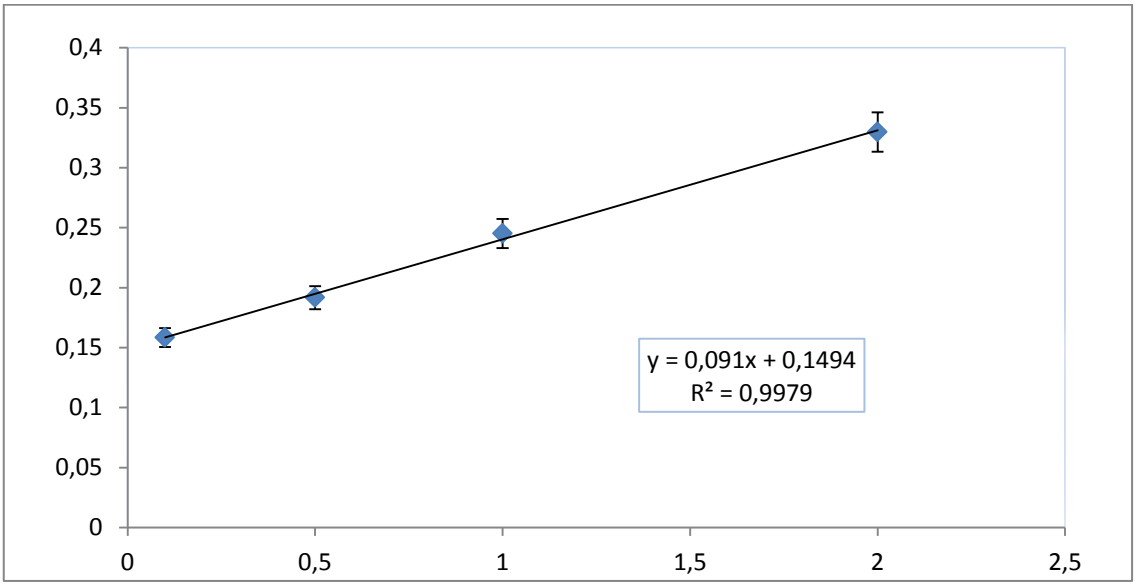

Figure B.1. Standard Curve for BSA

B.7. Coomassie Brilliant Blue G-250 (CBB G-250) Dye

- ✓ 100 mg Coomassie Brilliant Blue G-250 (CBB G-250)
- ✓ 50 ml Absolute Ethanol
- ✓ 100 ml 85% Phosphoric Acid

The above-mentioned mass of CBB G-250 was weighed and dissolved in 50 ml of absolute ethanol. Then, 100 ml of 85% Phosphoric Acid was added to this solution. After the overall solution was filtered through filter paper, the filtered solution (~15-20 ml) was diluted to 1 liter with ultrapure water and stored at 4^{o}C.

B.8. Separating Gel (0,375M Tris pH 8.8)

Table 3.B. Preperation Seperating Gel

	% 12
Acrylamide/bis	40.0 ml
Ultrapure water	33.5ml
1,5M Tris-HCl	25.0ml
%10 SDS	1.0 ml
%10 ammonium persulfate	0.50 ml
TEMED	0.050 ml
TOTAL	**100 ml**

B.8. Stacking Gel (0,125M Tris pH 6.8)

Table 4. B. Preperation Stacking Gel

	% 4.0
Acrylamide/bis	1.3 ml

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Table 4. B.(cont.)

Ultrapure water	6.1 ml
0,5M Tris-HCl	2.5 ml
%10 SDS	0.1 ml
%10 ammonium persulfate	0.05 ml
TEMED	0.01 ml
TOTAL	**10 ml**