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IN VITRO INVESTIGATION OF FOSCAN MEDIATED PHOTODYNAMIC THERAPY ON PRIMER BREAST CANCER CELL LINE

SAADET AKBULUT

MSc THESIS
BIOMEDICAL ENGINEERING PROGRAMME

İSTANBUL, FEBRUARY / 2014

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THESIS ADVISOR
ASST. PROF. DR. HAŞİM ÖZGÜR TABAKOĞLU

İSTANBUL, ŞUBAT / 2014

T.C.
FATİH ÜNİVESİTESİ
BİYOMEDİKAL MÜHENDİSLİK ENSTİTÜSÜ

FOSCAN GÜDÜMLÜ FOTODİNAMİK TERAPİNİN PRİMER MEME KANSERİ HÜCRE HATTI ÜZERİNDEKİ ETKİSİNİN *İN VİTRO* ARAŞTIRILMASI

SAADET AKBULUT

YÜKSEK LİSANS
BİYOMEDİKAL MÜHENDİSLİĞİ PROGRAMI

DANIŞMAN
YRD. DOÇ. DR. HAŞİM ÖZGÜR TABAKOĞLU

İSTANBUL, ŞUBAT / 2014

T.C.

FATIH UNIVERSITY

INSTITUTE OF BIOMEDICAL ENGINEERING

Saadet Akbulut, a MSc student of Fatih University Institute of **Biomedical Engineering** student ID 52011122, successfully defended the thesis/dissertation entitled "***In Vitro* Investigation of FOSCAN Mediated Photodynamic Therapy On Primer Breast Cancer Cell Line**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor: **Yrd. Doç. Dr. Haşim Özgür TABAKOĞLU** **...…… …….**

Fatih University

Jury Members: **Prof. Dr. Sadık KARA** .....

Fatih University

Doç. Dr. Oğuzhan KARATEPE .....

Bezmialem University

Date of Submission: 07 February 2014

Date of Defense: **07 February 2014**

TO MY FAMILY…

This study supported by The Bezmialem Foundation University and The Fatih University, was executed between May 2013 and December 2013.

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February 2014 Saadet AKBULUT

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LIST OF SYMBOLS

% Percent
γ Gama
μ Micro

LIST OF ABBREVIATIONS

ATP-TCA : Adenosine Triphosphate Tumor Chemosensitivity
CAM : Chorioallantoic Membrane
CO_2 : Carbon Dioxide
H&E : Hematoxylin and Eosin
HpD : Hematopophyrin derivatives
H_2O_2 : Hydrogen Peroxide
FDA-USA : Food and Drug Administartion - United States of America
nm : Nanometer
PDT : Photodynamic Therapy
PS : Photosensitizer
rmp :Rounds per Minute
ROS : Reactive Oxygen Species
S_0 : Ground State
S_1 : Exited Singlet State
T_1 : Triplet State
TCER : Tumor Cell Extraction Reagent
TDE : Tumor Digestion Enzyme
$^1O_2$: Singlet Oxygen
NY : New York

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SUMMARY

IN VITRO INVESTIGATION OF FOSCAN MEDIATED PHOTODYNAMIC THERAPY ON PRIMER BREAST CANCER CELL LINE

Saadet AKBULUT

Biomedical Engineering Programme

MSc Thesis

Advisor: Yrd. Doç. Dr. Haşim Özgür Tabakoğlu

Photodynamic therapy has a promising future to treatment for several cancer types. Photodynamic therapy is minimal invasive treatment protocol. Photodynamic therapy is in need of a light-activated agent, light source and oxygen which are converted into singlet oxygen during the treatment.

In this study, the cytotoxic effect of FOSCAN mediated Photodynamic Therapy (PDT) and some different types of chemotherapy drugs which are Paclitaxel, Doxorubicin, 5-Flourouracil were investigated in one 96-well plate with 10^4 cells/well for primary breast cancer tissue which admitted to Bezmialem Foundation University Faculty of Medicine the Department of General Surgery with proven invasive ductal breast carcinoma. The efficacies of treatments were evaluated with ATP tumor chemosensitive assay (ATP-TCA) which is based on detection of ATP.

Result from this study showed that FOSCAN mediated PDT is effective treatment for primary breast cancer cell line. Viability rate was decreased up to 4 % by FOSCAN mediated PDT. FOSCAN mediated PDT proven to be better treatment modality than chemotherapeutic drugs for breast cancer cell toxicity.

Keywords: Breast Cancer, PDT, Primary Cell Culture.

ÖZET

FOSCAN GÜDÜMLÜ FOTODİNAMİK TERAPİNİN PRİMER MEME KANSERİ HÜCRE HATTI ÜZERİNDEKİ ETKİSİNİN *İN VİTRO* ARAŞTIRILMASI

Saadet AKBULUT

Biyomedikal Mühendisliği Programı
Yüksek Lisans

Danışman: Yrd. Doç. Dr. Haşim Özgür Tabakoğlu

Fotodinamik Terapi (FDT) birçok kanserin tedavisi için umut verici bir geleceğe sahiptir. FDT minimal invaziv bir tedavi protokolüdür. FDT ışığa duyarlı bir ajana, ışık kaynağına ve ortamda oksijene ihtiyaç duymaktadır.

Bu çalışmada Bezmialem Vakıf Üniversitesi Tıp Fakültesi Genel Cerrahi Anabilim Dalı tarafından invaziv duktal karsinoma tanısı konulan meme kanseri dokuları üzerinde FOSCAN güdümlü Fotodinamik Terapi, Paklitaksel, Doksorubisin ve 5-Flourorasil gibi kemoterapi ilaçların sitotoksik etkileri 96'lık plaka üzerinde *in vitro* olarak araştırıldı. Uygulanan tedavilerin etkinlikleri ATP-TCA ile belirlendi.

Bu çalışmanın sonuçları, FOSCAN güdümlü FDT'nin primer meme kanseri hücre hattı üzerinde etkin bir tedavi olduğunu gösterdi. Canlılık oranın FOSCAN güdümlü PDT ile % 4'e kadar düştüğü ve FOSCAN güdümlü PDT'nin primer meme kanseri toksisitesi için kemoterapi ilaçlarından daha iyi bir tedavi yöntemi olduğu yapılan bu çalışma ile kanıtlanmıştır.

Anahtar kelimeler: Meme Kanseri, FDT, Primer Hücre Kültürü.

CHAPTER 1

INTRODUCTION

1.1 Motivation

Each year 11 million people are got cancer in the world and 150 thousand people are got cancer in Turkey. Surgery, chemotherapy and radiotherapy have been used for cancer treatment. The aims of cancer treatments are avoid healthy tissue damage which is possible with surgery. Cancer has become metastasis which is decreased performance of treatment (2). Chemotherapy is used different drug combination that has many side effects at certain intervals according to tumor type and stage. However, high dose chemotherapy regimens can occur seriously toxicity for patient. Therefore personalized chemotherapy regimens have been developed. Varieties of tumor chemosensitive assay have been used to test response of therapy based on in vitro toxicity assay. One of them is Adenosine Triphosphate Tumor Chemosensitive Assay (ATP-TCA) that is used to determine tumor growth inhibition. ATP-TCA is confidential, standardized, reproducibility, high sensitive evaluation method (3; 4).

Photodynamic Therapy (PDT) which has a promising future to treatment for several cancer types. PDT is preferred to have minimal invasive, low toxicity, localization and inexpensive treatment protocol (5).

At the present time, new investigations are made to development of PDT. New photosensitive agents (photosensitizer, PS) and treatment protocols have been developed. The FOSCAN has been widely used for the treatment of head and neck cancer in PDT. The FOSCAN which is approved by the European Union in 2001 has

strong absorption peak in the red part of spectrum (652 nm) which means deeper light penetration than HpD (6; 7; 8).

1.2 Objective

The appropriate photosensitizer is the most important factor to success of PDT. However, accomplished photosensitizer is not defined in literature for PDT with breast cancer. The treatment response can vary according to different molecular phenotype and complex interaction from patient to patient because of primary cell culture is used to determine of toxicity. In this study, FOSCAN that is approved by the European Union for treatment of head and neck cancer, mediated PDT and some different types of chemotherapy drugs these are doxorubicin, paclitaxel, 5-fluorouracil have been applied *in vitro* on primer breast carcinomas cell line and then have been investigated the percentage of viability by ATP-TCA assay (9; 10; 11). Hematoxylin and eosin (H&E) stain have been used to evaluate the grade, molecular subtype, Ki67 levels, diameter, and ER receptors in all samples.

The efficacies of applications were evaluated with ATP tumor chemosensitive assay (ATP-TCA). The chemotherapeutic drugs and FOSCAN mediated PDT responses' were predicted thanks to ATP tumor chemosensitive assay (12; 13; 14).

CHAPTER 2

CANCER

Cancer is a major health problem in the World that is recognized by disorganized cell growth and aggression that is overspread to the other sides from the origin in body. Variety of cancer types have been discriminated according to the tissue of origin. Cancers that are originated in epithelial cells are called carcinomas. Cancers derived from mesoderm cells are called sarcomas and cancers of glandular tissue are called adenocarsinomas. The treatment method must be performed differently according to the type of cancer. It can be obviously seen that there are six apparent features between cancer cells and normal cells. Uncontrolled cell division, angiogenesis, invasion and metastasis, protection of the length of telomeres, evasion of growth inhibitory signals and evasion of apoptotic cell death are imperative for carcinogenesis (15; 16; 17). Benign tumor and malignant tumor are separate from each other according to invasion and metastasis. Malignant tumor is characterized by rapid proliferation and by metastasis to the other parts of body to form secondary tumor. After the treatment, malignant tumor commonly reoccurs because of spread of surrounding tissue (18). Therefore benign tumor is not cancer, but all malignant tumors are cancerous (15).

According to data of American Cancer Society, each year 1.3 million people are diagnosed with breast cancer and 450 thousand people are died from breast cancer (19). Breast cancer is the most common cancer in women and annual incidence is increased with age. Breast cancer constitutes almost 30% of malignant disease and almost 16% of cancer related to deaths (20). A women's risk of contracting cancer is 12% of during life. In addition risk of death is 5% in women because of breast cancer. Early detection

of breast cancer, the development of adjuvant chemotherapy and endocrine therapy lead to decrease mortality rates. Although the development of diagnosis and treatment, breast cancer is a major reason for mortality and morbidity in women because of breast cancer is prone to metastasis. When the tumor is early detection, prognosis is good and expectation of survival is high without disease. When cancer cells become spread of the other side of body, cytotoxic drugs are used for treatment (21; 22).

2.1 Cancer Therapies

Early detection is the most important factor to decrease the mortality rate in cancer. Surgery, chemotherapy and radiotherapy are commonly used for cancer treatment. In addition to these, novel treatment methods are improved like immunotherapy and gene therapy (17). These treatment methods can be used alone or combined with together.

Ionizing radiation is used widely in several cancer treatments more in particularly the form of high energy γ-radiation. Radiotherapy is caused prostration, nausea and throwing up and these reduce the labor force. Certain variations can be occurred in application area some of these are dehydration, color change and hair loss on skin. Besides sensation loss and weakness can be occurred on nerve track (23). High tumor destruction and lowest toxicity is observed in ideal radiotherapy regimens (24).

2.1.1 Chemotherapy

Cancer has not metastasis or in stage 1-2, it can be treated completely with surgery. On the other hand cancer has metastasis that is spread the other side of body; it is impossible. Therefore chemotherapy and radiotherapy have been used to inhibit cellular growth and proliferation or eradicate the cancer cells. Traditional chemotherapy uses anticancer drugs that are directed against DNA and proteins. The damaged proteins and DNA affect the process of cell division (17; 15). Chemotherapy is more effective on rapidly dividing cells. However cancer stem cells are naturally resistant to chemotherapy and divided slowly by mitosis because of that cancer stem cells are protected from the effect of chemotherapy thus the treatment fail and tumor cells continue uncontrolled proliferation (25; 26).

There are different types of chemotherapeutic agents which are classified according to chemical structure, relationship to another drug and affect specific chemical substances within cancer cells (27; 28).

2.1.1.1 Doxorubicin

Doxorubicin is an antibiotic which is anthracycline derivative and it is the first anticancer drug in this group. Doxorubicin has been used to treatment of leukemia, lymphoma, soft tissue and bone sarcomas, neuroblastoma, hepatoblastoma. The effects of Doxorubicin demonstrate in different ways. One of them, it binds with DNA thus induce fragmentation on DNA. Besides, Doxorubicin is interacted with enzymes that are assignee in DNA replication and RNA transcription thus inhibits DNA replication and RNA transcription. Doxorubicin binds with cell membrane thus disrupts membrane function. However, Doxorubicin can generate Reactive oxygen species (29; 30; 31; 32).

Doxorubicin is given intravenous and swiftly decays in body. Its use restricted by cardiotoxicity. Doxorubicin can be combined with 5-Fluorouracil and cyclophosphamide thus success rate increase from 60 % to 80 % in advanced breast cancer (29; 31). The chemical structure of Doxorubicin is given Figure 2.1.

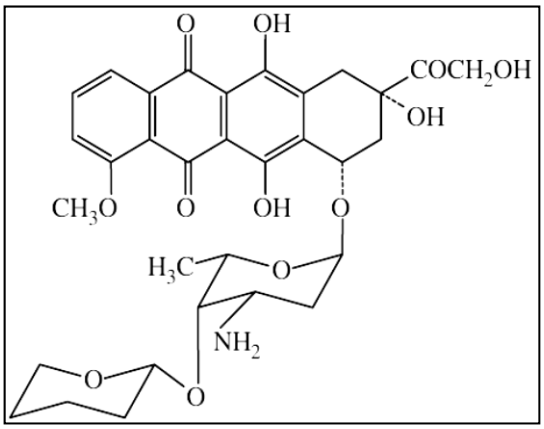

Figure 2.1 The Structure of Doxorubicin.

2.1.1.2 Paclitaxel (TAXOL®)

Paclitaxel that have been approved by FDA for treatment of breast cancer in 1994 is antineoplastic chemotherapeutic agent. The microtubules are collected and stable microtubules communities are constituted thanks to Paclitaxel binds microtubules. The

reformatted microtubules are inhibited mitosis and tumor cells die in the course of G1 phase and in passing from G2 to M (28; 33; 34).

Paclitaxel is given intravenous and half life is 5-8 hour. Paclitaxel can be combined with platinum agents and applied for treatment of breast cancer. Paclitaxel cannot pass the blood brain barrier, induces neurotoxicity and generate drug resistance for these reasons its use restricted (33). The chemical structure of Paclitaxel is given Figure 2.2.

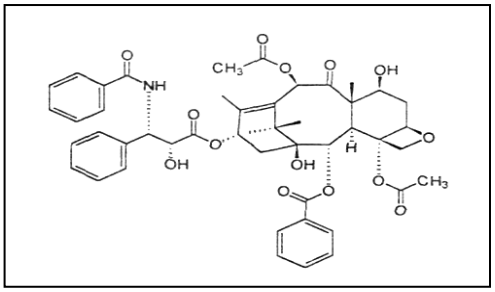

Figure 2.2 The structure of Paclitaxel.

2.1.1.3 5-Fluorouracil

5-Fluororacil that has been approved for treatment of actinic keratosis, basal cell carcinoma, breast cancer, colorectal cancer, gastric adenocarsinom, pancreatic cancer, squamous cell carcinoma of the head and neck cancer by FDA before 1984, is antineoplastic chemotherapeutic agent (35; 36). 5-Fluorouracil is need to phosphorylation for active. 5-Fluorouracil is blocked thymidylate synthetase enzyme thus inhibits DNA replication. However, 5-Fluorouracil can participate into the RNA structure as pseudonucleotide (37; 38). 5-Fluorouracil activate caspase-6 thus induces the cancer cell apoptosis. 5-Fluorouracil is given intravenous. Its use restricted by cardiotoxicity. 5-Fluorouracil can be combined with other chemotherapeutic agents (36). The chemical structure of 5-Fluorouracil is given Figure 2.3.

Figure 2.3 The Structure of 5-Fluorouracil.

2.2 Photodynamic Therapy

At the end of ninetieth century the healing power of light was noticed and sun light is used for the treatment of certain diseases that is called heliotherapy. Vitilligo, psoriasis, cancer and even psychosis were treated with sun light in Ancient Egyptian, Indian and Chinese. However, at the beginning of twentieth century modern light therapy was developed and Niels Finsen was won the Nobel Prize in 1903 through his work on phototherapy. Finsen was used red light and ultraviolet light that was generated an arc lamp in this work to treat smallpox and cutaneous tuberculosis (39; 40; 41).

At the same time, RAAB was investigating the effect of light-activated chemical compounds (acridine red molecule) on the organism paramecia. In this study, RAAB was discovering that the light-activated acridine was need oxygen to die paramecia in medium that was called '*Photodynamisch Wirkung*'. In 1966 LIPSON et al. was the first clinical application on breast cancer and xenon arc lamp was used as light source and hematoporphyrin was used as photosensitizer in this application (42). In 1975 Dougherty et al. was a trial on mouse mammary tumor with hematoporphyrin derivative (HpD) and red light, they were completely treated the lesion (43). Photodynamic therapy has been used the treatment of tumors since 1978 (44; 45). In 1987 Photofrin® was approved FDA-USA that was developed by Dougherty et al. (46). In 1990 significant developments have been obtained in PDT thanks to developed second generation of light-sensitive agents (47).

PDT has a promising future to treatment for several oncological, cardiovascular, dermatological, and ophthalmic diseases. PDT is in need of a photosensitive agent (photosensitizer, PS), light source and oxygen to treatment. The principle of PDT based on topically or systemically is performed photosensitizer which accumulates in target lesion and then target lesion is illuminated with light source which has appropriate wavelength. Initially, the photosensitizer interacts with light to reveal the Reactive Oxygen Species (ROS). This reaction is trigged cell death. There are three fundamental mechanisms of photodynamic therapy to eradicate the tumor cells which can show an alteration according to selected photosensitizer and the nature of tumor tissue. Certain photosensitizers localize in specific subcellular site or organelles which are targeted cellular damage by means of apoptosis and necrosis. Another PDT induced response is vascular damage by means of vascular stasis, vascular hemorrhage, and hypoxia.

Inflammatory that leads to tumor destruction is indirect action of PDT. (48; 49; 50; 51; 52; 1). The PDT procedure is given Figure 2.4.

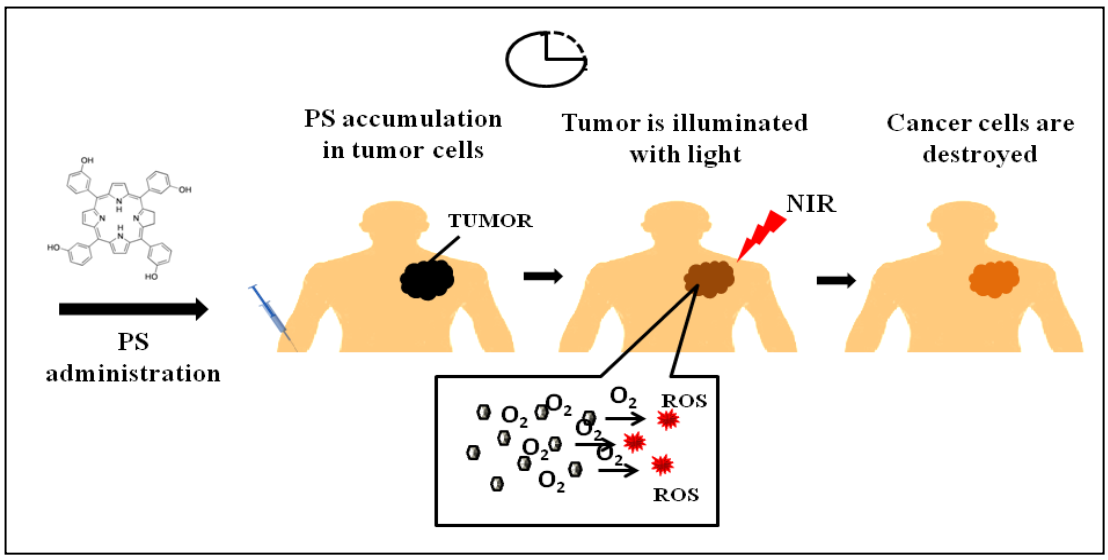

Figure 2.4 PDT procedure. Firstly PS are administrated and accumulated in tumor cells. And then this field is illuminated with light therefore destroying tumor cells probably through formation of singlet oxygen.

PDT has lots of advantage by comparison with traditionally treatment methods such as localized treatment in order words minimal toxicity healthy tissue, reproducible, low cost and ease of use. PDT is efficacious alternative methods in clinical. It is proved with successful result from clinical trials (53; 54; 1; 55).

2.2.1 Photosensitizer

Photosensitizers are chemical compounds which has not curative effect without illumination. Photosensitizers absorb light and generate reactive oxygen species. Photosensitizer is given topical or systemically and accumulation in target tissue. Photosensitizer that is in the ground state (S_0) is illuminated with red or near infrared light source which has appropriate wavelength and it transpose from ground state to excited singlet state (S_1) and then it is transpose from excited singlet state to excited triplet state (T_1) via intersystem crossing. The photosensitizer reacts with substrate (type 1 reaction) to generate superoxide or other radical species (H_2O_2) or the energy transferred to molecular oxygen (type 2 reaction) to generate singlet oxygen ($^1O_2$). The type 2 reaction is expected to occur during the PDT. The photochemical reaction occurs in the tumor cells thus singlet oxygen ($^1O_2$) is emerged which is eradicated the tumor

cells without affecting normal tissue. Accumulation of singlet oxygen leads to oxidative stress or injures the vasculature of tumor or surrounding healthy vessels or induces to damage on DNA in tumor cells (56; 57; 58; 59). The Photooxidation mechanism is abstracted in Figure 2.5.

New and impressive photosensitizers have been improved to get best outcome in PDT. Photosensitizers should be not toxicity in dark, available to selectively accumulate in tumor tissue, hydrophobic due to rapidly penetrate the cell membrane, pure chemical compound, absorb at a wavelength in therapeutic window because of the penetration of light increases in tissue (60; 61; 1).

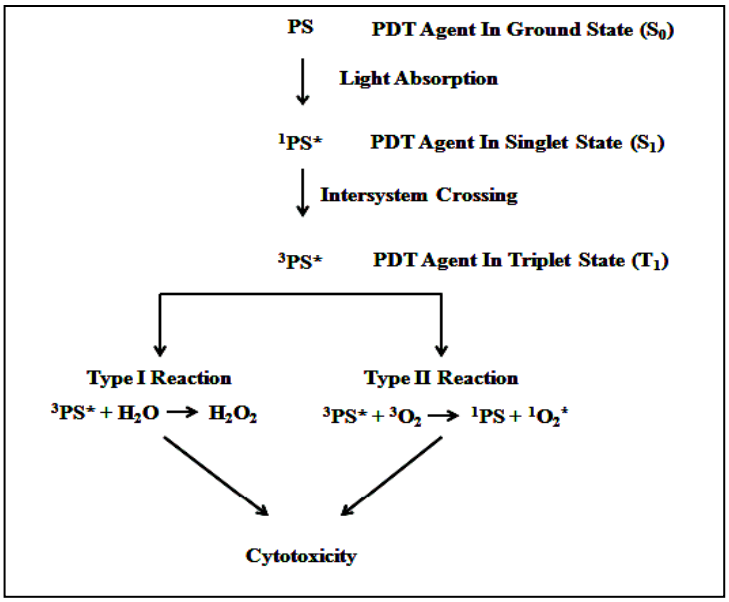

Figure 2.5 Photooxidation Mechanism (1).

Frequently, several PS are used as first, second and third generation PS in PDT. The first generation photosensitizers are porphyrin based and included Hematoporphyrin and its derivatives (HpD). Porphyrin is first photosensitizer approved by FDA for PDT. The second generation photosensitizers are chemical pure and simple (61) such as 5-aminolevulinic acid (ALA), benzoporphyrin derivatives (BDP), m-tetrahtdroxyphenylchlorin (mTHPC) and talaporfin sodium. Third generation photosensitizer are reoccurred that proper drugs are modified with antibodies or protein/receptor systems or a radioactive tag (62).

2.2.1.1 FOSCAN®

FOSCAN® is approved for treatment of head and neck squamous cell carcinoma by the European Union in 2001 which is second generation photosensitizers. FOSCAN® has strong absorption peak in the red part of spectrum (652 nm) which means deeper light penetration than HpD. FOSCAN® is a hydrophobic chlorine derivates thus it can readily penetrate with cell membrane (8; 61; 63; 1). The molecular structure of FOSCAN® is shown in Figure 2.6.

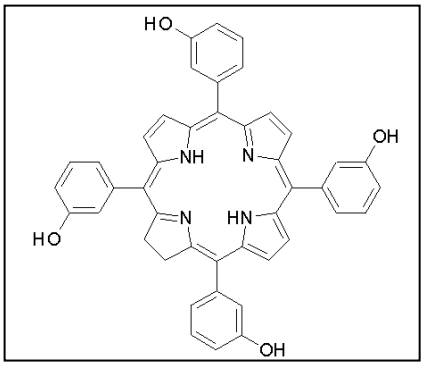

Figure 2.6 FOSCAN® chemical structure.

2.2.2 Light Sources

A variety of light sources have been used in PDT these are lasers, light emitting diodes (LEDs) and filtered lamps. The lasers are more preferred in PDT application because of monochromatic by this mean is the chosen wavelength can exactly match the absorption spectra of chosen photosensitizer, coherent by this mean is the light can focus to specific site, intense by this mean is treatment take less time. However, lasers can applied for small skin lesion because of spot size, whereas nonlaser light sources can applied for the treatment of large skin lesion. The laser light was delivered directly into the inaccessible body site thanks to optical fiber (60; 64; 46; 40; 65; 66; 67; 68). The noncoherent light sources are the first light source in PDT which are safer, ease to use and low cost. However, hyperthermia and low light intensity are limited to use in PDT (55).

The chosen light sources have to provide intensity up to 200mW/cm^2 for oncological indication in PDT. However, when light source have over 150mW/cm^2 fluence rate, it can induced hyperthermia. For this reason, the effective light intensity should be used in short treatment period. The light is interact with skin which is occur chromophors

(melanin, hemoglobin) and they are decreased the penetration of light into skin. The between 600-700 nm region of the spectrum are most penetrate 50-200 % more than the between 400-500 nm region of the spectrum in most tissue. The between 600-1300 nm region of spectrum are absorbed chromophors that is called therapeutic window/optical window (69; 5; 40) (Figure 2.7).

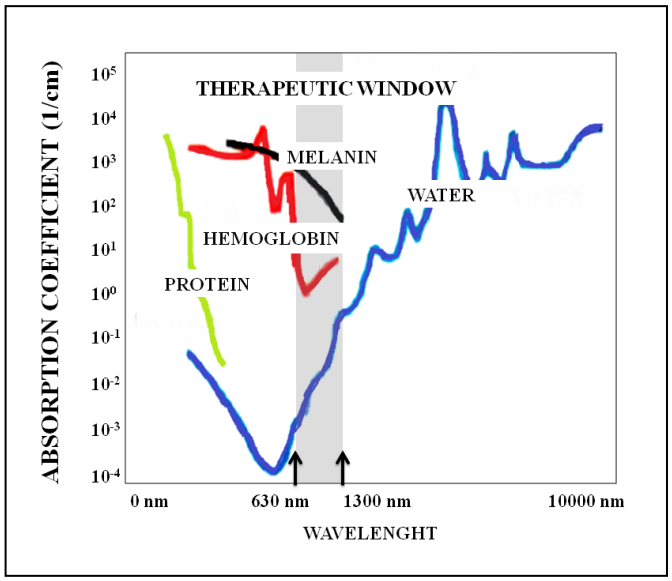

Figure 2.7 The Absorption Spectra for Biological Tissue.

CHAPTER 3

MATERIALS AND METHODS

This study supported by The Bezmialem Foundation University and The Fatih University, was executed between May 2013 and December 2013.

This research was made with permission No.71306642/050-01-04-72 and date of 25.03.2013 by The Bezmialem Foundation University of Ethical Committee.

3.1 Patient Profile

We studied sample from 8 female patients which admitted to Bezmialem Foundation University Faculty of Medicine the Department of General Surgery with proven invasive ductal breast carcinoma. Patients were evaluated according to grade, stage, levels of Ki67, diameter and molecular status by means of luminal A and luminal B and estrogen receptors. The breast cancer patients' properties are abstracted in Table 3.1.

Table 3.1. The breast cancer patients' properties

Patient	Molecular Subtype	ER Receptor	Grade	Ki67	Diameter
1	Luminal B	90	3	40	16
2	Luminal B	80	3	40	35
3	Luminal B	95	2	20	40
4	Luminal B	90	3	25	35
5	HER 2	90	3	25	38
6	Luminal B	95	3	30	45
7	Luminal B	90	3	20	45
8	Luminal A	90	1	15	35

The summary of study was given in Figure 3.1.

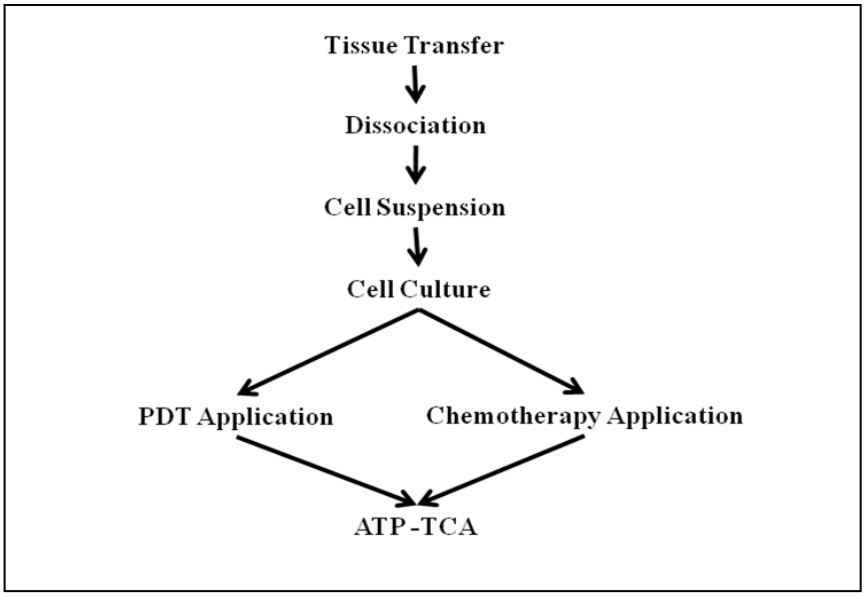

Figure 3.1 The Summary of Study.

3.2 Tissues Transfer

The samples were taken from 8 patents who were underwent surgical operation. The samples were transferred in specific medium supplemented with 1 % penicillin/streptomycin and 1 % HEPES (Gibco, Grand Island, NY, USA). The samples were mechanically digested with lancet and then incubated with Tumor Digestion Enzyme (TDE) which was used 2.5 mL and was diluted with 2.5 mL Chorioallantoic Membrane (CAM) medium overnight at 37 $^{\circ}$C and 5% CO_2 in a humidified incubator.

3.3 Cell Culture

Homogenized sample were centrifuged at 800 rpm for 8 min and then supernatant was aspirated with a pasteur pipet without disturbing the cell pellet. The cell pellet was resuspended with 1mL CAM and then CAM was added to reach volume of 5mL and then cell count was performed. The $1x10^4$ /90 µL cells were plated per each well in the 96 U-bottom well plates and then incubated 72 hour at 37 $^{\circ}$C and 5 % CO_2 in a humidified incubator. The plate layout and groups were summarized in Figure 3.2.

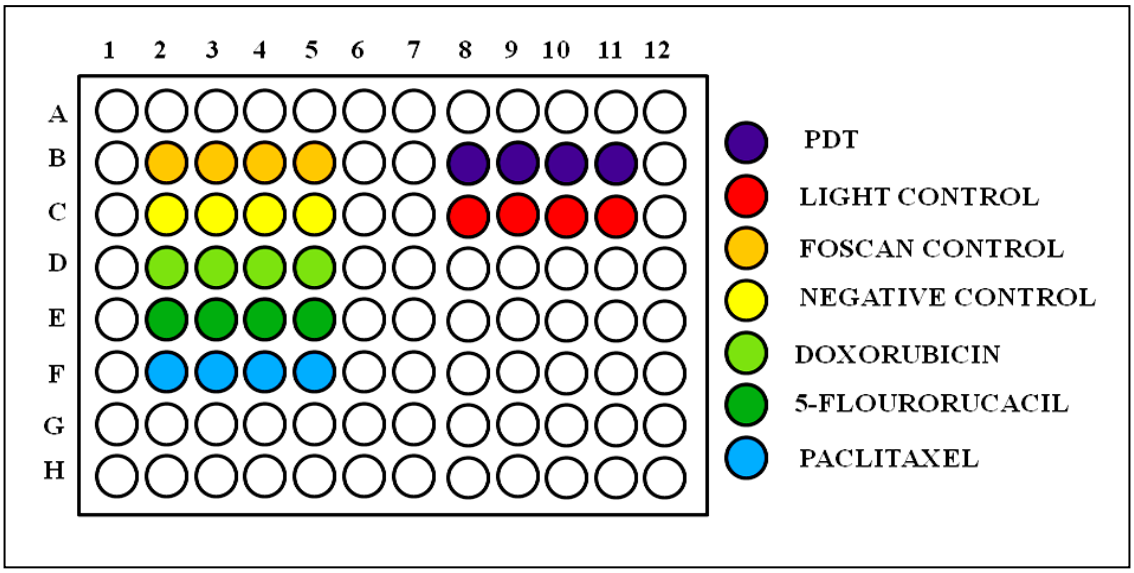

Figure 3.2 Plate layout.

3.4 PDT Application on Primary Breast Cancer

3.4.1 Light Source

Laser system was manufactured by Optotronics, Canada, USA which has infrared spectrum at 635nm wavelength with output power from 100 mW to 400 mW. The laser high stability output of within less than 5% over 8 hours. The laser diameter at aperture is 3X4 mm. The laser working mode is continuous wave. The laser is used thermoelectric cooling system to ensure stable output over long periods (Figure 3.3).

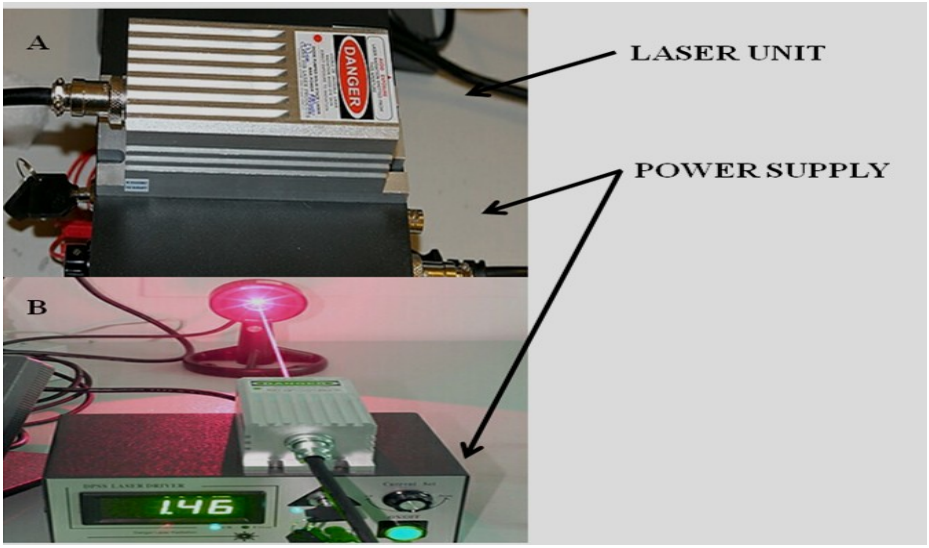

Figure 3.3 The light source. A) Side view of laser and power supply. B) Front view of power supply and laser unit and its during power measurement.

3.4.2 PDT Protocol

The FOSCAN® was supplied by Biolitec GmpH, Jena, Germany. The stock solution concentration was 4 mg/mL and was aliquoted to store at +4 ˚C in the dark. The 2,67 µL of FOSCAN® from stock solution was involved with 297,33 µL CAM to reach final concentration of 5µM of FOSCAN®. 10 µL prepared FOSCAN® was added in the FOSCAN control and PDT groups and then they were incubated for 24 hour at 37 ˚C and 5% CO_2 in a humidified incubator. At the same time, light control and negative control groups were added fresh 10 µL CAM medium. After the incubation, the cells were irradiated with 635 nm diode laser (Optotronics, Canada, USA) via bare fiber (Thorlabs GmbH Dachau/Munich, Germany) for 5 min at fluence rate 18 J/cm^2 at room temperature and then the cells were incubated 72 hour at 37 ˚C and 5% CO_2 in a humidified incubator. The PDT application was summarized in Figure 3.4 and Figure 3.5.

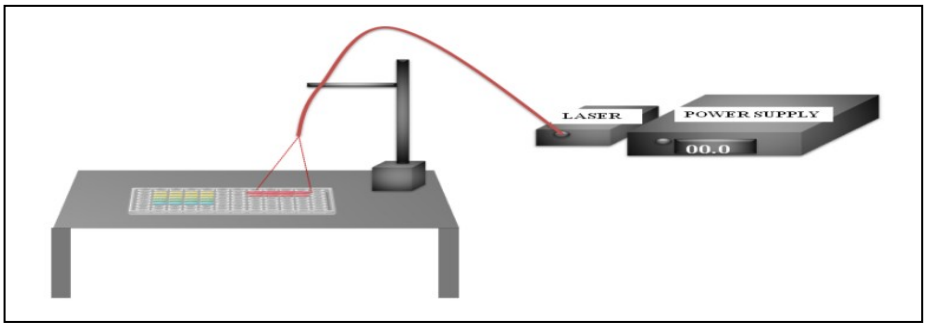

Figure 3.4 The detailed drawing of the *in vitro* treatment set-up for FOSCAN mediated PDT.

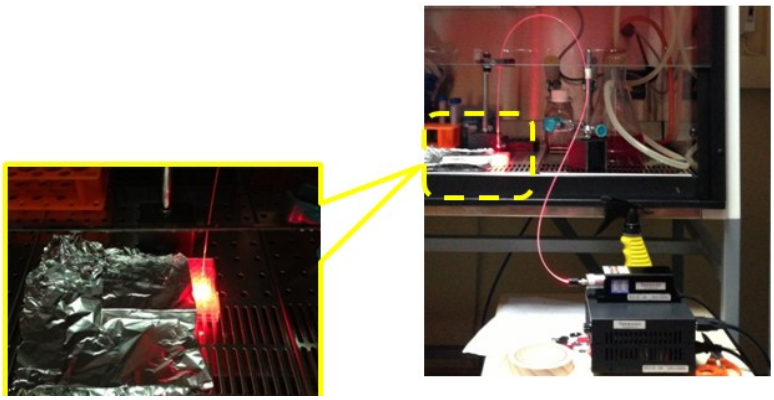

Figure 3.5 The cells were incubated 72 hours with FOSCAN. PDT and light control groups were illuminated with light at 635 nm for 5 min. Aluminum foil is used to avoid other groups' interference from laser.

3.5 Chemotherapy Application On Primary Breast Cancer

The Paclitaxel was supplied by Orna Ilaç, Turkey. The stock solution was stored in aliquot at +4 ˚C. The 90,5µL of Paclitaxel from stock solution was involved with 5 µL CAM to prepare 800 % test drug concentration (TDC) and then 100 µL diluted serially down to reach 100 % TDC. The 10 µL of prepared Paclitaxel was added in Paclitaxel groups and then they were incubated for 72 hour at 37 ˚C and 5% CO_2 in a humidified incubator.

The Doxorubicin was supplied by Med-Ilaç, Turkey. The stock solution was stored in aliquots at -20^{o}C. The 10 µL of Doxorubicin from stock solution was involved with 5 µL CAM to prepare 800 % test drug concentration (TDC) and then 100 µL diluted serially down to reach 100 % TDC. The 10 µL of prepared Doxorubicin was added in Doxorubicin groups and then they were incubated for 72 hour at 37 ˚C and 5% CO_2 in a humidified incubator.

The 5-Flourorucacil was supplied by Koçak Farma, Turkey. The stock solution was stored at room temperature in a cool and dark place. The 18 µL of 5-Flourorucacil from stock solution was involved with 5 µL CAM to prepare 800 % test drug concentration (TDC) and then 100 µL diluted serially down to reach 100 % TDC. The 10 µL of prepared 5-Flourorucacil was added in 5-Flourorucacil groups and then they were incubated for 72 hour at 37 ˚C and 5 % CO_2 in a humidified incubator.

3.6 ATP-TCA Assay

The ATP-TCA (DCS Innovative Diagnostika-Systeme, Hamburg, Germany) assay was used to examine viability. Three days after treatment, the cellular ATP levels were determined with ATP-TCA assay. All equipments were reached to room temperature before the ATP-TCA assay. The 50 µL of Tumor Cell Extraction Reagent (TCER) was added and mixed to each well to was extracted ATP in the cells and then were allowed to stand for 20-30 min at room temperature. The 50 µL of medium and the 50 µL luciferin–luciferase counting reagent were transferred to white plate. The white plate was measured in luminometer for 10-20 seconds.

CHAPTER 4

RESULTS

The cells of eight breast cancer patients have been treated with FOSCAN® mediated PDT, Paclitaxel, Doxorubicin and 5-Fluororacil and then analyzed using ATP-TCA assay. Figures 14-21 are depicted FOSCAN® mediated PDT on primer breast cancer cells viability.

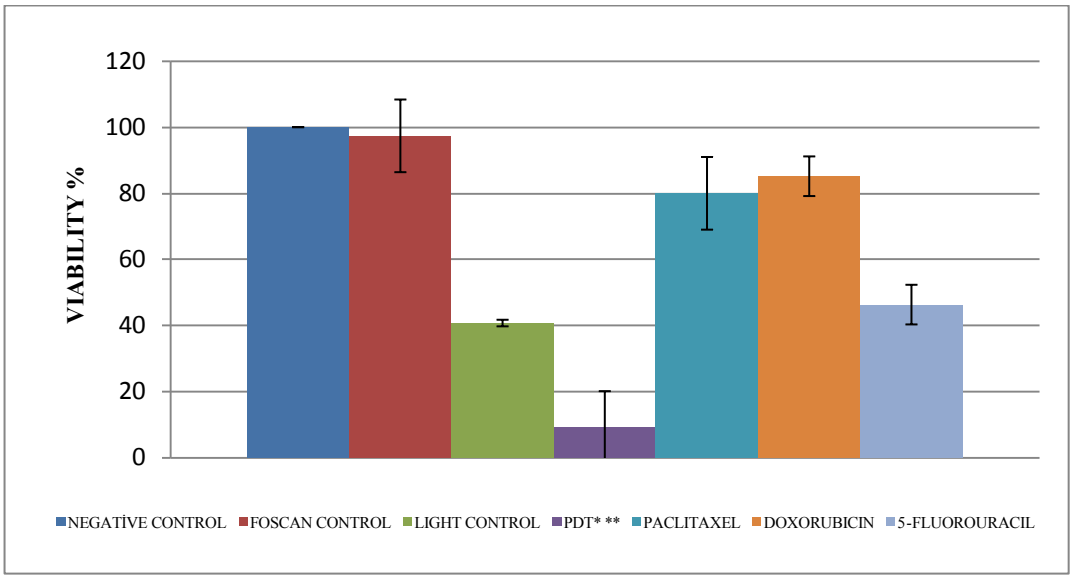

Figure 4.1 ATP-TCA assay. Percentage of viability produced by applied chemotherapeutic drugs and FOSCAN® mediated PDT on primer breast cancer cells viability that have been incubated for 24 h and then exposed to 18 J/cm^2. (*) Represents significant difference between PDT and control groups: $p<0.01$. (**) Represents significant difference between PDT and chemotherapeutic drug groups: $p<0.01$.

The ATP-TCA assay results of first patient were depicted in Figure 4.1. The bar chart illustrates the viability of four groups' namely negative control, FOSCAN® control, light control, PDT, Paclitaxel, Doxorubicin and 5-Flourouracil. It was observed that when the cells were applied with FOSCAN®, percentage of viability approximately was 97 % at a concentration of 10 µM. However, when the cells were illuminated with 652 nm diode laser at fluence rate 18 J/cm^2 for 5 min, percentage of viability was 40%. Finally, when the cells were treated with FOSCAN® mediated PDT; percentage of viability is 9%. The cells were treated with Paclitaxel, Doxorubicin and 5-Flourouracil; percentage of viability is respectively 79,97 %, 85,14 % and 46,27 % at the 100 TDC.

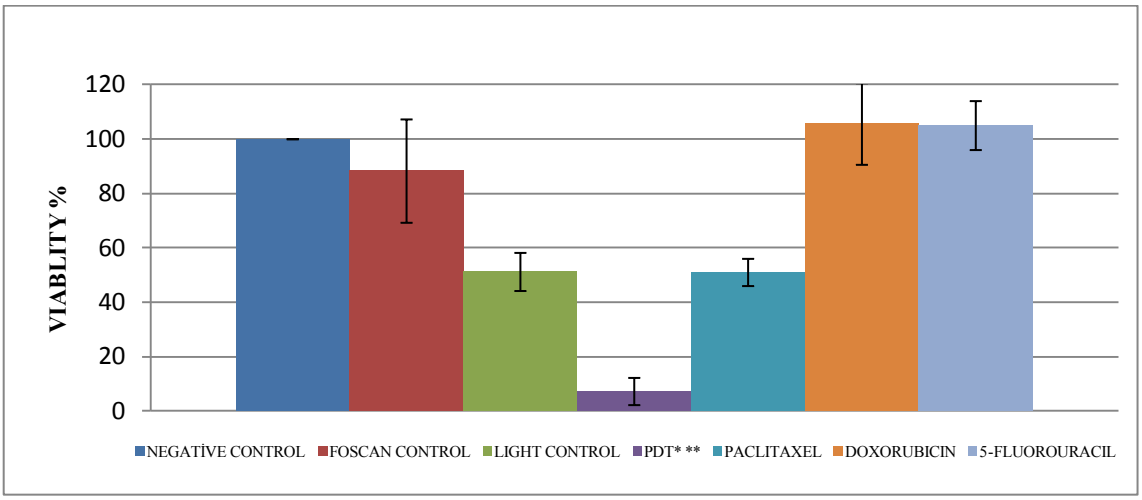

Figure 4.2 ATP-TCA assay. Percentage of viability produced by applied chemotherapeutic drugs and FOSCAN® mediated PDT on primer breast cancer cells viability that have been incubated for 24 h and then exposed to 18 J/cm^2. (*) Represents significant difference between PDT and control groups: $p<0.01$. (**) Represents significant difference between PDT and chemotherapeutic drug groups: $p<0.01$.

The ATP-TCA assay results of second patient were depicted in Figure 4.2. The bar chart illustrates the viability of four groups' namely negative control, FOSCAN® control, light control, PDT, Paclitaxel, Doxorubicin and 5-Flourouracil. It was observed that when the cells were applied with FOSCAN®, percentage of viability approximately was 88 % at a concentration of 10 µM. However, when the cells were illuminated with 652 nm diode laser at fluence rate 18 J/cm^2 for 5 min, percentage of viability was 51 %. Finally, when the cells were treated with FOSCAN® mediated PDT; percentage of

viability is 7 %. The cells were treated with Paclitaxel, Doxorubicin and 5-Flourouracil; percentage of viability is respectively 51 %, 105 % and 105 % at the 100 TDC.

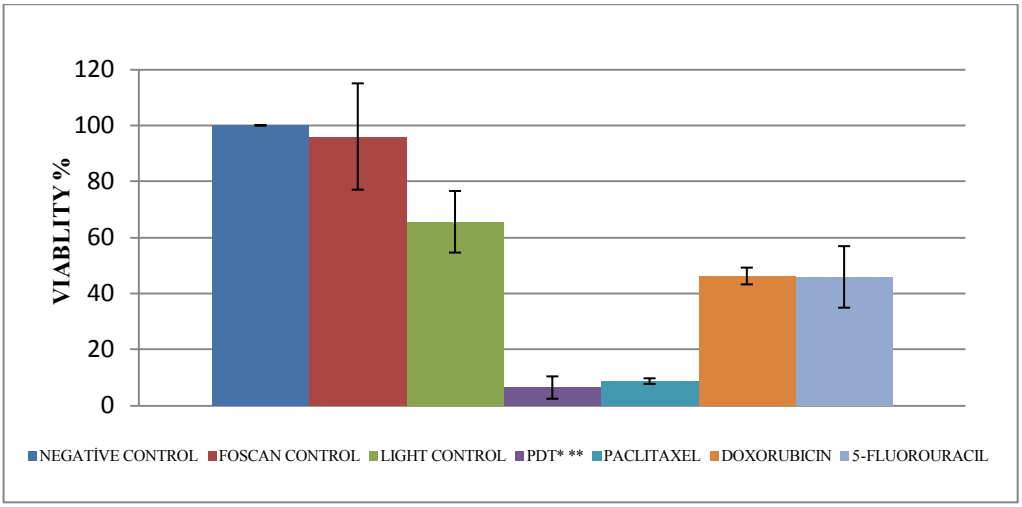

Figure 4.3 ATP-TCA assay. Percentage of viability produced by applied chemotherapeutic drugs and FOSCAN® mediated PDT on primer breast cancer cells viability that have been incubated for 24 h and then exposed to 18 J/cm^2. (*) Represents significant difference between PDT and control groups: $p<0.01$. (**) Represents significant difference between PDT and chemotherapeutic drug groups except for Paclitaxel: $p<0.01$.

The ATP-TCA assay results of third patient were depicted in Figure 4.3. The bar chart illustrates the viability of four groups' namely negative control, FOSCAN® control, light control, PDT, Paclitaxel, Doxorubicin and 5-Flourouracil. It was observed that when the cells were applied with FOSCAN®, percentage of viability approximately was 96 % at a concentration of 10 µM. However, when the cells were illuminated with 652 nm diode laser at fluence rate 18 J/cm^2 for 5 min, percentage of viability was 65 %. Finally, when the cells were treated with FOSCAN® mediated PDT; percentage of viability is 6 %. The cells were treated with Paclitaxel, Doxorubicin and 5-Flourouracil; percentage of viability is respectively 8,60 %, 46,16 % and 45,84 % at the 100 TDC.

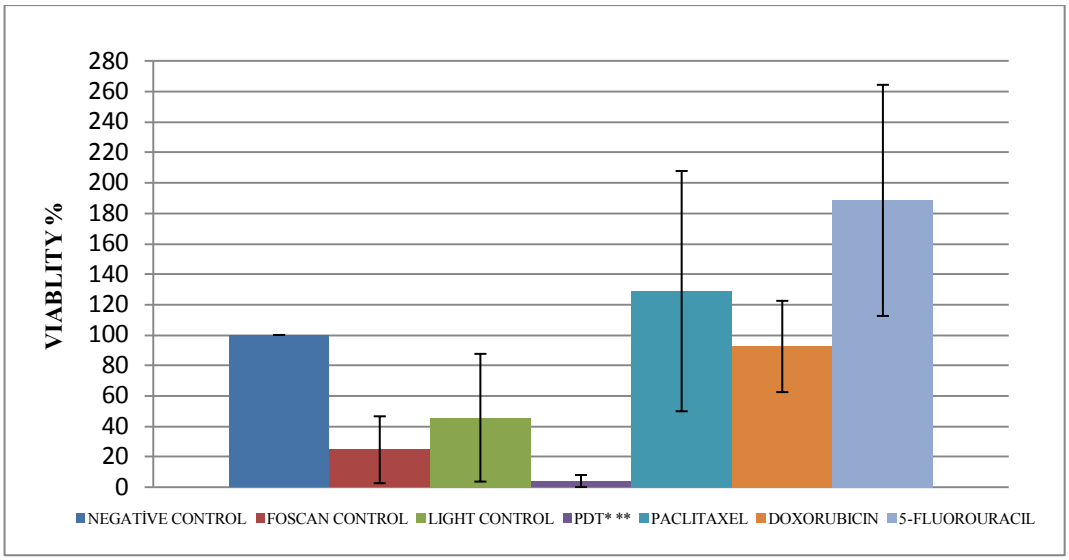

Figure 4.4 ATP-TCA assay. Percentage of viability produced by applied chemotherapeutic drugs and FOSCAN® mediated PDT on primer breast cancer cells viability that have been incubated for 24 h and then exposed to 18 J/cm^2. (*) Represents significant difference between PDT and control groups: $p<0.01$. (**) Represents significant difference between PDT and chemotherapeutic drug groups: $p<0.01$.

The ATP-TCA assay results of fourth patient were depicted in Figure 4.4. The bar chart illustrates the viability of four groups' namely negative control, FOSCAN® control, light control, PDT, Paclitaxel, Doxorubicin and 5-Flourouracil. It was observed that when the cells were applied with FOSCAN®, percentage of viability approximately was 24 % at a concentration of 10 µM. However, when the cells were illuminated with 652 nm diode laser at fluence rate 18 J/cm^2 for 5 min, percentage of viability was 45 %. Finally, when the cells were treated with FOSCAN® mediated PDT; percentage of viability is 3 %. The cells were treated with Paclitaxel, Doxorubicin and 5-Flourouracil; percentage of viability is respectively 128,83 %, 92,44 % and 188,50 % at the 100 TDC.

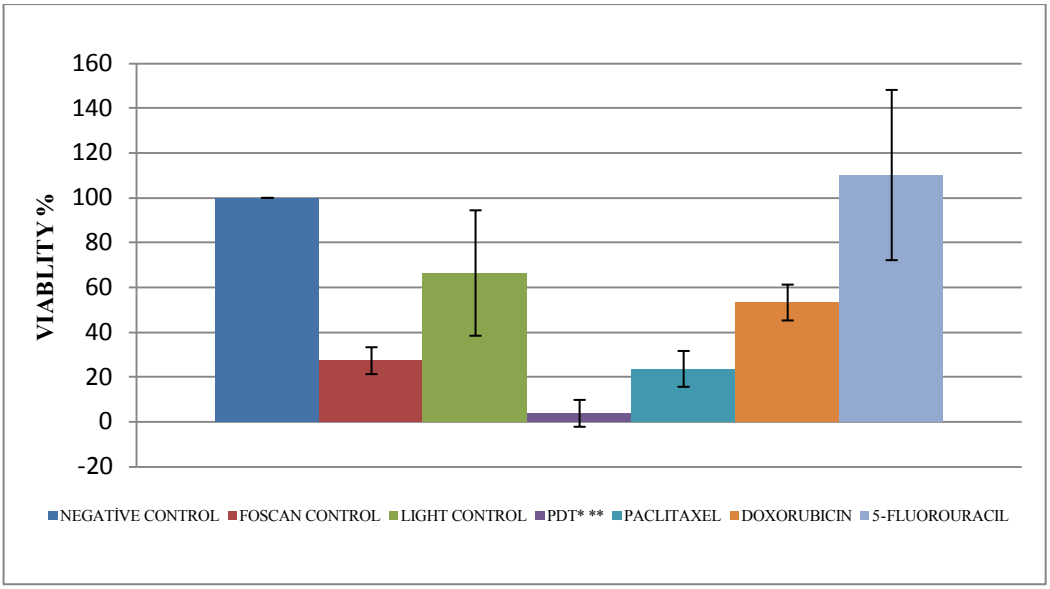

Figure 4.5 ATP-TCA assay. Percentage of viability produced by applied chemotherapeutic drugs and FOSCAN® mediated PDT on primer breast cancer cells viability that have been incubated for 24 h and then exposed to 18 J/cm^2. (*) Represents significant difference between PDT and control groups: $p<0.01$. (**) Represents significant difference between PDT and chemotherapeutic drug groups: $p<0.01$.

The ATP-TCA assay results of fifth patient were depicted in Figure 4.5. The bar chart illustrates the viability of four groups' namely negative control, FOSCAN® control, light control, PDT, Paclitaxel, Doxorubicin and 5-Flourouracil. It was observed that when the cells were applied with FOSCAN®, percentage of viability approximately was 27 % at a concentration of 10 µM. However, when the cells were illuminated with 652 nm diode laser at fluence rate 18 J/cm^2 for 5 min, percentage of viability was 66 %. Finally, when the cells were treated with FOSCAN® mediated PDT; percentage of viability is 3 %. The cells were treated with Paclitaxel, Doxorubicin and 5-Flourouracil; percentage of viability is respectively 23,67 %, 53,30 % and 110,20 % at the 100 TDC.

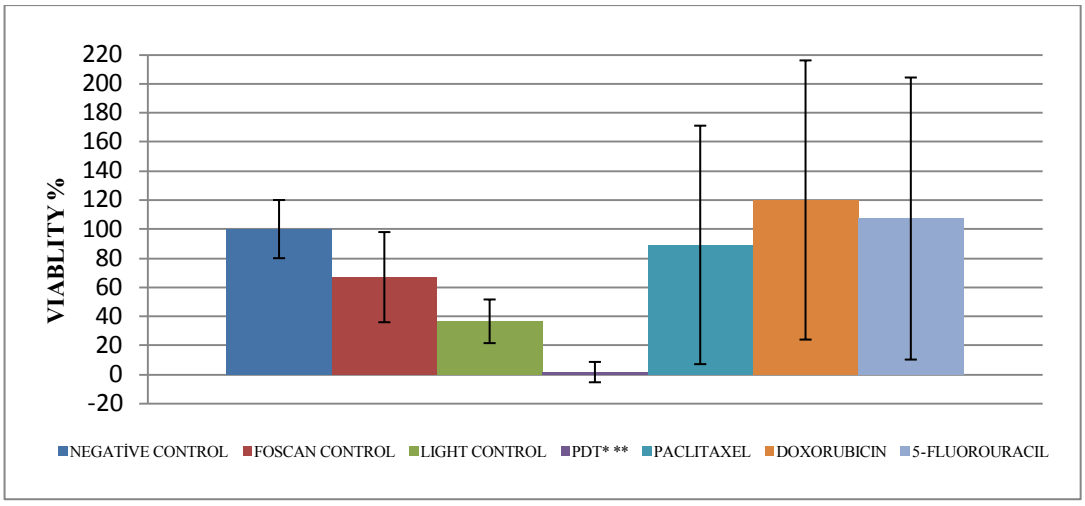

Figure 4.6 ATP-TCA assay. Percentage of viability produced by applied chemotherapeutic drugs and FOSCAN® mediated PDT on primer breast cancer cells viability that have been incubated for 24 h and then exposed to 18 J/cm^2. (*) Represents significant difference between PDT and control groups: $p<0.01$. (**) Represents significant difference between PDT and chemotherapeutic drug groups: $p<0.01$.

The ATP-TCA assay results of sixth patient were depicted in Figure 4.6. The bar chart illustrates the viability of four groups' namely negative control, FOSCAN® control, light control, PDT, Paclitaxel, Doxorubicin and 5-Flourouracil. It was observed that when the cells were applied with FOSCAN®, percentage of viability approximately was 66 % at a concentration of 10 µM. However, when the cells were illuminated with 652 nm diode laser at fluence rate 18 J/cm^2 for 5 min, percentage of viability was 36 %. Finally, when the cells were treated with FOSCAN® mediated PDT; percentage of viability is 1 %. The cells were treated with Paclitaxel, Doxorubicin and 5-Flourouracil; percentage of viability is respectively 89,13 %, 120,02 % and 107,27 % at the 100 TDC.

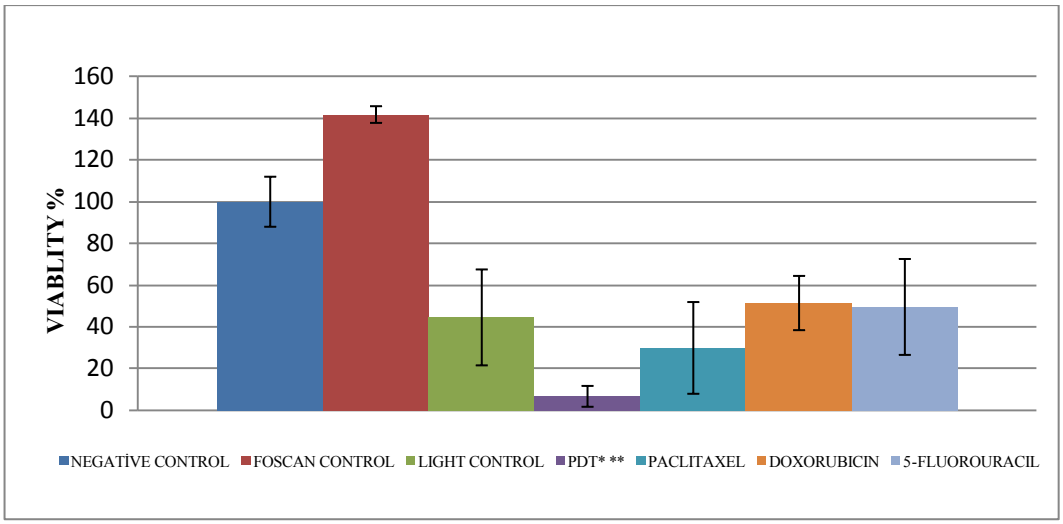

Figure 4.7 ATP-TCA assay. Percentage of viability produced by applied chemotherapeutic drugs and FOSCAN® mediated PDT on primer breast cancer cells viability that have been incubated for 24 h and then exposed to 18 J/cm^2. (*) Represents significant difference between PDT and control groups: $p<0.01$. (**) Represents significant difference between PDT and chemotherapeutic drug groups: $p<0.01$.

The ATP-TCA assay results of seventh patient were depicted in Figure 4.7. The bar chart illustrates the viability of four groups' namely negative control, FOSCAN® control, light control, PDT, Paclitaxel, Doxorubicin and 5-Flourouracil. It was observed that when the cells were applied with FOSCAN®, percentage of viability approximately was 141 % at a concentration of 10 µM. However, when the cells were illuminated with 652 nm diode laser at fluence rate 18 J/cm^2 for 5 min, percentage of viability was 44 %. Finally, when the cells were treated with FOSCAN® mediated PDT; percentage of viability is 6 %. The cells were treated with Paclitaxel, Doxorubicin and 5-Flourouracil; percentage of viability is respectively 29,90 %, 51,42 % and 49,55 % at the 100 TDC.

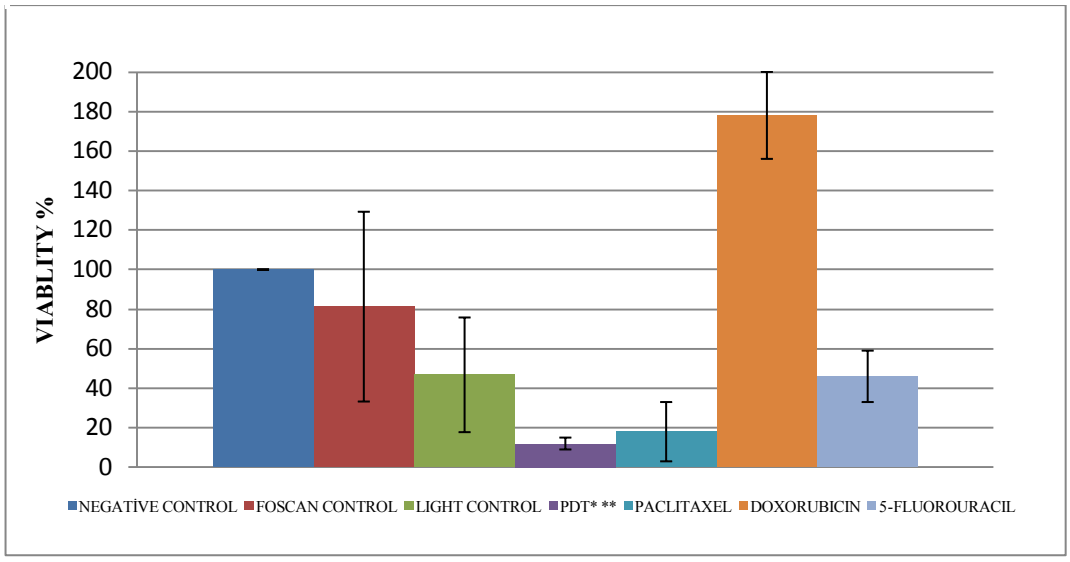

Figure 4.8 ATP-TCA assay. Percentage of viability produced by applied chemotherapeutic drugs and FOSCAN® mediated PDT on primer breast cancer cells viability that have been incubated for 24 h and then exposed to 18 J/cm^2. (*) Represents significant difference between PDT and control groups: $p<0.01$. (**) Represents significant difference between PDT and chemotherapeutic drug groups except for Paclitaxel: $p<0.05$.

The ATP-TCA assay results of seventh patient were depicted in Figure 4.8. The bar chart illustrates the viability of four groups' namely negative control, FOSCAN® control, light control, PDT, Paclitaxel, Doxorubicin and 5-Flourouracil. It was observed that when the cells were applied with FOSCAN®, percentage of viability approximately was 81 % at a concentration of 10 µM. However, when the cells were illuminated with 652 nm diode laser at fluence rate 18 J/cm^2 for 5 min, percentage of viability was 46 %. Finally, when the cells were treated with FOSCAN mediated PDT; percentage of viability is 12 %. The cells were treated with Paclitaxel, Doxorubicin and 5-Flourouracil; percentage of viability is respectively 18 %, 178 % and 46 % at the 100 TDC.

CHAPTER 5

DISCUSSION AND CONCLUSION

The recent advances in PDT make it a promising cure method for several cancer treatments in the clinical implementation. FOSCAN® is proper PS and has been used for head and neck cancer therapy. The antigrowth effect of FOSCAN® mediated PDT, Paclitaxel, Doxorubicin, 5-Fluorouracil was investigated on primary breast cancer cell lines by the ATP-TCA in this study.

Cell viability of first patient and second patient who were treated with Paclitaxel, having same pathological features of Luminal B, grade 3 and 40 % of Ki-67 labeling index, was decreased to 79 % and 51 % respectively. Ki67 presents in progressing and growing cells during of G1, G2 and S phases of cell cycle. When the cells are dividing to proliferate, Ki67 level is increased (70). Paclitaxel is effective especially during G1 phase of tumor cells. Ki67 levels are demonstrated that many of tumor cells are in G1 and G2 for these patients. Therefore, Paclitaxel was more effective than other chemotherapeutic drugs Tumor tissue diameters were 16 mm and 35 mm for first and second patient respectively. The effect of Doxorubicin and 5-Fluorouracil was inversely proportional with tumor diameter**.** Results of the FOSCAN® toxicity trials proved that concentration of 5 µM of FOSCAN was not toxic for these patients. The effect of FOSCAN mediated PDT was not altered according to molecular subtype, grade, diameter and the value of Ki67 levels

Fourth and fifth patients having same pathological features of Grade 3 and 25 % of Ki-67 labeling index, but only molecular subtype was different, these are Luminal B and HER 2 respectively The clinical progress of HER 2 breast cancers are unrecoverable and have poor prognosis. The clinical trials demonstrated that free survival rate was increased with chemotherapy regimens combined with Paclitaxel, carboplatin and

trastuzumab (71). Cell viability was decreased to 23 % in fifth patient by Paclitaxel but it was increased up to 128 % in fourth patient with Luminal B plus the value of 25% of Ki67 labeling index because of characteristics of Paclitaxel. Doxorubicin was impotent (P=0,862) as well, cell proliferation was increased up to 188%. Due to anthracycline resistance in fourth patient with Luminal B, breast cancer treated with combined Docetaxel and Doxorubicin were avoided (72). However Doxorubicin positively affected the survival rate in fifth patient with HER 2. On the other hand these patients were not affected by 5- Fluorouracil. The effect of FOSCAN mediated PDT was not altered according to molecular subtype, grade, diameter and the value of Ki67 levels decreased viability to 3 % in these patients.

Third patient with Luminal B, Grade 2, the 25 % of Ki-67 labeling index and eighth patient with Luminal A, Grade 1, the 15% of Ki-67 labeling index were sensitive to chemotherapy induced with Paclitaxel (Eighth Patient of P= 0,001; Third Patient P= 0,000008) and 5-Fluorouracil that decreased the viability to 45% and 49% respectively. Doxorubicin was impotent (P=0,313) due to anthracycline resistance in eighth patient but viability was decreased to 46% in high grade with Luminal B. The effect of FOSCAN mediated PDT was not altered based on the clinical behavior and pathological features in these patients.

Sixth patient with Luminal B, Grade 3, 30% of Ki-67 labeling index; seventh patient with Luminal B Grade 3, 20% of Ki-67 labeling index were more sensitive to chemotherapy induced with Paclitaxel than Doxorubicin and 5-Fluorouracil. Paclitaxel inhibiting mitosis decreased cell viability up to 89% and 29% respectively. Paclitaxel may increase fatal effect on cells by combined regimens with Gemcitabin. In clinical trials it was proved that combination of Paclitaxel and Gemcitabin was more effective than only Paclitaxel on metastatic breast cancer (73). Doxorubicin was impotent due to anthracycline resistance for these patients. The effect of FOSCAN® mediated PDT was not altered according to molecular subtype, grade, diameter and the value of Ki67 levels that it was decreased viability up to 1% in these patients.

The effect of only light was almost same for all patients who viability was decreased to 36%. It may have occurred because of red light treatment effect. Red light stimulates natural defense system and is used for several cancer treatments (74)

All patients were observed to be not sensitive to 5 µM concentration of FOSCAN® except for fifth patient with HER 2 and fourth patient with Luminal B. Reason might be that the number of patient was not enough, or heterogeneity of patient groups,

FUTURE RECOMMENDATIONS STUDIES

FOSCAN® mediated PDT has been used to treat head and neck cancer that is approved by the European Union in 2001. Our study has shown that FOSCAN® mediated PDT is decreased viability up to 4% on primary breast cancer (P=0,0273). However, there were not represent significant differences between chemotherapeutic drugs; Paclitaxel (P= 0,17358), Doxorubicin (P= 0,7505), and 5-Fuorouracil (P= 0,3043) and negative control. 5µM of FOSCAN® was not toxic in dark.

In future studies, in order to enhance the efficacy of FOSCAN®-PDT, PS can be modified in the form of nanoparticules to increase uptake and distinctive selectivity of PS into cancerous tissue. PDT can combined with other treatment methods to increase prognosis and free survival rate.

In clinical trials it was proved that combination of chemotherapeutic drugs is more effective than alone on breast cancer treatment (75).

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CURRICULUM VITAE

Name Surname: **Saadet Akbulut**

Place and Date of Birth: **11.07.1989**

Address: **Fatih University/Biomedical Engineering Institute 34500, Büyükçekmece/İstanbul**

E-Mail: **akbulut_saadet@hotmail.com**

B.Sc.: Sakarya University, Faculty of Arts & Sciences, Departmants of Biology

Professional Experience and Rewards:

07.2010 - 08.2010 Worked as trainee of biologist in the ISKI Water Quality Laboratuary in TURKEY

06.2012 – 07. 2012 Worked as traniee of biologist in the Istanbul Okmeydanı Education and Research Hospital Pathology Laboratuvary in TURKEY

04.2013 – 12.2013 Worked as project assistant in entitled 'Fotodinamik Terapi ve Kemoterapinin Eşzamanlı in vitro Uygulamasının ex vivo Primer Meme Tümörlerindeki Etkisi' supported by Bezmialem Foundation University and Fatih University in TURKEY

05.2013- Continue Working as project assistant in entitled ‘MCF-7 Meme Tümörü Üzerinde ICG Molekülünün in vivo Fotodinamik Terapi Etkisinin Moleküler Analiz ve Doku Karakterizasyonu İle Belirlenmesi’ support by Tübitak in TURKEY.

List of Publications and Patents:

Akbulut S., and Uzun A., 2011: ‘Sapanca Gölü’nün Toplam Koliform ve Fekal Koliform Bakteri Yönünden İncelenmesi’ Undergraduate Thesis. June, 2011 Sakarya, TURKEY.

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Bireller S., Rencizogulları C., **Akbulut S.,** and Cakmakoglu B., Investigation of Hesperidin and Photodynamic Therapy On U87 Glioblastoma Cell Line November, 2013, TURKEY.

Kiris A., Kiris T., **Akbulut S**., Malya U., Gucin Z., Karatepe O., Tabakoglu O. H., Çift Toplayıcı Küre Sistemi İle Tümörlü ve Sağlıklı Pankreas Dokusunun Optik Özelliklerinin Karakterizasyonu: Prospektif Klinik Çalışmanın Erken Dönem Sonuçları, November, 2013 Antalya, TURKEY.