T.C.
FATIH UNIVERSITY
INSTITUTE OF BIOMEDICAL ENGINEERING

PAMAM modified porphyrin mediated Photodynamic Therapy effects on AGS stomach cancer cell lines: in vitro study

TUĞBA KİRİŞ

MSc THESIS
BIOMEDICAL ENGINEERING PROGRAMME

İSTANBUL, JUNE / 2013 (DEFENSE)

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THESIS ADVISOR
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İSTANBUL, JUNE / 2013 (DEFENSE)

T.C.
FATİH ÜNİVESİTESİ
BİYOMEDİKAL MÜHENDİSLİK ENSTİTÜSÜ

Pamam modifikasyonlu porfirin molekülünün AGS mide kanseri hücreleri üzerindeki fotodinamik terapi etkisinin in vitro araştırılması

TUĞBA KİRİŞ

YÜKSEK LİSANS TEZİ
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Tuğba Kiriş, a **MSc** student of Fatih University **Institute of Biomedical Engineering** student ID **52011103**, successfully defended the **thesis** entitled "**PAMAM modified porphyrin mediated Photodynamic Therapy effects on AGS stomach cancer cell lines: in vitro study** ''which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my dear family who supported me unquestioning on any ground,

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LIST OF SYMBOLS

®	Registered trademark symbol
μ	Micro
ν	Frequency
λ	Wavelength
h	Planck constant
l	Liter
M	Molar

ABBREVIATIONS

PDT	:Photodynamic Theraphy
PS	:Photosensitizer
PSs	:Photosensitizers
μM	:Micromolar
cm	:Centimeter
DMEM	:Dulbecco Modification Eagle Medium
DMSO	:Dimethyl Sulfoxide
FBS	:Fetal Bovine Serum
FCS	:Fetal Calf Serum
Hp	:Hematoporphyrin
hr	:Hour
i.v.	:Intravenous
J	:Joule
kg	:Kilogram
LED	:Light Emitting Diodes
mg	:Milligram
mm	:Millimeter
min	:Minute
nm	:Nanometer
PBS	:Phosphate Buffered Saline
PpIX	:Protoporphyrin IX
0PS	:Non-Excited Ground State Photosensitizer
1PS	:Excited Singlet State Photosensitizer

3PS	:Excited Triplet State Photosensitizer
ROS	:Reactive Oxygen Species
O2	:Oxygen
1O2	:Singlet Oxygen
3O2	:Triplet State Oxygen
-OH	:Hydroxyl Radical
CI	:Cell Index
UK	:United Kingdom
USA	:United States of America
US-FDA	:United States Food and Drug Administration

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SUMMARY

PAMAM modified porphyrin mediated Photodynamic Therapy effects on AGS stomach cancer cell lines: in vitro study

Tuğba KİRİŞ

Biomedical Engineering Programme

MSc Thesis

Advisor: Assist Prof. Dr. Haşim Özgür TABAKOĞLU

Stomach cancer is one of the most common cancer related death cause, according to the estimations 21,600 women and men will diagnosed and 10,900 of them are expected to die because of stomach cancer in 2013.

Early diagnosis of gastric cancer is somehow problematic, especially due to lack of specific symptoms and well defined risk factors. Recently use of endoscopic treatment procedure which take place of traditional surgical method outcomed in better survival rates. Currently laser ablation, photodynamic treatment, endoscopic mucosal resection and endoscopic stent application are the most common techniques.

One of the trending treatment option, photodynamic theraphy, was focused on for stomach cancer in this thesis study. AGS human stomach cancer cells which was obtained from Fatih University Cell Culture Laboratory has been targeted for the treatment. Aim of this study is the real time analysis of photodynamic theraphy application on AGS stomach cancer lines with Poly(amido amine) (PAMAM) which confined in dendrimer structure. Using the photosensitizers which modified with PAMAM is fairly new application. PAMAM which confined in dendrimer structure of the molecule is enable to easily uptake in cells and this way it shows effects. Some photosensitezers which don't target specific cell groups may show toxicant effect and can lead to transform healthy cells into cancer cells. We hypothesized that, dendrimer structures enable advantages and can penetrate in tumor tissue easily. Different energy

doses with different drug concentrations have been tested on tumor cells and investigated by real time cell viability, apoptosis and invasion.

Results from our experiments demonstrated that 50 µm and 75 µm are the best density amounts for PDT applications on in vitro AGS stomach cancer cell lines. Increasing the step of amination of molecule lead to cell death and increases the PDT effect. Poly(amido amine) can increase porphyrin uptake in cell more easily.

Keywords: PAMAM, Photodynamic theraphy, AGS, Porphyrin, Cancer treatment, LED .

FATIH UNIVERSITY - INSTITUTE OF BIOMEDICAL ENGINEERING

ÖZET

PAMAM modifikasyonlu porfirin molekülünün AGS mide kanseri hücreleri üzerindeki fotodinamik terapi etkisinin in vitro araştırılması

Tuğba KİRİŞ

Biyomedikal Mühendisliği Programı

Yüksek Lisans Tezi

Danışman: Yrd.Doç. Dr. Haşim Özgür TABAKOĞLU

Mide kanserinin insidansı son yıllarda azalmasına rağmen halen kansere bağlı ölüm sebeblerinde ilk sıralarda yer almaktadır. Mide kanseri, dünyada erkeklerde 2., kadınlarda 4. en sık görülen kanserdir. 2013 yılı istatistiklerine göre 21.600 kadın ve erkeğe mide kanseri tanısı konması ve 10.900 'ünün mide kanseri sebebiyle hayatlarının son bulması beklenmektedir.

Mide kanserinin özgün semptomlarının olmaması ve risk faktörlerinin iyi tanımlanmaması erken tanıyı zorlaştırmaktadır, tanı sıklıkla ileri evrede konmaktadır. Günümüzde erken tanının mümkün olduğu durumlarda morbidite ve mortalitesi yüksek, aynı zamanda daha pahalı olan cerrahi müdahale yerine endoskopik tedavi yöntemleri tercih edilmeye başlamıştır. Özellikle lazer ile uygulanan ablasyon ve koagülasyon teknikleri, endoskopik mukozal rezeksiyon ve fotodinamik terapi gibi minimal invaziv yöntemlere yönelim artmıştır.

Bu tez çalışmasında mide kanserinin fotodinamik terapi yöntemi ile tedavisi üzerine çalışıldı. Çalışma *in vitro* olarak Fatih Üniversitesi hücre kültürü laboratuvarı tarafından temin edilen AGS insan mide kanseri hücreleri üzerinden yürütülmüştür.

Çalışmanın amacı Poliamido amin (PAMAM) modifikasyonu yapılmış protoporfirin IX (PpIX) molekülünün AGS mide kanseri hücre hatları üzerindeki fotodinamik terapi etkisinin gerçek zamanlı olarak araştırılmasıdır. PAMAM modifikasyonu oldukça yeni bir uygulamadır. PAMAM modifikasyonu molekülü dendrimer yapısına hapsederek hücre içine alımı kolaylaştırmaktadır. Modifikasyon sayesinde ajan özellikle hedef bölgede birikmekte ve çevre sağlıklı dokulara toksik etki oluşturmamaktadır.

Hipotezimiz, denrimer yapının avantajını kullanarak ajanın tümör içine alımını kolaylaştırarak penetrasyon derinliğini arttırmaktır böylece fotodinamik terapinin etkinliğini en düzeye çıkarmaktır. Zaman değişken alınıp aynı güç değerinde, 5 farklı ajan konsantrasyonu denenerek optimum parametreler belirlenmeye çalışıldı. Gerçek zamanlı ölçümün avantajı olarak hücreler üzerine yapılan tüm işlemler aralıksız olarak kaydedildi. Böylece ilk an etkisini de gözlemlemek mümkün olmuştur.

Deney sonuçları göstermektedir ki 50 µm ve 75 µm fotodinamik terapi uygulamamız için ideal doz konsantrasyonlarıdır. Aminleme basamakları arttıkça (P_0, P_1, P_3, P_4) porfirin molekülünün hücre yaşamsallığı üzerindeki FDT' nin etkisini de arttığı böylece daha çok hücre ölümüne sebep olduğu görülmüştür.

Anahtar kelimeler: PAMAM , Fotodinamik terapi , AGS, porfirin, LED, Kanser.

FATİH ÜNİVERSİTESİ -BİYOMEDİKAL MÜHENDİSLİK ENSTİTÜSÜ

CHAPTER 1

1.1 Purpose of the Thesis

Photodynamic theraphy (PDT) is an emerging non surgical clinical treatment modality for solid tumors and many other nononcologic diseases that includes the administration of a photosensitizer drug and followed by irradiation with light which has a specific wavelength(s). In the presence of molecular oxygen a serial photochemical reactions occurs. This serial reactions lead to apotosis, immune response and vascular damage or direct tumor cell death.

The purpose of this thesis study is the real time analysis of photodynamic theraphy application of Poly(amido amine) (PAMAM) modified protoporphyrin (PpIX) molecules on stomach tumor cells (AGS cells). Different energy doses with different drug concentrations have been tested on tumor cells and investigated by real time cell viability, cytotoxicicity, and invasion.

1.2 Motivation

Cancer has become one of the most important public problem all around the worldwide. According to a World Health Organization (WHO) report which published in 2008, 7.4 million people die of cancer in 2004 and 83.2 million people will die in 2015. As regards the American Cancer Society estimations for United States 1,660,290 new cancer cases and 580,350 cancer deaths expected for 2013 [1-3]. Early diagnosis is crucial for extended survival time and quality.

For that reason, researchers have focused on understanding the molecular base of different cancers to make possible earlier diagnosis and to generate new treatment modalities. The best well-known treatment options are surgery, chemotherapy and radiotherapy. A fairly new and promising United States Food and Drug Administration (US-FDA) approved modalitiy with the use of light sources and photosensitizing substance is the Photodynamic Therapy (PDT) [4, 5]. The main advantages of PDT over other treatment methods are; its low systemic toxicity, particular tumor cell fatality without harmful the surrounding healthy tissue, minimal invasiveness and low-cost application. The best attractive point is that PDT can be well-tolerated and repeatable in case of recurrence areas [6-9]. However, it is fairly new method and there isn't enough background information for applications.

Part of this thesis was devoted to this essential need to investigate PDT effect on AGS human stomach cancer cells and to find best effective parameters for treatment to improve PDT responsiveness.

Another major challenge in PDT treatment is to develop new photosensitizers that can be used at wavelengths to treat deep tissue tumors and can be uptaken easily. In consequence of Poly(amido amine) PAMAM modification of PpIX molecule, penetration depth and increased cell uptake have been enabled. Thus, PAMAM modified porphyrin mediated PDT on stomach cancer cells were tested to assess whether this method presents an alternative tumor treatments in the future.

CHAPTER 2

2.1 Cancer

Cancer in medical term malignant neoplasm is a genetic diseases which considered by uncontrolled enlargement and proliferation of anomalous cells depend on acquisition of function of oncogenes and loss of function of tumor suppressors [3, 10-15]. Cancer is not just one disease but it is a group diseases. There are more than 100 different types of cancer. Generally each one is classified by the type of cell that is firstly affected.

Cancer commonly forms as a tumor which can be malignant or benign. Benign tumors rarely are life threatening and they grow within a confident capsule which borders their size and preserve the characteristics of the cell of origin. Malignant tumors occupy nearby tissues, than spread and deposits to other parts of the body it is also entitled metastasis [11, 12, 16, 17].

2.2 Cancer Therapies

An elaborated examination of survival rates of cancer patients all around the world has shown that most cancers are curable if early diagnosed. Early detection of cancer can highly increase the success of treatment. Thanks to early diagnosis of cancer increased interest in the conservation of normal tissue in cancer treatment methods [10, 18-20].

First and most common approach surgery gives place to non-invasive or minimally invasive therapeutic methods by force of development of medical technology, but surgery still has an significant place in diagnosis and also staging of cancer [7, 21].

Chemotherapy is another option for treatment of cancers with anticancer drugs. Chemotherapeutic agents destroy tumor tissue by restraining proliferation but these drugs also have cytotoxic side effects for healthy tissues. Due to this undesired side effects patients can feel uncomfortable and may result to review or early termination of therapy [22-24].

Radiation therapy is another method [6, 25, 26] for the damage of cancer cells especially solid tumors by use of high energy particles or waves such as x-ray, proton, gamma rays. Like chemotherapy, radiotherapy has a number of side effects armpit anxiety, chest pain, heart diseases, lowered white blood cell counts, fatigue, lung problems and diarrhea [27].

In addition to these commonly used methods; angiogenesis inhibitors, bone marrow transplantation, immunotherapy, ultrasound treatment [28-30], cryotherapy [29, 31] , gene therapy [20, 32] and photodynamic therapy are other alternative options for cancer treatment.

PDT is a FDA approved minimally invasive treatment option for the especially treatment of solid mass of cancer cells by using a light source and photosensitizing chemical agent. Main advantages of PDT over other therapy options include; localized treatment, minimal side effects to healthy tissue, repeatable and relatively less expensive cost, promising results have been obtained in a various of clinical trials PDT as an considerably improved the standart of living and life expectancy of patients and invaluable alternative for cancer therapies [6-8, 33-40].

2.3 Photodynamic Therapy

Light has been used for a treatment way more than 3 thousand years [41]. The scientific origin for modern light therapy begins with Danish physician Niels Finsen at the end of the nineteenth century [5, 42]. Finsen used ultraviolet light from the sun to cure cutaneous tuberculosis, smallpox and lupus vulgaris which are very common seen in Scandinavia at that times. In 1903 Fins got Nobel Prize for his discovery of Phototheraphy [43]. Almost at the same time a German student Oscar Raab informed that definite wavelengths of light were lethal for the organism "paramecia" exposed to

the chemical compound "acridine" in 1900 [44]. Herman von Tappeiner and A Jensionek later tried another chemical topically applied "eosin" to treat skin cancer under the white light. They called this phenomena "photodynamic action" in 1907 [45, 46].

Porphyrins which are the largely explored type of chemical compounds in PDT, were investigated by Friedrich Meyer-Betz beginning of 1913 [5, 6, 46]. PDT began to form in 1960s by the studies of Richard Lipson and Baldes [47]. They worked on the tumor accumulation of hematoporyrin derivative (HpD) which prepared by Dr. Samual Schwartz. Thanks to Dougherty and his colleagues HpD was further developed purpose of scientific and laboratory researces in 1970s to 1980s.

FDA approved the first photosensitizing drug the porfimer sodium Photofrin® in 1987 [48]. At the present time the use of PDT has been accepted for use in clinical treatment in the USA, EU, Canada, Russia and Japan. As As a treatment option for Barret´s esophagitis, obstructive tracheobroncheal carcinoma using the photosensitizer Porfimer sodium (®Photofrin) was approved by FDA. For actinic keratosis to use of 5-aminolevulinic acid, 5-ALA (®Levulan) and 5-ALA in alcohol solution (®Kerastick) also approved. Verteporfrin (®Visudyne) can be applied for macular degeneration. Besides to the above the European Union also approved the use of meta-tetrahydroxy-phenyl chlorine (mTHPC), also called as temoporfrin. The treatment of early and palliative cases of head and neck carcinomas (®Foscan) approved as a PS for PDT. Methyl aminolaevulinat (®Metvix) was accepted as PS prodrug for squamous- and basal cell carcinomas [49].

Three essential constituents are needed for PDT; a PS, a light supply and presence of oxygen. Cancer treatment with PDT [7, 47, 50-52] requires a systemic or topical administration of a photosensitizer [7, 53, 54] than irradiation of targeted tumor loci with proper wavelength of light source [55] (Figure 2.1) which when effectively united generate a photodynamic reaction [56].

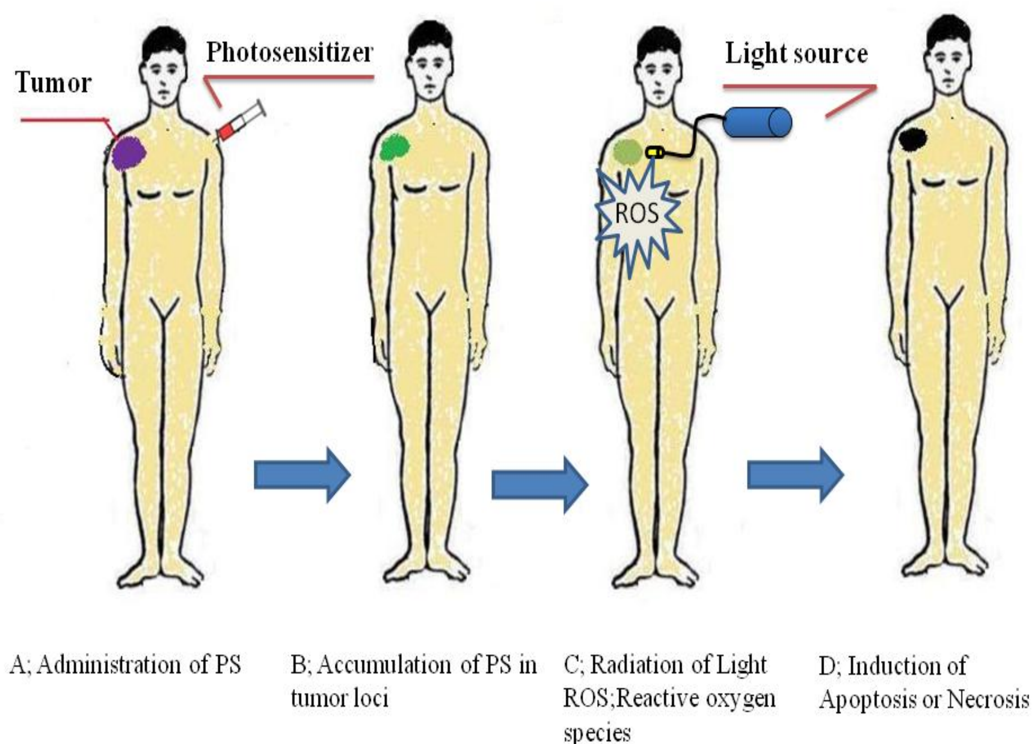

Figure 2.1 Profile of PDT treatment. PDT involves the choosy uptake and preservation of a PS in a tumour loci , the latter irradiation with light source, and formation of singlet oxygen induce to cell death via apoptosis or necrosis [41]

2.4 Photochemistry and Photophysics of Photodynamic Theraphy

The real biological and physical mechanism and interactions of PDT and its use are unclear [53]. Figure 2.2 shows possible photochemical mechanisms. S_0, S_1, and S_2 shows singlet electronic states of the molecule. Excitation of the absorbing molecule from the ground singlet state, S_0, to the first excited singlet state, S_1 is caused by absorption of a of a photon (hν). S1 directly or from the first triplet excited state, T1, which is generated after intersystem crossing may be causes for photochemistry. The molecule can rest back to S_0 from either S_1 or T_1 radiatively or nonradiatively. k_{nr}, k_{isc}, k_f, and k_p represent rate constants for nonradiactive decay, intersystem crossing, fluorescence, and phosphorescence.

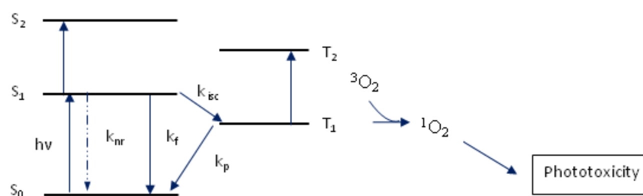

Figure 2.2 Energy level chart for the photo excitation of a molecule [57]

After absorption of light energy by a PS, the energy of its electrons are amplified rendering the photosensitizer excited. By emitting fluorescence, the excited photosensitizer can rest back to its ground state or to a triplet state through a process called intersystem crossing, from which it can rest by emitting phosphorescence. Energy of excited photosensitizer can be transferred to molecular oxygen that is one of the rare compounds which have triplet ground state, and the two molecules relax to respective singlet states in triplet state. Sharman and friends say that in the singlet state molecular oxygen, $^1O_2$, is excited, highly reactive and thereby responsible for the majority of lesions generated during PDT (Figure 2.3) [58].

Excited PS on transferring its overload energy returns to its ground state to admit extra photons or becomes photochemically degraded in a process may called as photobleaching. On the other hand, an excited PS may react directly with biomolecules to form free radicals that further react with molecular oxygen producing superoxide radical anion, hydrogen peroxide or hydroxyl radical. Superoxide radical anion is generated for instance by excitation of porphyrins in the presence of falling substances [55, 59].

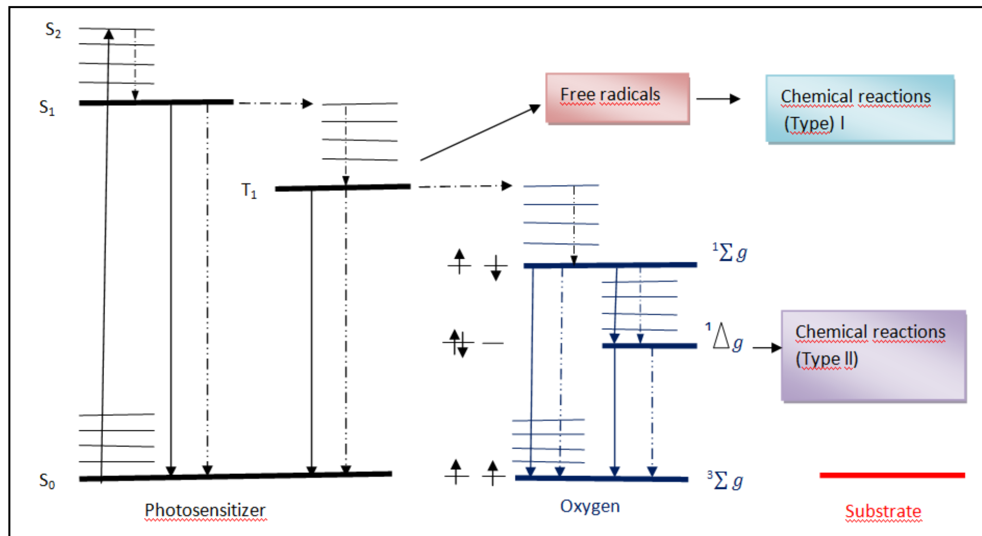

Figure 2.3 Jablonski Diagram [60]

The triplet state photosensitiser can react with biomolecules with two mechanism, Type I and Type II reactions shown in Figure 2.4.

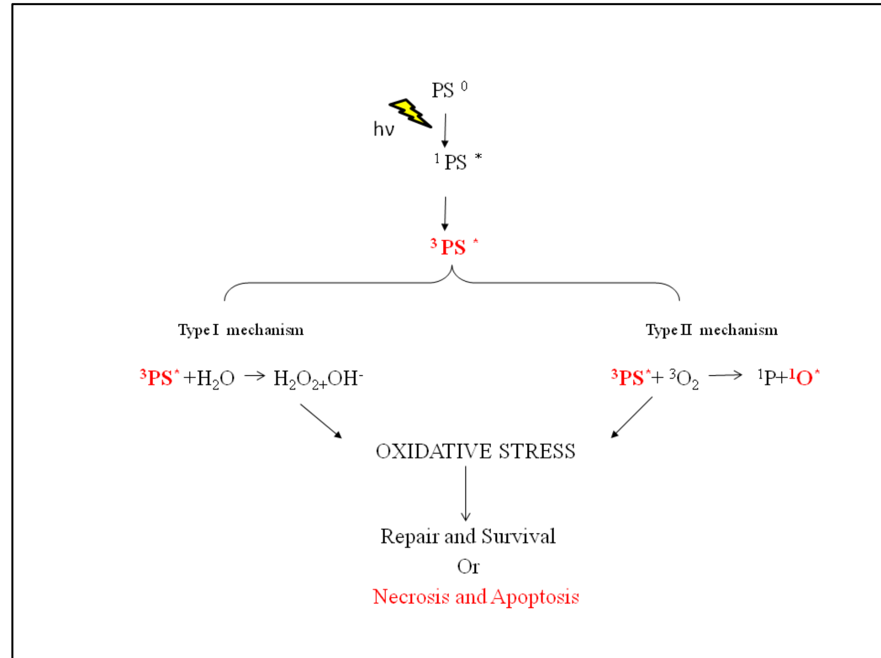

Figure 2.4 Type I and Type II mechanisms [57]

The photosensitizer in a singlet ground state (^0PS) becomes triggered to an excited singlet state ($^1PS^*$) which goes after by intersystem crossing to an excited triplet state ($^3PS^*$). Energy transfer from $^3PS^*$ to biological molecules and molecular O_2 via Type I and Type II reactions produces reactive oxygen species ($^1O_2$, $^{\cdot-}O_2$, $^{\cdot-}OH$ and H_2O_2) that generates different cellular actions. Since very reactive radicalic oxygen species are used, type II mechanism is the common mechanism [51].

2.5 Biological Mechanism of Cell Death

The biological effects of photodynamic theraphy cover a large number of effects, many of which are interrelated. At the subcellular level, PDT effects are particularly noted on membranes, additionally on cell surfaces, lysosomes, mitochondria and other cell organelles. Direct effects on DNA have been known, and DNA and RNA polymerase inhibition has been exposed. On the other hand, these effects are repairable to a large extend. Individual cell death effects rely on the cell line type, the PS type and a little the rate of cell growth and its physical demonstration, *e.g.*, the quantity of cell clusters [61].

Antitumor effects of PDT comprise of 3 interconnected mechanisms: direct tumor cell kill, injure to the vasculature, and initiation of an tough inflammatory reaction that can outcome in to the development of immune response also shown in figure 2.5 [62].

The relative input of these mechanisms relys on largely the type and dose of photosensitizer used, timing between PS administration and light contact, total light dose and its fluence rate, tumour oxygen concentration. [63, 64].

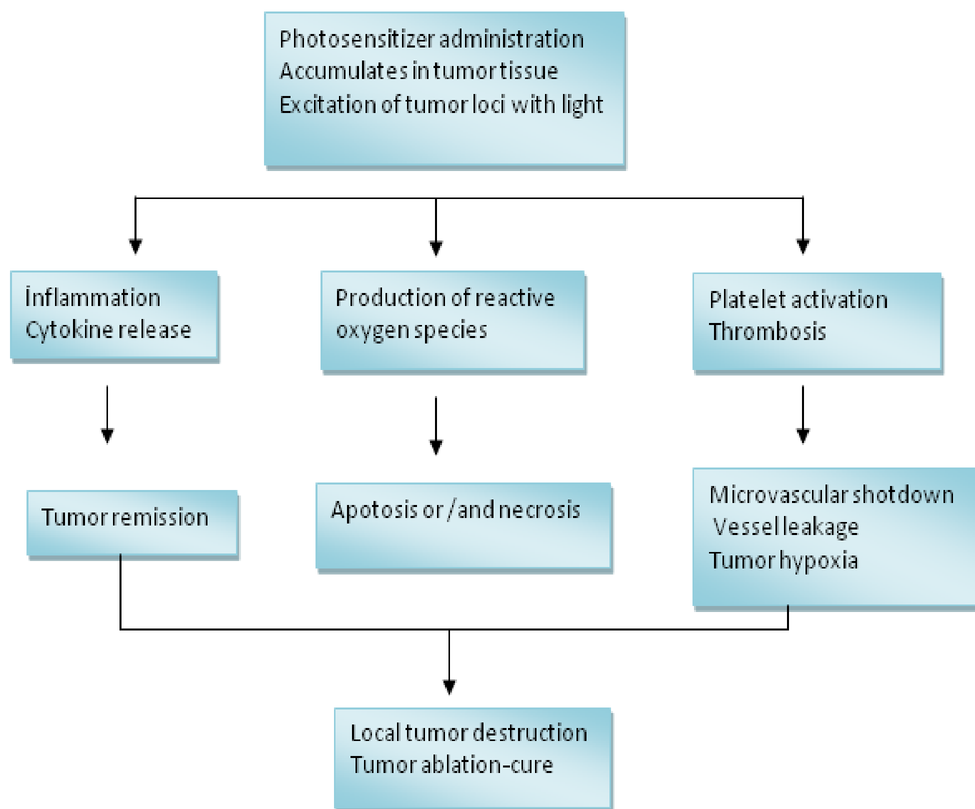

Figure 2.5 : Pathways of PDT caused tumor destruction depicting possible contributions from direct tumor cell death, vascular injury and immune response [9]

2.6 Photosensitizers

Photosensitizers in PDT allow for the transferal and translation of light energy into a type II chemical reaction [56]. A great number of photosensitizing dyes have been studied in PDT. Chemically, the only requirement for a ‘‘good’’ PS is a high quantum yield for singlet oxygen formation and proper solubility. Solubility in water is not necessity as many ‘‘carrier vehicles’’, for instance dendrimers and nanocarriers have been developed for PS [16,17].

Photosensitizers can be classified depending on the chemical structure. Table 2.1 summaries the PS families currently applied in clinics or/and under clinical survey.

Table 2.1 Photosensitizer families [56]

Family Name	**Photosensitizers**
Porphyrin platform	HpD, HpD-based, BPD, ALA, Texaphyrins
Chlorophyll platform	Chlorins, Purpurins,Bacteriochlorins
Dyes	Phtalocyanine, Napthalocyanine

The oldest PS in clinical use is hematoporphyrin. Chemically consist of ether and ester linked oligomers with n up to 9 (Fig. 2.6). Trade name is Photofrin®. The active ingredient is called porfimer sodium. Canada was the first country that approved porfimer sodium for bladder cancer. At the present time FDA approved Photofrin® for esophageal cancer, endobronchial and early stage cervical cancer and highgrade dysplasia in Barrett’s esophagus [65].

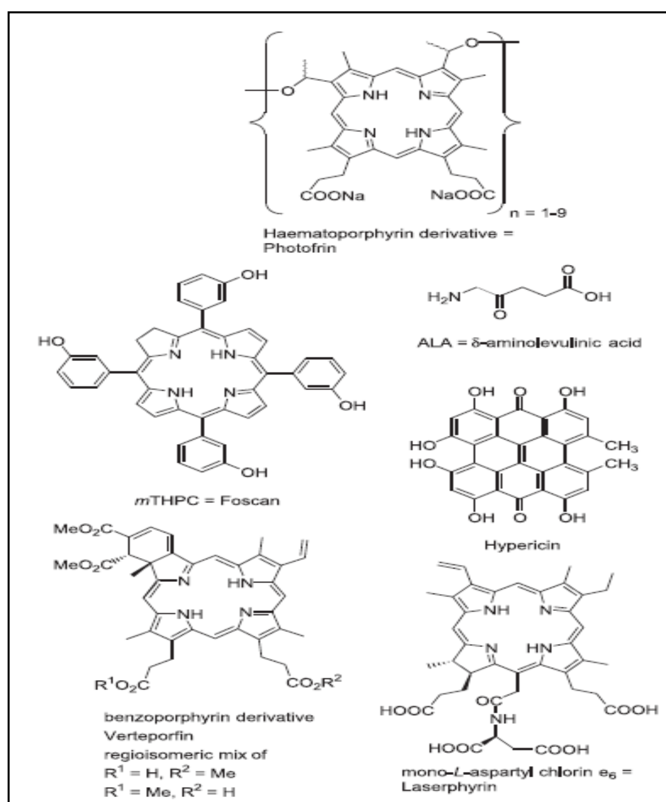

Figure 2.6 Selected photosensitizers in clinical use [65]

Figure 2.7 shows clinically available photosensitizers. 5,10,15,20-Tetra(3-hydroxyphenyl)chlorin usually abbreviated as mTHPC, also called Temoporfin. Trade names of different formulations are: Foscan®, Fospeg®, and Foslip®. For the clinically treatment of head and neck cancer MTHPC approved in Europe [66].
Aminolevulinic acid abbreviated as ALA. ALA is the biosynthetic precursor of heme, this way the intracellularly active substance is endogenously formed protoporphyrin IX. Trade names are Levulan®, Metvix® (for methyl aminolevulinate) and Hexvix® (Europe) or Cysview® (USA) (hexaminolevulinate) [67]. Levulan is FDA approved for acticinic keratosis and has orphan drug status for esophageal dysplasia. For the treatment of non-melanoma skin cancer Metvix approved in many countries.

For imaging bladder cancer Hexvix is used for an imaging agent . Benzoporphyrin derivative monoacid ring Verteporfin® is drug name, and Visudyne® is trade name, approved for treatment of age-related macular degeneration, although treatment with the monoclonal antibody fragment Ranibizumab or Bevacizumab today appears to be outstanding. Mono-L-aspartyl chlorin e_6, Talaporfin® and NPe_6 all are synonyms . For the cure of lung cancer Laserphyrin® approved in Japan .

Table 2.2 Clinically available photosensitizers [4]

Platform	Drug	Substance	Manufacturer
Porphyrin	Photofrin®	HpD	Axcan Pharma Inc.
Porphyrin	Photogem®	HpD	Moscow Research Oncological Institute
Porphyrin	Levulan®	ALA	DUSA Pharmaceuticals, Inc.
Porphyrin	Metvix®	M-ALA	PhotoCure ASA
	Hexvix®	H-ALA	PhotoCure ASA
Porphyrin	Visudyne®	Verteporfin	Novartis Pharmaceuticals
Texaphyrin	Antrin®, Lu-Tex	Lutexaphyrin	Pharmacylics
Chlorine	Foscan®	Temoporfin	Biolitec Pharma Ltd
Chlorine	LS11, Photolon®, Litx™, Apoptosin™, Laserphyrin	Talaporfin	Light Sciences
Chlorine	Photochlor	HPPH	RPCI
Phthalocianines	Photosens®	Phthalocyanine	General Physics Instute
	Pc4	Phthalocyanine	CWRU
Padoporfin	Tookad	Bacteriochlorophyll	The Weisman Institute of Science

Many other photosensitizers are under the investigation at different clinical phases. Some of them are Antrin, Photochlor, Radochlorin, Texaphyrin (Motexafin), Photosens, Purlytin, azadipyrromethenes, silicon phthalocyanine PC-4, and others. A Pd(II) bacteriopheophorbide derivative Tookad® is worthy because it specifically targets the vascular system using short drug - light intervals [61]. High molar extinction coefficient and high absorbance is one of the important selection criteria for PS, especially in red and near infrared spectral regions (600-810 nm). This criteria is related to with light penetration depth into the tissue, which is connected with wavelength. For the PDT treatment of deeper tumors, light source at longer wavelengths should be applied with compatible PS.
The updated research in PDT has been focused on the enhancement of replacement new-generation PSs with improved physical, chemical and therapeutic features [11, 68].

2.6.1 **Porphyrin**

Porphyrin which derived from the Greek word porphura is a purifed mixture of HpD and the molecular structure with absorbtion spectra are shown in Figure 2.6 [69]. Porphyrins are a ubiquitous class of naturally occurring molecules involved in a broad sort of important biological processes ranging from oxygen transport to photosynthesis, from catalysis to pigmentation changes. The widespread property of all these molecules is the fundamental structure of the porphine macrocycle, which originate of a 16-atoms ring containing 4 nitrogen atoms, acquired by linking 4 tetrapyrrolic subunits with 4 methine bridges [69].

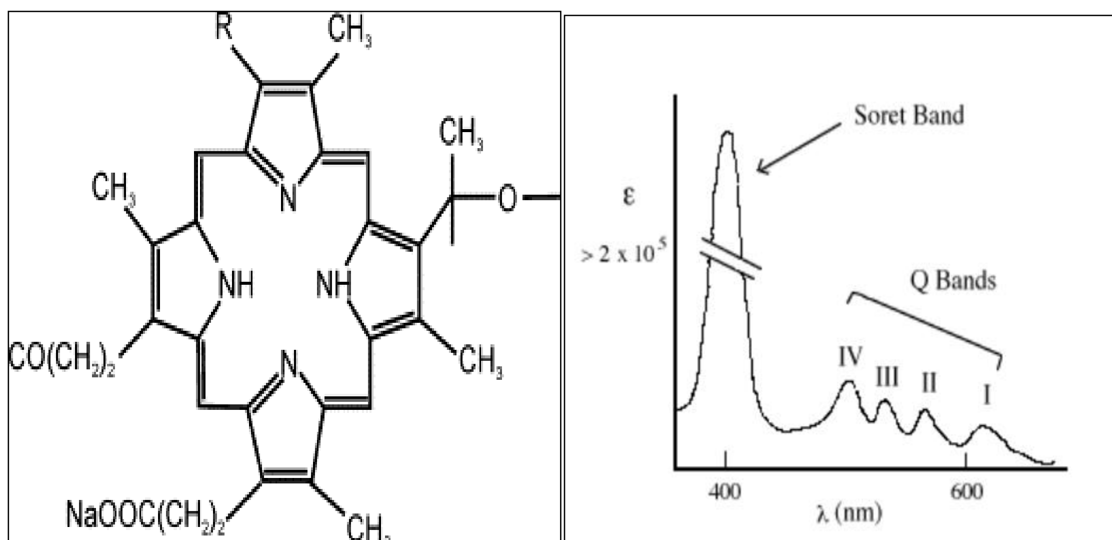

Figure 2.7 Molecular structure of Photofrin (PH), typical UV-visible absorption spectrum of porphyrins [69]

2.7 LIGHT SOURCES

It is already known that the irradiated light is one of the essential component of PDT, and the choice of light sources has a vital importance for succes of PDT studies [55]. Photons that are delivered into living tissue can either be absorbed or scattered. Scattered photons will finally be absorbed or will escape from the tissue in the form of diffuse reflection. Light propagates through the tissue (Figure 2.8) [6], however light is absorbed by the dominant chromophores; such as hemoglobin, melanin, lipids, aminoacids and water in the tissue which limits how deep the light will penetrate. Each chromophore absorbs light at specific wavelength(s)

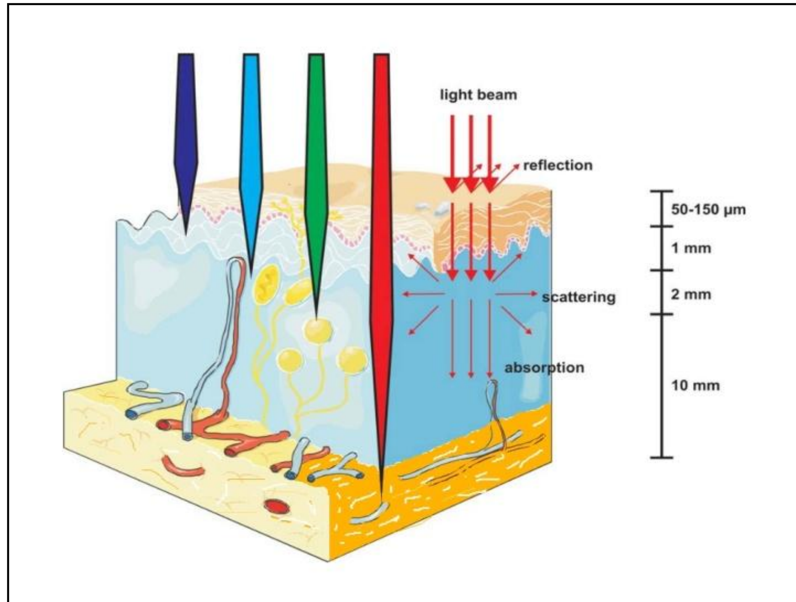

Figure 2.8 Light Propagation Through the Tissues [6]

Therapeutic window also known "optical window" of living tissue is between 600-1300 nm. Beacuse of this reason in order to reach the optimal depths, the photosensitizers are selected that absorb at the longest wavelength possible (Figure 2.9).
Quite apparently the light source and light delivery are two of the vital subjects in PDT. The choice of light source for PDT can be required by the loci of the tumor, by the light dose convey and by the choice of PS [70].

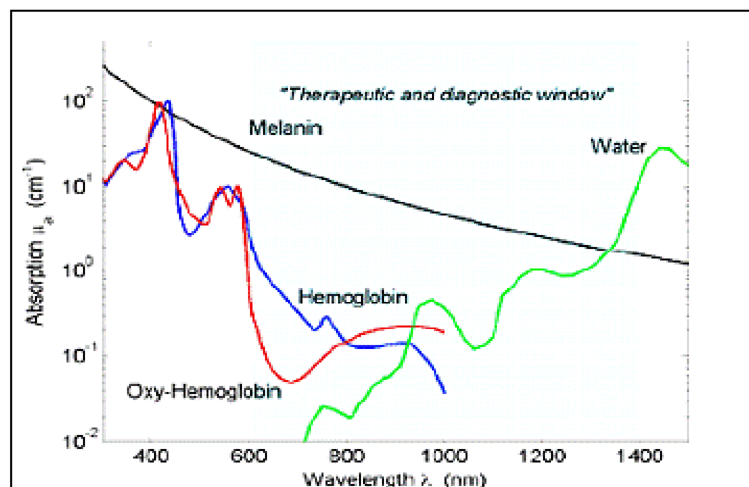

Figure 2.9 Absorption spectra for the main constituents of biological tissues [71]

Laser and nonlaser light sources have both been employed to perform PDT studies. One main advantage of lasers is their monochromaticity, which gives the greast efficiency of photoactivation during treatment. While PDT has been conservatively performed using lasers, the availability of lamps is challenging the use of lasers where light can be directly delivered to the tumour (skin, nasal cavity, etc.) without any necessity to couple the source to an optical fibre [55]. Laser light sources are not only very expensive, but also a specifically tailored optical design is necessity to expand the beam for the irradiation of extend area [72].

Optical fibers are good option for illumination at inaccessible locations into the body. The fibre optic tip can be modified for irradiation of the target lesion. For superficial illumination optic fibers with a lens tip are used to spread the light over the target area. In hollow organs illumination is frequently performed with cylindrical diffusers combined with inflated balloons for uniform light distribution. Black coating of one way of the balloon is enable to protection of normal tissue especially for delicate illumination areas [46, 48].

In more recent times light-emitting diodes (LEDs) became a practical technology for PDT purposes . LEDs ordering the advantages over lasers of being inexpensive, stable, easy to operate, requiring little maintenance, less hazardous, thermally nondestructive, do not need an external cooling system, and readily available. Besides LEDs can be arranged in arrays flexibly to irradiate wide area according to the geometry of target area [34].

2.8 Clinical Applications of Photodynamic Theraphy

PDT is commonly performed on an outpatient treatment and has major advantages over other cancer treatment options. In comparison, typical radiotherapy regimes include daily irradiation for a total of 5 to 7 weeks. Dosage limit brings application and repetition limit in radiotherapy. In addition, development of drug resistance significantly limits the success of chemotherapy in cancer patients and contributes to cancer recurrence and high mortality rates and chemotherapy schedules typically last for

several months with violent side effects [73]. Other treatment method; surgery, mostly consist a single procedure with general anesthesia and requires hospital treatment for 3 to 4 weeks. In the surgery, the best standard is that 0.5 to 3 cm part of tissue resected beyond lesion margins including the tumor visually detected and/or biopsy confirmed. So that in most cases, tissue or organ dysfunction is observed [74]. One of the advantage of PDT is that the treatment can be repeated in case of areas where high recurrence risk probable. Such retreatment is extremely difficult for either surgery or radiotherapy, without the risk of severe normal tissue damage and due to limits of radiation dose for tissues, organs. Among those other treatment methods, PDT is cost-effective, have very minimal side effects and provide increased life expectancy and life quality for cancer patients. PDT spares tissue nature, providing a matrix for regeneration of normal tissue, because it doesn't harm subepithelial collagen and elastin, and this way protects supporting elements. Successful clinical results have been showed that PDT can be curative and a good alternative for treatment of cancer [5-8, 42, 75, 76].

PDT is becoming an attractive treatment option especially for localized cancers. Furthermore, there is higly increasing interest and research developing clinical PDT treatment techniques in brain, breast, cardiovascular, gastroenterological, urological and gynecological cancers [35, 77-88]. Current research focused on the improvement of next generation photosensitizers with better performance. Nanotechnogical methods have been employed for development targeted PDT [8, 39]. Another rising area of research is the combination of PDT with different theraphies such as chemotherapy, radiation therapy and immunotherapy [35, 74, 89-95].

CHAPTER 3

3.1 Cell Culture

3.1.1 Thawing AGS Cells

AGS human stomach cancer cells obtained from Fatih University Biology Department Cell Culture Laboratory .

Before thawing process, 10 ml of pre-warmed Dulbecco's Modified Eagle Medium (DMEM from GIBCO) to 37ºC was added into 15 ml falcon tubes. Then cryovial tubes were taken from the nitrogen tank and transferred to 37ºC water bath. As soon as possible liquid content in the tube was transferred to the tube containing medium and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the pellet of the cells was resuspended in 10 ml medium and centrifugation was repeated once more in order to get rid of DMSO. All of the cells in the pellet were seeded with DMEM containing 20 % FBS and the next day medium was refreshed in order to get rid of dead cells.

3.1.2 Seeding and Subculture of AGS cells

After seeding of cells into culture flasks, cells were attached to the surface of flasks. When cells became 80-90% confluent, they were subcultured. Before the subculture, water bath was used to warm DMEM, FBS, PBS and trypsin to 37°C. Medium in the flask was removed by a sterile pipette and then 5 ml of calcium and magnesium free Phosphate Buffered Saline (PBS, Biochrom) to remove residual medium. After removal of PBS, 4 ml of pre-warmed 0.25% Trypsin/EDTA (GIBCO) was added to the

flask and kept at room temperature for 1-2 min. Then cells were observed under invert microscope. When cells were detached from the surface of the flask, 1 ml of FBS added to the flask to inactivate the function of trypsin. The cells in the flask with trypsin and FBS was transferred into a new 15 ml falcon tube and centrifuged at 1500 rpm for 10 min at room temperature. After centrifugation, supernatant was discarded by leaving about 0.5 ml of the cell suspension at the bottom. Pellet was finger mixed and volume was up to 10 ml with DMEM medium in order to remove the remaining any trypsin. Centrifugation step was repeated once more and then cells were counted by hemocytometer. After counting, cells were seeded at a density of 1 x 10^4 cells/cm^2 with 10% FBS containing DMEM for expansion. Subculture of cells was repeated at about 4-5 days intervals.

3.1.3 Cell treatment

AGS human stomach cancer cells (1 x 10^4) were seeded in 96-well plate and incubated overnight for cells to settle down. Plates were divided into 2 groups (Control (C) and PDT). Control group was replaced with fresh complete media. PDT group was incubated in dilution ratio of 1:1, 1:10, 1:25, 1:100 and 1:250 to four generation of synthesized material (P_0, P_1, P_2, P_3) for 24 hour. Synthesis material generations entitled PpIX0, PpIX1, PpIX2 and PpIX3 in well plate figure 3.1.

Figure 3.1 : Well ID, Cell-Type, Cell-Number, Compound Name, Concentration Unit(μM) have shown for PAMAM and PpIX molecules at 96 well-plate PDT application

The next day Control group was kept at dark for 20 minute and PDT group was treated under 465 nm LED for 20 minute with a power density 21 mW. Same protocol repeated for 10 minute experiments.

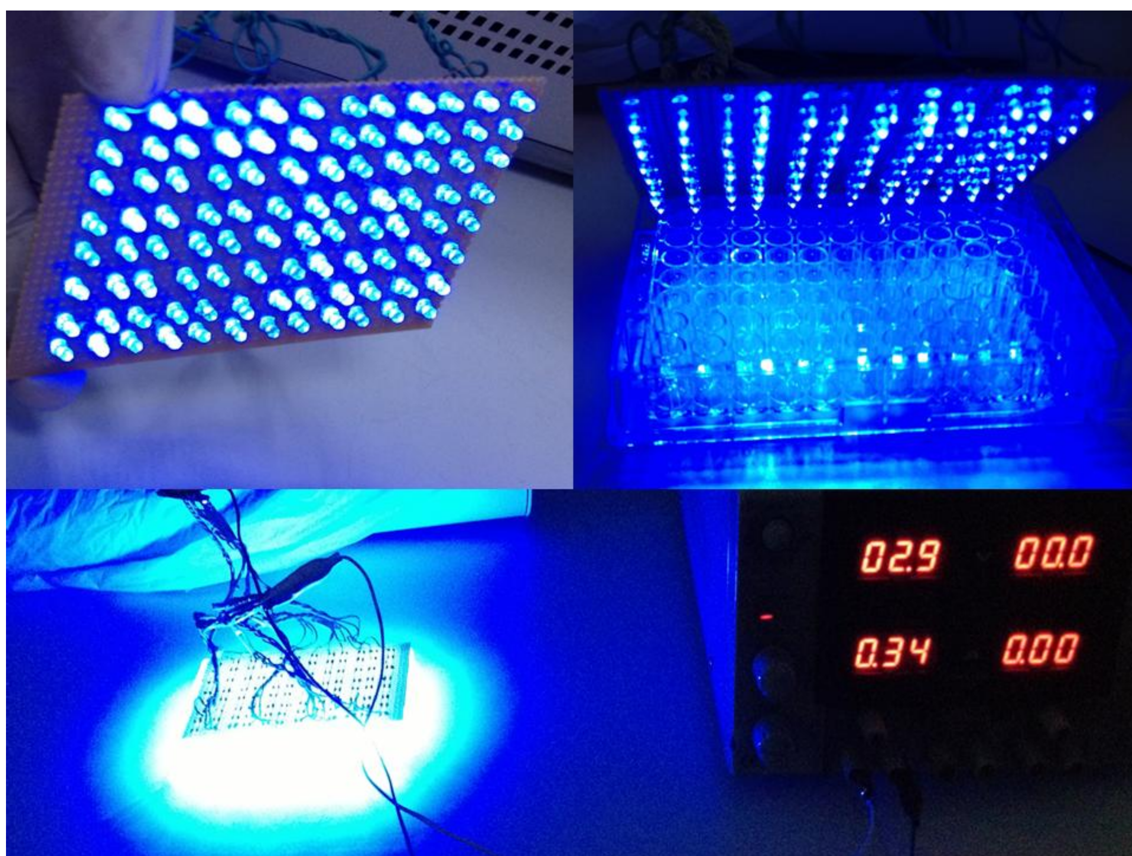

Figure 3.2 Pictures shows LED based 96 well plate illumination system

3.2 Light source

The irradiation experiments were performed with composed of a 12x8 LED array system (Figure 3.2). Every well is paired with a LED which is fixed on a perforated plate. LED system is connected to ARDUINO® control card that can be controlled by a computer. LEDs irradiate at 465 nm nm peak wavelength and have 21 mW optical powers measured by optical power and energy meter (S121C Standard Photodiode Power Sensor, Si, 400 - 1100 nm, 500 mW, PM200 Optical Power and Energy Meter Thorlabs). System can be modulated below values for 250 ms on and 250 ms off. It didn't show any temperature increase with continuous opening in 30 minutes on LED's.

3.3 Synthesis of PAMAM-Porphyrin Derivatives

Porphyrin molecule (figure 3.3) was purchased from Porphyrin Systems GbR [5,10,15,20-Tetrakis-(4-carboxyphenyl)-21,23H-porphyrin 97%, Molecular Formula: $C_{48}H_{30}N_4O_8$, Molecular Weight: 790.78, Composition: C(72.91%) H(3.82%) N(7.09%) O(16.18%)].

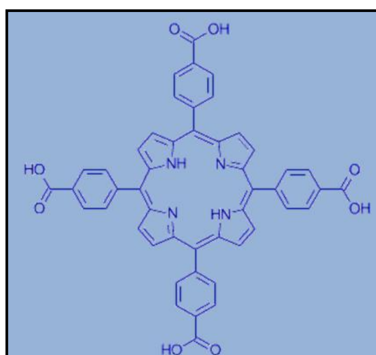

Figure 3.3 : 5,10,15,20-Tetrakis-(4-carboxyphenyl)-21,23H-porphyrin 97% [96]

Dendrimers are polymeric, 3-dimensional macro molecules comes from applying mathematical progressions to organic synthesis. Because of the cascade character of the

chemistry used for preparing these molecules, and the control that can be exercised over their preparation, a somewhat good understanding of the interior exists, leaving the gross exterior surface topology stil majorly a enigma. Dendrimers of generations 3 and 4 commonly used for the reason that they appear to have wanted characteristics for use in in vivo studies [97]. Poly(amidoamine) PAMAM dendrimers are remarkably branched, spherical macromolecules, characterized by a narrow size distribution and a high degree of molecular uniformity [98]. The first complete dendrimer family to be synthesized, characterized and commercialized were PAMAM dendrimers [99].

In order to synthesize PAMAM dendrimer on porphyrin core, carboxylic acid functional porphyrin was reacted with $SOCl_2$ to obtain acyl chloride. Acyl chloride functional porphyrin was dissolved in dichlorometane and mixed slowly with solution of trietilamine and ethilendiamine which was also dissolved in dichlorometane. This mixture was stirred about 8 hours at room temperature (Figure 3.4).

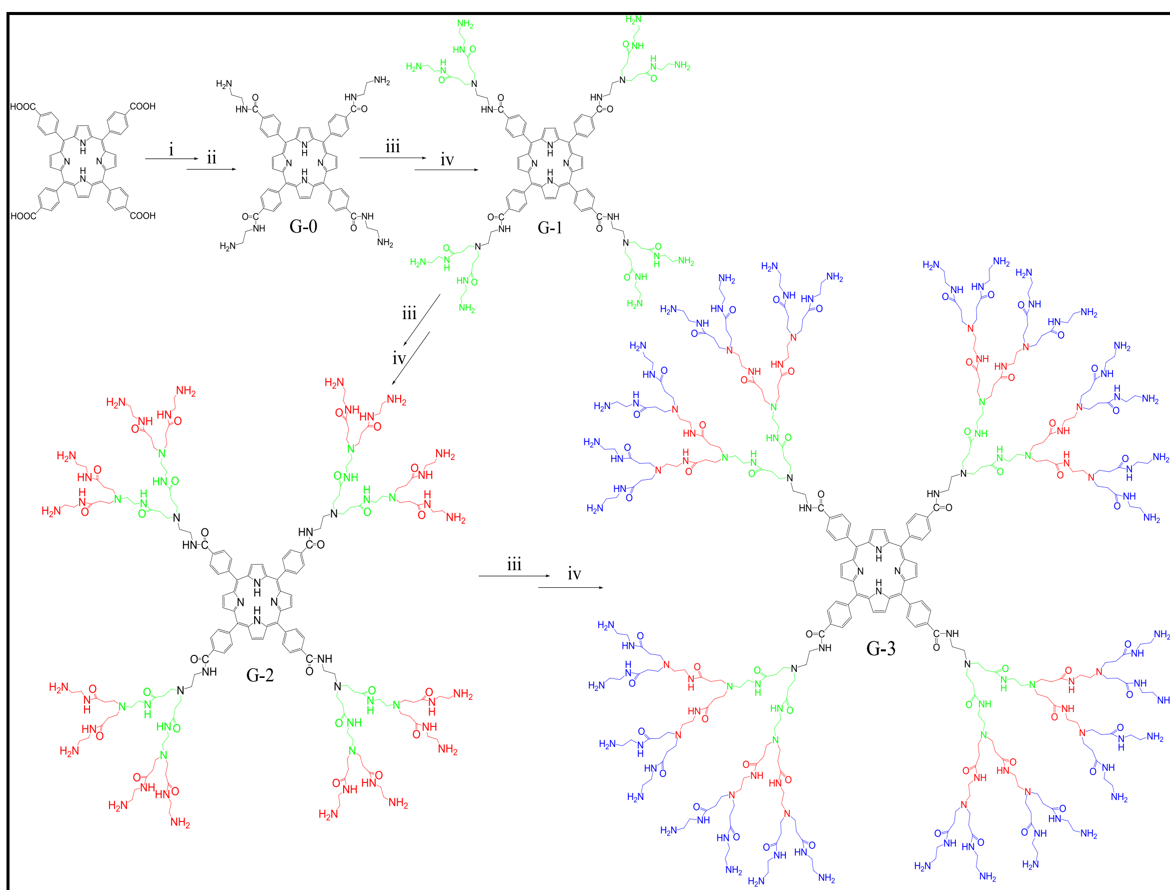

Figure 3.4 : Schemes shows synthesis of PAMAM covered porphyrin derivates. After the first amination that grow by swinging the branches. Every branch enables to way for connection.

Than, excess etilenaiamin and solvent was removed by rotary evaporator. The amine-terminated porphyrin cored PAMAM dendrimers was synthesized divergently by initial Michael addition of methanolic solution of ferrocene amine with excess methyl acrylate (1:10 molar ratio). The reaction mixture was stirred for three days at room temperature. The excess methylacrylate was removed under vacuum at 40–50 °C temperature to afford the ester-functionalized derivative. The reaction mixture was next submitted to the reaction sequence leading to the next generation porphyrin-PAMAM dendrimer, consisting of the exhaustive amidation of the ester functionalized porphyrin-PAMAM dendrimers to ethylenediamine (1:30 molar ratio), followed by Michael addition of the resulting amine with methylacrylate (20 equiv of G0.5). Excess reagents were removed under vacuum at 60–70 °C temperature. Repetition of this two-step procedure ultimately leads to the next generations of porphyrin-PAMAM dendrimer (G1, G2 and G3).

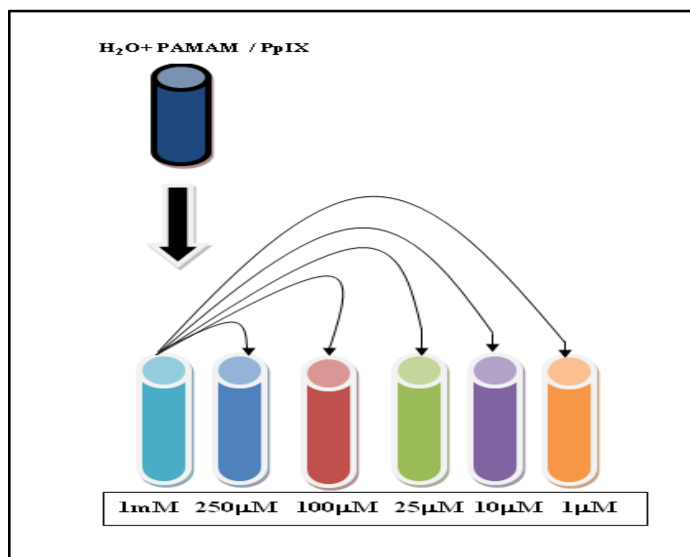

Figure 3.5 : Serial dilutions of the chemicals.

3.4 Real time Monitoring

The xCELLigence system real-time cell analyzer was purchased from ROCHE®. The xCELLigence System monitors cellular events in real time devoid of the integration of labels.

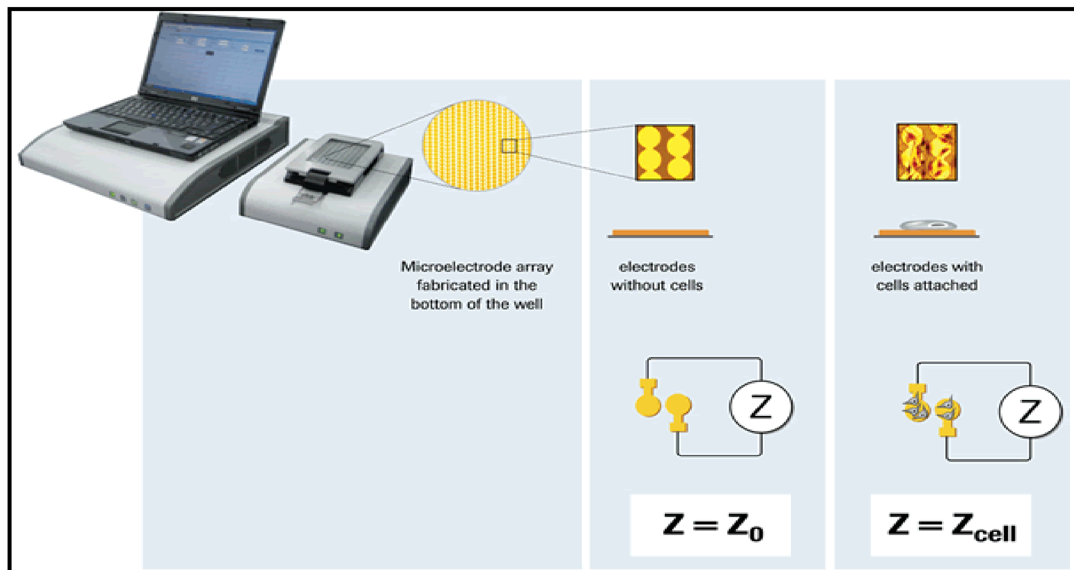

Figure 3.6 : The xCELLigence system real-time cell analyzer [100]

The System measures electrical impedance (Figure 3.6) across interdigitated micro-electrodes integrated on the bottom of tissue culture E-Plates (Figure 3.7). The impedance measurement yields quantitative information about the biological position of the cells, including cell number, viability, and morphology [100, 101].

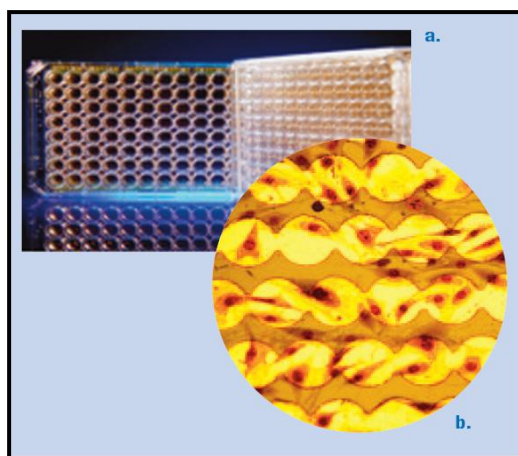

Figure 3.7 : The E-Plate. The 96 well plate is an SBS standard dimension tissue culture plate (a). The bottoms of each well have 80% of the surface area covered by interdigitated gold microelectrodes (b) [101].

3.5 Cytotoxic Analysis

AGS human stomach cancer cells (1 x 10^4) were seeded in 96-well plate and incubated overnight for cells to settle down. Plates were divided into 2 groups (Control (C) and PDT). Control group was replaced with fresh complete media. PDT group was incubated in dilution ratio of 1:10, 1:25, 1:100 and 1:250 to four generation of PAMAM molecule for 24 hour (figure 3.5). PAMAM generations entitled PAMAM0, PAMAM1, PAMAM2 and PAMAM3 in well plate .

The next day Control group was kept at dark for 20 minute and PDT group was treated under 465 nm LED for 20 minute with a power density 21 mW. Same protocol repeated with 10 minutes irradiation.

3.6 Cell Morphology

AGS cell lines were seeded in duplicate 96 well plates (1×10^4 cells / well) in 100 µl medium and let incubation with serial dilutions of the chemicals modified

protoporphyrin IX (PpIX) and PAMAM were prepared at a ratio 1:1, 1:10, 1:25, 1:100 and 1:250 (figure 3.5) to four generation of synthesized material (P_0, P_1, P_2, P_3). The morphology of AGS cells was observed under an inverted microscopy (Nicon Eclipse Ti-U LH-M100C-1) after 24 hours incubation.

3.7 Statistical Analysis

Statistical analysis was performed using a two-tailed paired Student's t-test to determine statistical differences for groups as indicated. Differences with $p<0.05$ were

regarded as significant.

CHAPTER 4

RESULTS AND DISCUSSION

PAMAM modified PpIX on AGS cancer cell lines viabilty results measured along the PDT and also after PDT with xCELLigence system real-time cell analyzer (Roche). Graphs observed in the figure represent four independent experiments. PAMAM modified PpIX caused membrane damage and reduced the proliferation of cancer cells depends on the generation.

All graphs obtained from xCELLigence system real-time cell analyzer (Roche). The Cell Index (CI) at each time point is defined as (Rn -Rb)/15, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the media alone.

PpIX0 shows protoporphyrin IX without modification with PAMAM.

PpIX1 shows protoporphyrin IX with PAMAM modification, indicates first generation.

PpIX2 shows protoporphyrin IX with PAMAM modification, indicates second generation.

PpIX3 shows protoporphyrin IX with PAMAM modification, indicates third generation.

AGS human stomach cancer cells were used to examine the cytotoxic effects of PpIX and PAMAM modified PpIX with and without irradiaiton. Cell viability graph of the

irradiated and unirradiated samples revealed that cell viability of treated cells decreased with PAMAM modified PpIX generations.

Modified PpIX generations demonstrated similar inhibition effect on the proliferation of the cell lines. Uptaken by cell significantly easy to compare to unmodified PpIX (PpIX0) and water solubility increased with modification.

Without Light Irradiation Results;

For PpIX0; 250µM and 100µM concentrations showed highly toxic effect %94 ± of cells death without light irradation. With 25µM concentration %23 ± of cells death without light irradiation. 10µM and 1µM didn't show any significant reducing effect on cell viability without light irradiation.

For PpIX1 ; 250µM and 100µM concentrations showed highly toxic effect %92 ± and 25µM concentration showed %21 ± of cells death without light irradation. With 10µM and 1µM concentrations didn't show any significant reducing effect on cell viability without light irradiation.

For PpIX2 ; 250µM and 100µM concentrations showed highly toxic effect %95 ± of cells death without light irradation. With 25µM, 10µM and 1µM concentrations didn't show any significant reducing effect on cell viability without light irradiation

For PpIX3 ; 250µM and 100µM concentrations showed highly toxic effect respectively %95 ± and % 94 ± of cells death without light irradation. With 25µM, 10µM, 1µM concentrations didn't show any reducing effect on cell viability without light irradiation (Figure 4.1).

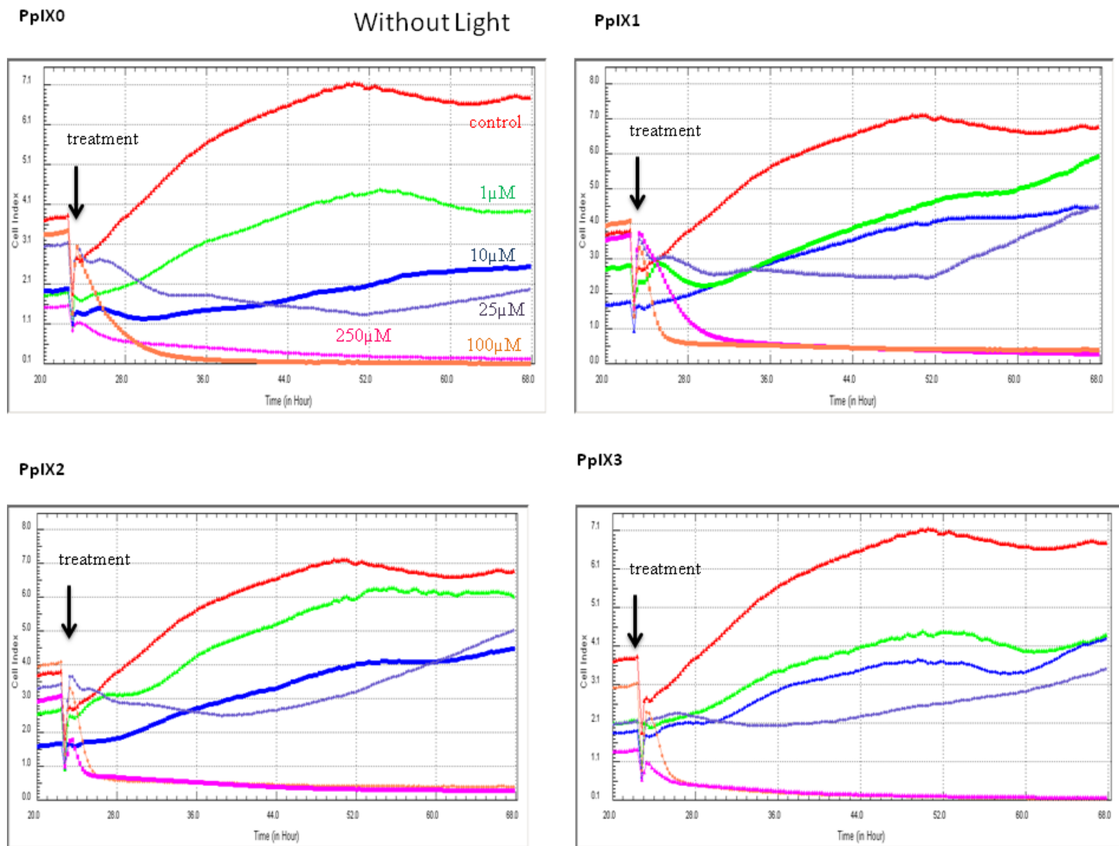

Figure 4.1 : 1 x 10^4 AGS cells seeded and incubated overnight for cells to settle down. After than cells incubated in dilution ratio of 1:1, 1:10, 1:25, 1:100 and 1:250 to four generation of PpIX to 24 hour kept at dark. Cell viability observed, (x) axis shows CI and (y) axis shows time (h). Arrow indicates the beginnig of the treatment (PS).

With 20 minutes Light Irradiation Results;

For PpIX0 ; 250µM and 100µM showed highly toxic effect respectively %92 ± and %96 ± of cells death before PDT has been applied. 25µM concentration showed %88 ± of cells death with 20 minutes light irradiation. With respectively %6.5 ± and %14 ± cell death have been shown with 10µM and 1µM PpIX incubation with 20 minutes light irradiation.

For PpIX1 ; 250µM concentration showed toxic effect. 100µM concentration could be said that best effective dose for first generation of PS, before PDT it isnt show any toxic effect, after PDT application cell viability decreased %70 ±. 25µM, 10µM, 1µM

concentrations didn’t show significant reducing effect on viability with PDT application.

For PpIX2 ; 250µM and 100µM showed highly toxic effect respectively %79 ± and %81 ± of cells death before PDT has been applied. 25µM concentration with PDT application viability decreased %23 ±. 10µM concentration with PDT application viability decreased %6 ±. 1µM concentrations didn’t show significant reducing effect on viability with 20 minutes PDT application.

For PpIX3; 250µM concentration showed highly toxic effect %98 ± of cells death before PDT has been applied. 100µM concentration with PDT application 20 minutes induced %50 ± of decreasing on viability. 25µM and 10µM concentration induced respectively % 16 ± and % 14 ± of cell death with 20 minutes PDT application. 1µM didn’t showed significant reducing effect on viability with 20 minutes PDT application (Figure 4.2).

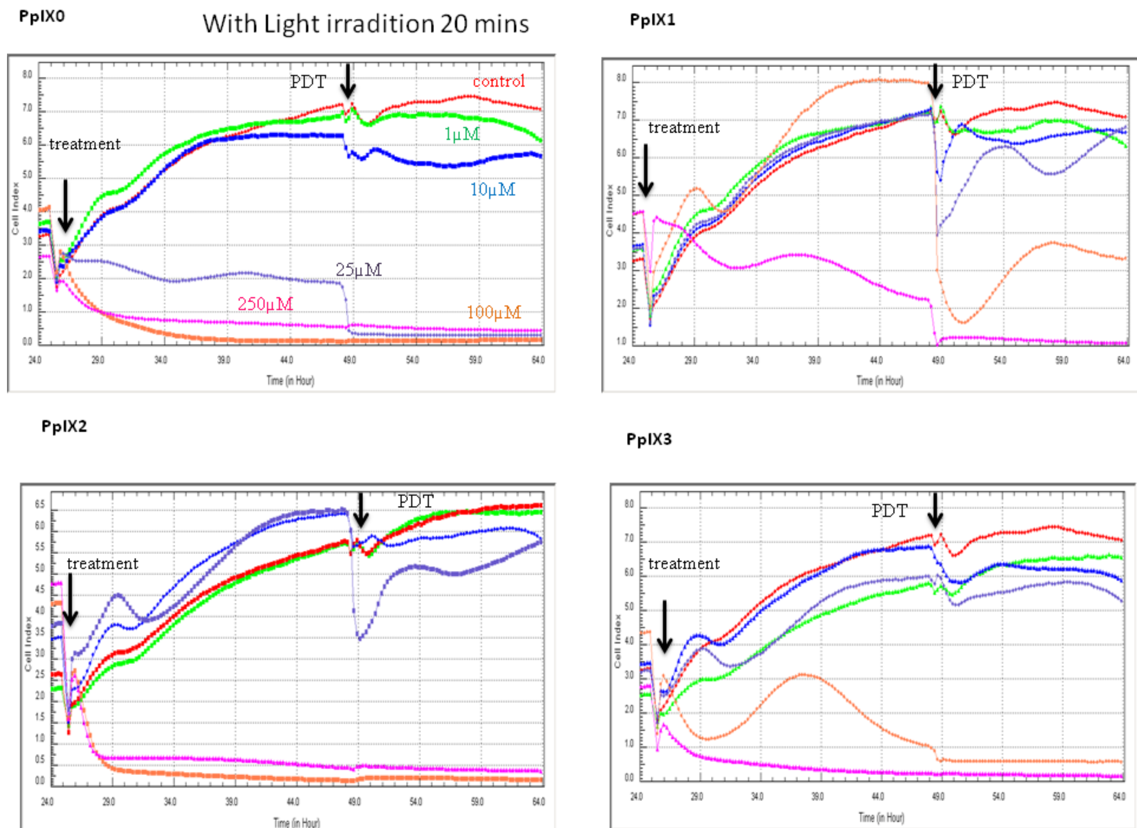

Figure 4.2 : 1 x 10^4 AGS cells seeded and incubated overnight for cells to settle down. Than cells incubated in dilution ratio of 1:1, 1:10, 1:25, 1:100 and 1:250 to four generation of PpIx to 24 hours. After 24 hours 20 minutes irradiation done . Cell viability observed, (x) axis shows CI and (y) axis shows time (h). First arrow indicates the beginnig of the treatment (PS) and second arrow indicates beginning of PDT (light irradation) .

With 10 minutes Light Irradiation Results;

For PpIX0 ; 250μM and 100μM showed highly toxic effect respectively %94 ± and %96 ± of cells death before PDT has been applied. 25μM concentration induced %50 ± of cells death with 10 minutes PDT application. 10μM and 1μM concentrations didn't show any significant decrease on viability with 10 minutes PDT application.

For PpIX1 ; 250μM and 100μM concentrations showed toxic effect. After PDT application viability decreased %50 ±. 25μM, 10μM and 1μM concentrations didn't show any significant reducing effect on viability, after 10 minutes PDT application cell viability continued increasingly.

For PpIX2 ; 250μM and 100μM concentrations showed toxic effect respectively %89 ± and %85 ± of cells death before PDT has been applied. 25μM,10μM and 1μM concentrations didn't show significant reducing effect on viability, after 10 minutes PDT application proliferation continued, viability increased.

For PpIX3 ; 250μM and 100μM concentrations showed toxic effect respectively %89 ± and %96 ± of cells death before PDT has been applied. 25μM, 10μM and 1μM concentrations didn't show significant reducing effect on viability, after 10 minutes PDT application, proliferation continued viability increased (Figure 4.3). It showed that 10 minutes isn't sufficient time duration for this study. After 10 minutes light irradiation unexpectedly viability increased. We can say that insufficient light duration may result increase of viability because it leads to occur an effect like biomodulation. In consequence of inadequate PS concentration or/and light duration if tumor cells survive to protect themselves, they proliferate quickly. As a result of this mechanism in graphs we have seen increases in cell number after PDT application.

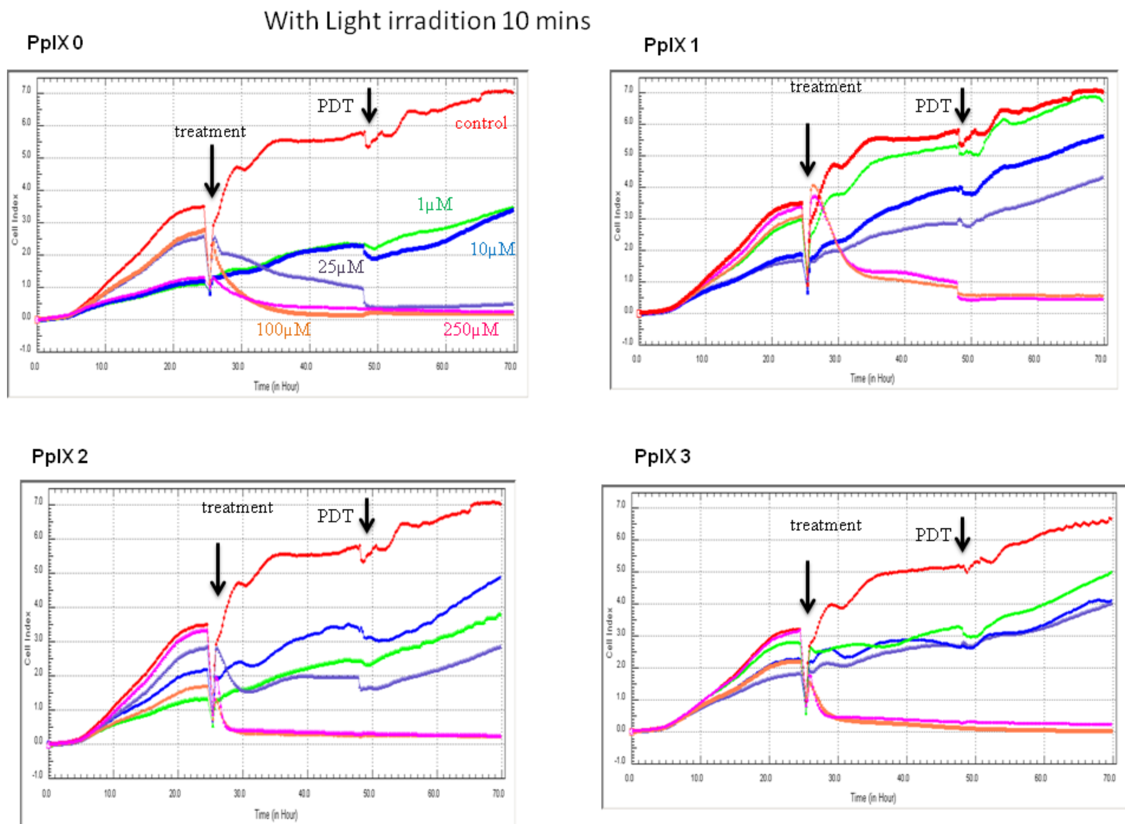

Figure 4.3 : 1 x 10^4 AGS cells seeded and incubated overnight for cells to settle down. After than cells incubated in dilution ratio of 1:1, 1:10, 1:25, 1:100 and 1:250 to four generation of PpIx to 24 hour. After 24 hours incubaton 10 minutes irradiation done . Cell viability observed, (x) axis shows CI and (y) axis shows time (h). First arrow indicates the beginnig of the treatment (PS) and second arrow indicates beginning of PDT (light irradation) .

Cytotoxic analysis of PAMAM ;

PAMAM0 ; 250µM concentration didn't show any reducing effect on viability. 100µM concentration didn't induced decrease on viability without light, after 20 minutes PDT application induced % 6 ± of cells death. 25µM and 10µM concentrations with 20 minutes PDT application induced % 6 ± of cells death. Except 250µM PAMAM didn't show any toxic effect. All concentrations lines below the control line.

PAMAM1 ; 250µM, 100µM, 25µM, 1µM concentrations didn't induced reduced effect on viability. Except 250µM PAMAM didn't show any toxic effect. All concentrations lines above the control line.

PAMAM2 ; 250µM and 100µM concentrations induced %15 ± of cells death with PDT 20 minutes application. Except 250µM and 100µM concentrations PAMAM didn't show significant toxic effect. Except 250µM other concentration lines above the control line.

PAMAM3 ; 250µM and 100µM concentration showed toxic effect before PDT has been applied. 25µM and 10µM concentrations didn't show significant toxic effect. 25µM and 10µM concentrations lines nearly same with control line.

Expectedly after 20 minutes PDT application proliferation increased, because of PAMAM doesn't have a photosensitizer property. Increases with generation number cytotoxic property of PAMAM lead to slight number of cell death . (Figure 4.4)

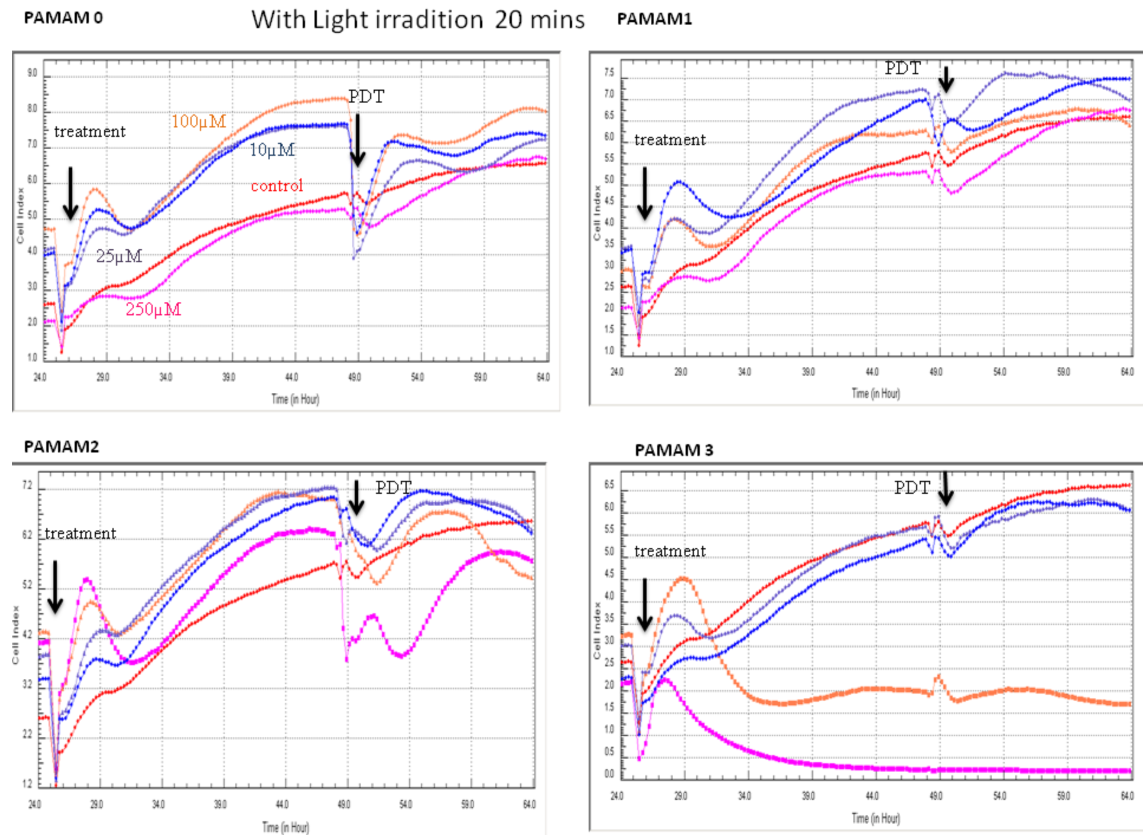

Figure 4.4 : 1 x 10^4 AGS cells seeded and incubated overnight for cells to settle down. After than cells incubated in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 to four generation of PAMAM to 24 hour. After 24 hours 20 minutes irradiation done . Cell viability observed, (x) axis shows CI and (y) axis shows time (h). First arrow indicates the beginnig of the treatment (PS) and second arrow indicates beginning of PDT (light irradation) .

PAMAM0 ; 250µM, 100µM, 25µM and 10µM concentrations didn't show significant toxic effect, proliferation continued. All concentration lines nearly same with control line.

PAMAM1 ; 250µM, 100µM showed slight toxicicity, 25µM and 10µM concentrations didn't show significant toxic effect, proliferation continued.

PAMAM2 ; 250µM, 100µM, 25µM, 10µM concentrations didn't showed toxic effcet with 10 minutes PDT application. Cell proliferation continued.

PAMAM3 ; 250µM and 100µM concentrations showed toxic effect respectively % 75± and %70 of cells death before PDT has been applied. 25µM, 10µM concentrations didn't showed toxic with 10 minutes PDT application, cell proliferation continued.

Expectedly after 10 minutes PDT application proliferation increased, because of PAMAM doesn't has photosensitizer property. Increases with generation number and concentration cytotoxic property of PAMAM lead to slight number of cell death (Figure 4.5).

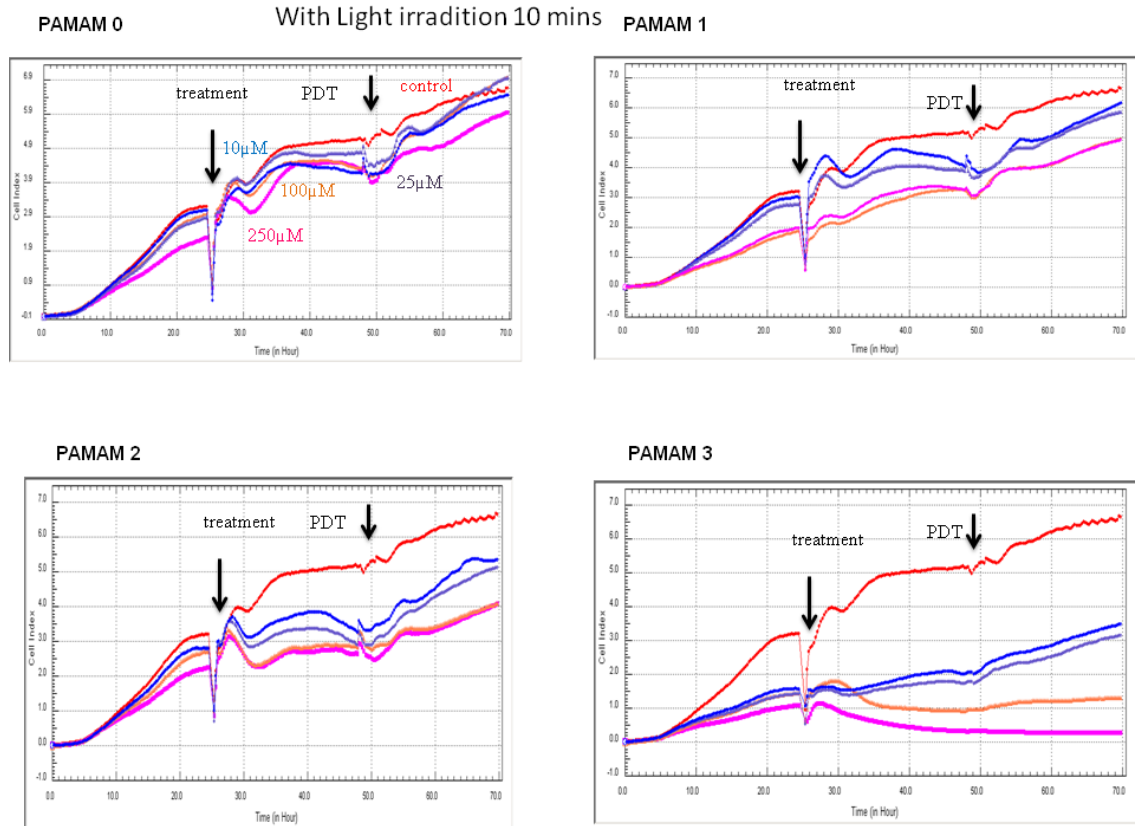

Figure 4.5 : 1 x 10^4 AGS cells seeded and incubated overnight for cells to settle down. After than cells incubated in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 to four generation of PAMAM to 24 hours. After than 24 hours 10 minutes irradiation done . Cell viability observed, (x) axis shows CI and (y) axis shows time (h). First arrow indicates the beginnig of the treatment (PS) and second arrow indicates beginning of PDT (light irradation) .

During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy. With cell shrinkage, the cells are getting smaller, the cytoplasm is dense and the organelles are more strictly packed. Pyknosis is the result of nucleus condensation and this is the most characteristic feature of apoptosis. Some of the major morphological changes that occur with necrosis include cell swelling; formation of cytoplasmic vacuoles; distended endoplasmic reticulum; formation of cytoplasmic blebs; condensed, swollen or ruptured mitochondria; disaggregation and detachment of

ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes; and finally disruption of the cell membrane [102].

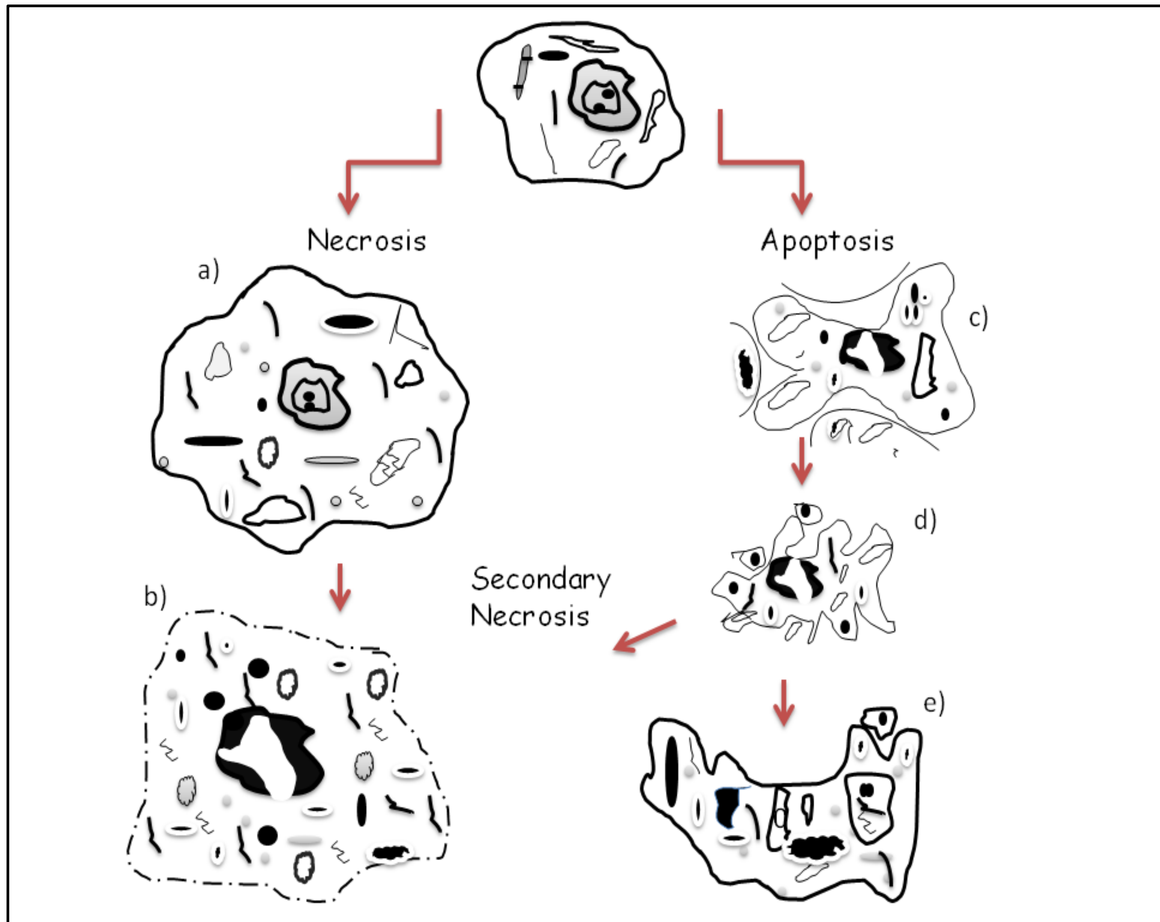

Figure 4.6 : Through necrosis the cell forms blebs, swells (a) and releases cytosolic constituents after permeabilisation of the plasma membrane (b), leading to an inflammation reaction in tissues. Apoptosis is characterised by a number of specific morphological and biochemical aspects that are different from necrosis. An apoptotic cell shrinks, forms blebs, and detaches from its neighbours, while the plasma membrane remain intact (c). In nucleus, the chromatin condenses at the nuclear membrane. After all, the cell disintegrates into apoptotic bodies (d) that are taken up by neighbouring cells (e). Only newly several techniques became present to recognize this form of cell death *in vitro* [103].

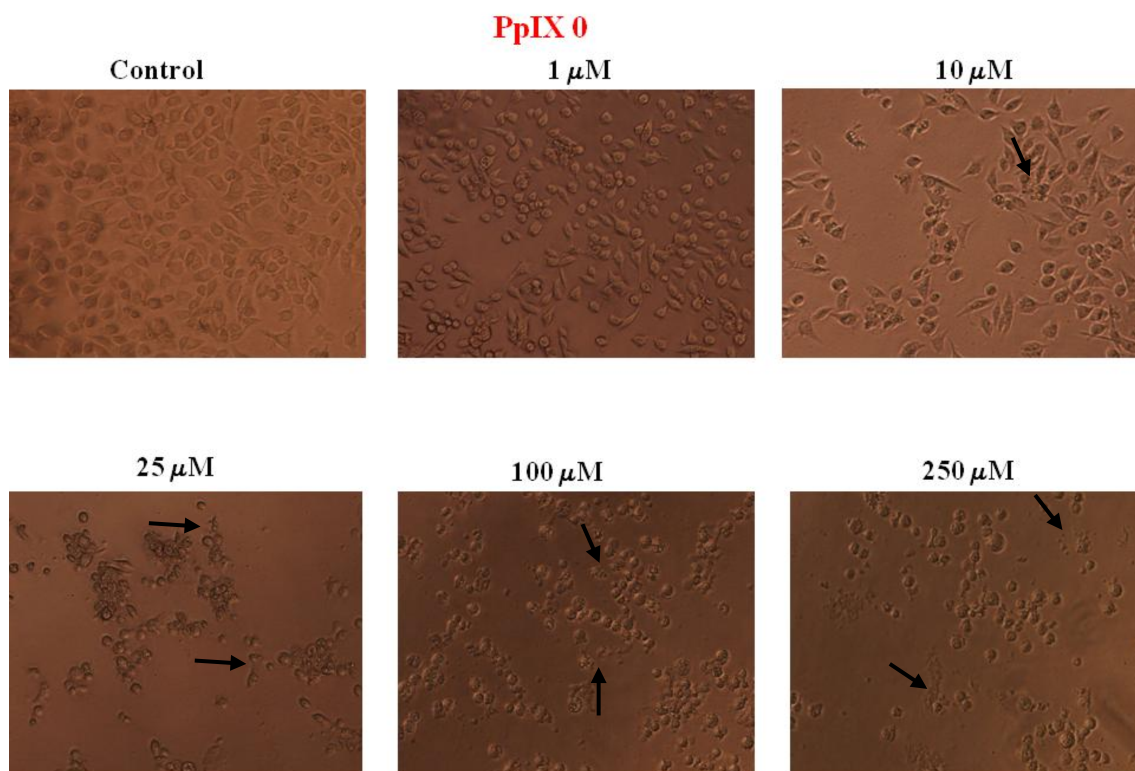

Figure 4.7 : 1 x 10^4 AGS cells seeded in 100 µl medium and let incubation with serial dilutions of the chemicals protoporphyrin IX (PpIX) in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_0 generation of synthesized material and incubated 24 hours at dark than observed under an invert light microscope. Images are at 10X

Morphology and real time monitoring results supports each other. There was no change in the morphology of the cells in dark for 1µM and 10µM concentrations but with increasing concentrations 25 µM to 250 µM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed (arrows indicates). As was also understood from pictures 25µM from to 250 µM showed toxic effect. Cell death has shown dose dependent manner at dark at unmodified PpIX (PpIX0) (Figure 4. 7)

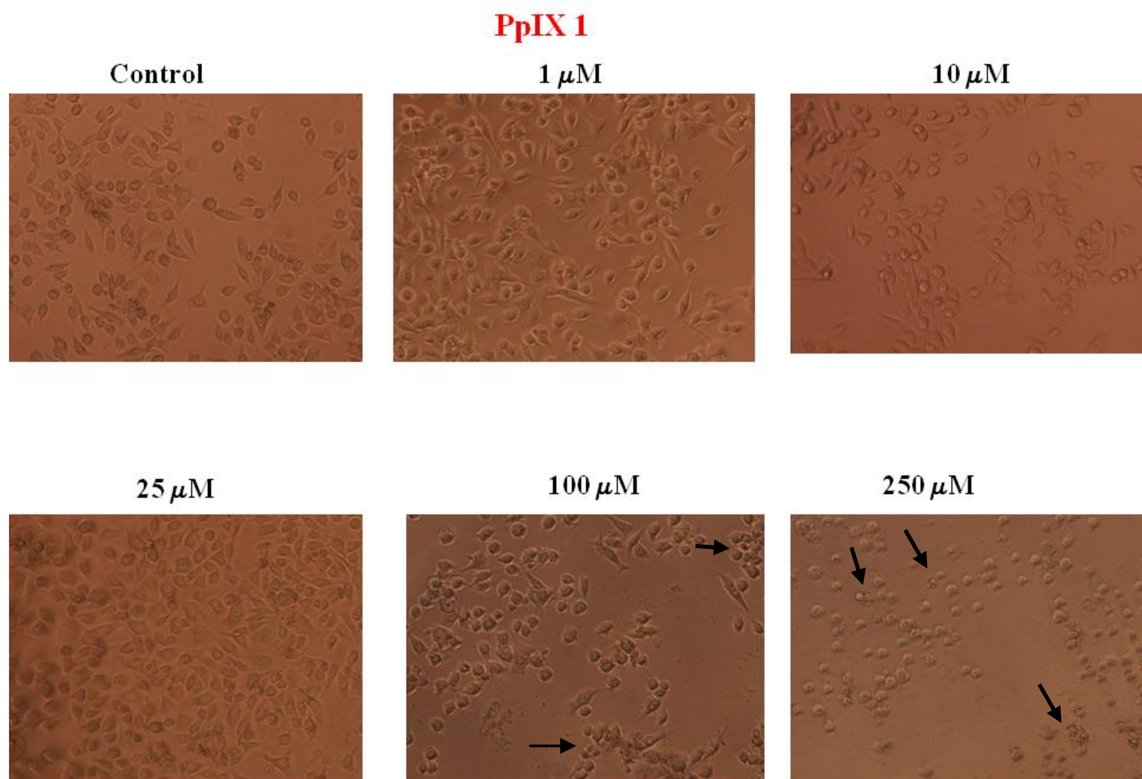

Figure 4.8 : 1 x 10^4 AGS cells seeded in 100 µl medium and let incubation with serial dilutions of the chemicals protoporphyrin IX (PpIX) in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_1 generation of synthesized material and incubated 24 hours at dark than observed under an invert light microscope. Images are at 10X.

Morphology and real time monitoring results supports each other. There is no change in the morphology of the cells at dark for 1µM and 25 µM concentrations. At 10µM concentration there is an unexpected result some of cells death. With increases concentrations 100 µM to 250 µM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed (arrows indicates). As is also understood from picture 250 µM showed toxic effect. Cell death didn't showed dose dependent manner at dark with first generation of PpIX1 (Figure 4.8)

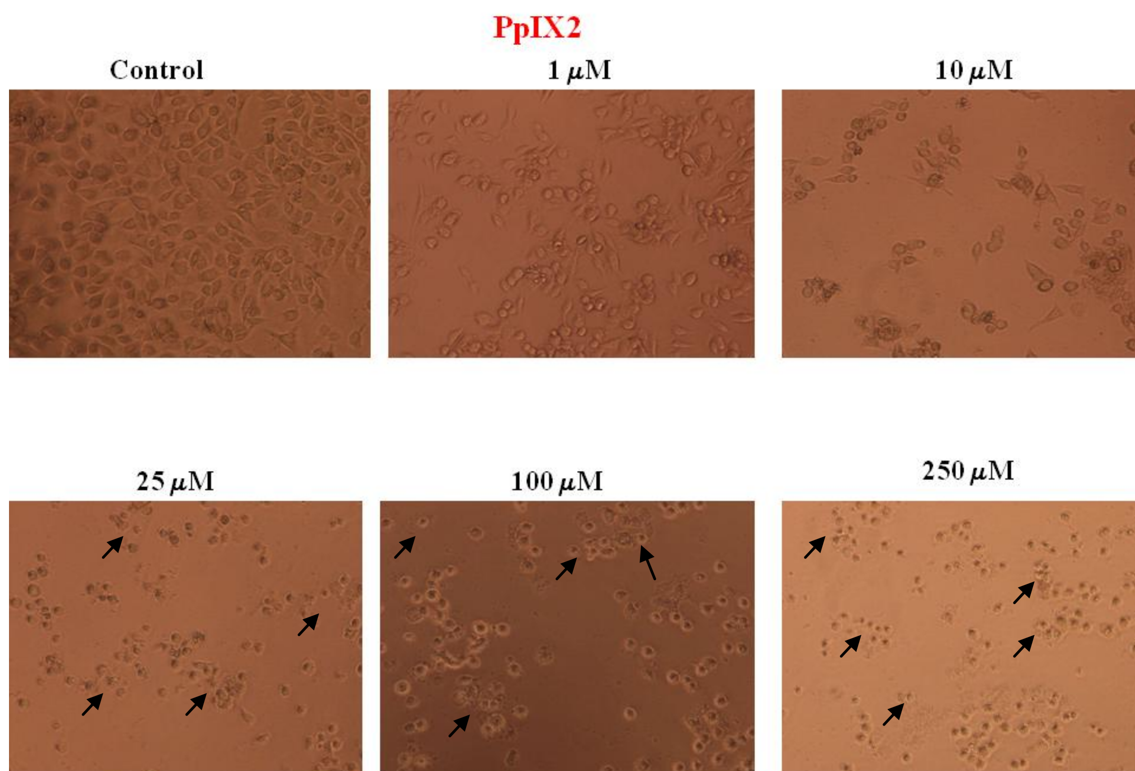

Figure 4.9 : 1 x 10^4 AGS cells seeded in 100 µl medium and let incubation with serial dilutions of the chemicals protoporphyrin IX (PpIX) in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_2 generation of synthesized material and incubated 24 hours at dark than observed under an invert light microscope. Images are at 10X.

Morphology and real time monitoring results supports each other. There is no change in the morphology of the cells at dark for 1µM and 10 µM concentrations. With increases concentrations 25µM to 250 µM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed (arrows indicates). As is also understood from picture 25µM, 100µM and 250 µM showed toxic effect. Cell death showed dose dependent manner at dark with second generation of PpIX2 (Figure 4.9).

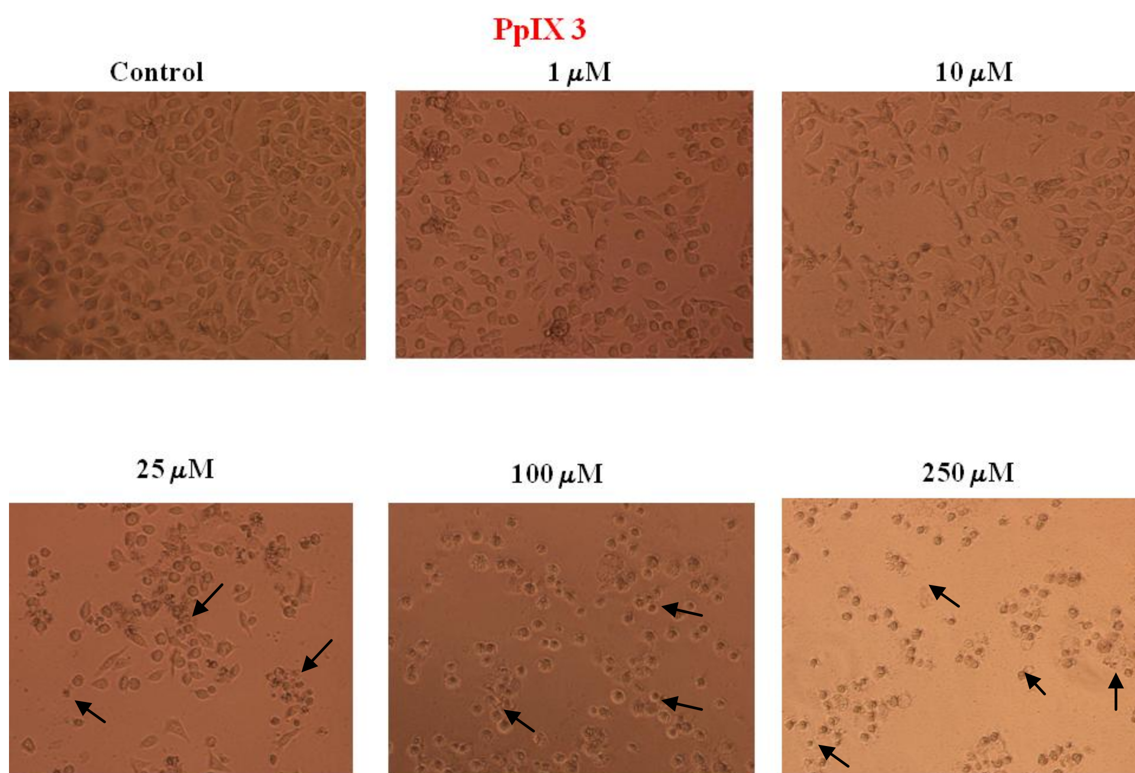

Figure 4.10 : 1 x 10^4 AGS cells seeded in 100 µl medium and let incubation with serial dilutions of the chemicals protoporphyrin IX (PpIX) in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_3 generation of synthesized material and incubated 24 hours at dark than observed under an invert light microscope. Images are at 10X.

Morphology and real time monitoring results supports each other. There is no change in the morphology of the cells at dark for 1µM and 10 µM concentrations. With increases concentrations 25µM to 250 µM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed (arrows indicates). As is also understood from picture 100µM and 250 µM showed toxic effect. Cell death showed dose dependent manner at dark with third generation of PpIX3(Figure 4.10).

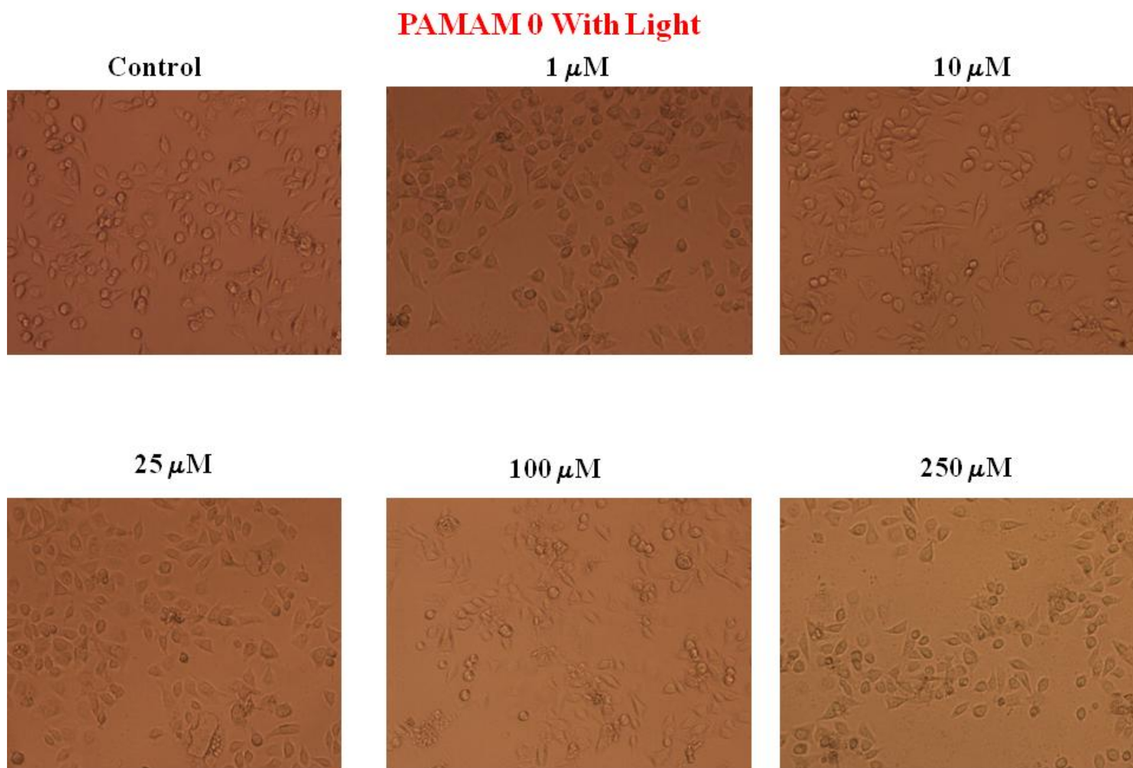

Figure 4.11 : 1 x 10^4 AGS cells seeded in 100 µl medium and let incubation with serial dilutions of the chemicals PAMAM in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_0 generation of synthesized material and incubated 24 hours than exposed to light irradiation 20 minutes and observed under an invert light microscope. Images are at 10X.

There is no change in the morphology of the cells for 1µM, 10µM, 25µM,100 µM and 250 µM. As is also understood from pictures PAMAM0 have no toxic effect on cells control groups and even treated group with 250 µM shown similar morphology (Figure 4.11).

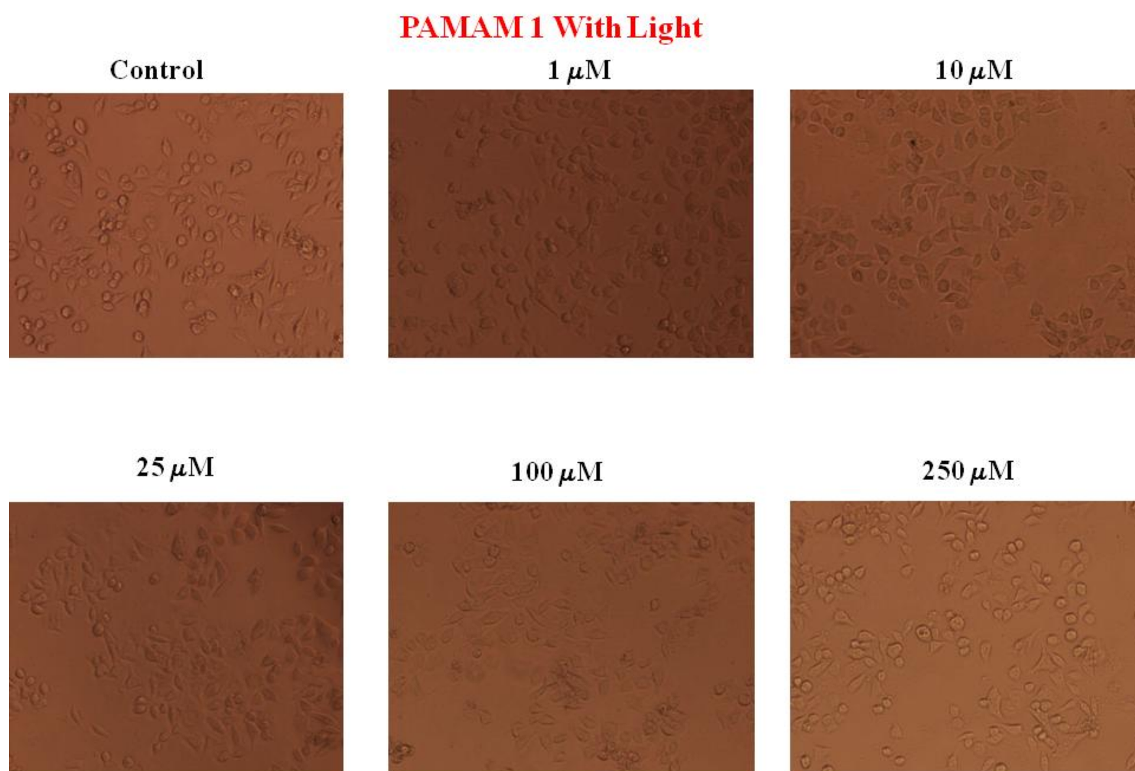

Figure 4.12 : 1 x 10^4 AGS cells seeded in 100 μl medium and let incubation with serial dilutions of the chemicals PAMAM in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_1 generation of synthesized material and incubated 24 hours than exposed to light irradiation 20 minutes and observed under an invert light microscope. Images are at 10X.

There is no change in the morphology of the cells for 1μM, 10μM, 25μM, 100 μM and 250 μM. As is also understood from pictures PAMAM1 have no toxic effect on cells morphology conserved (Figure 4.12)

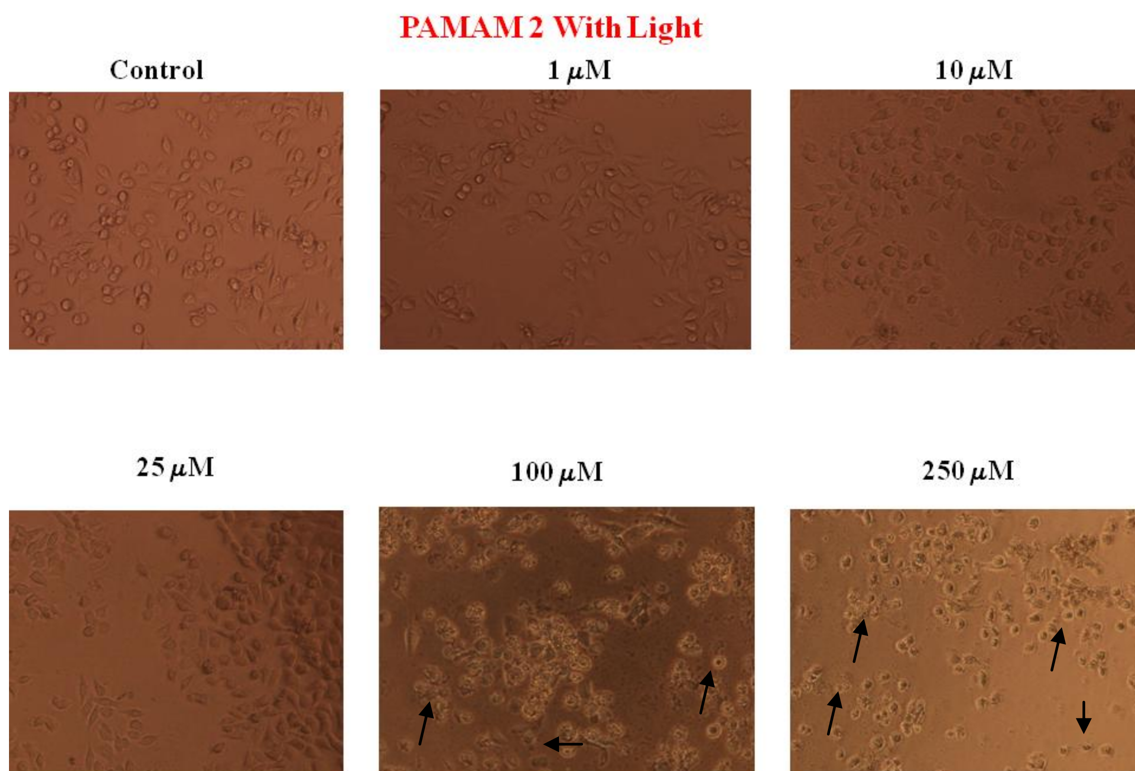

Figure 4.13 : 1 x 10^4 AGS cells seeded in 100 μl medium and let incubation with serial dilutions of the chemicals PAMAM in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_2 generation of synthesized material and incubated 24 hours than exposed to light irradiation 20 minutes and observed under an invert light microscope. Images are at 10X.

There is no change in the morphology of the cells for 1μM, 10μM and 25μM concentrations. With increases concentrations 100μM to 250μM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed (arrows indicates). As is also understood from picture 250 μM showed toxic effect. Cell death showed dose dependent manner at PAMAM2 (Figure 4.13).

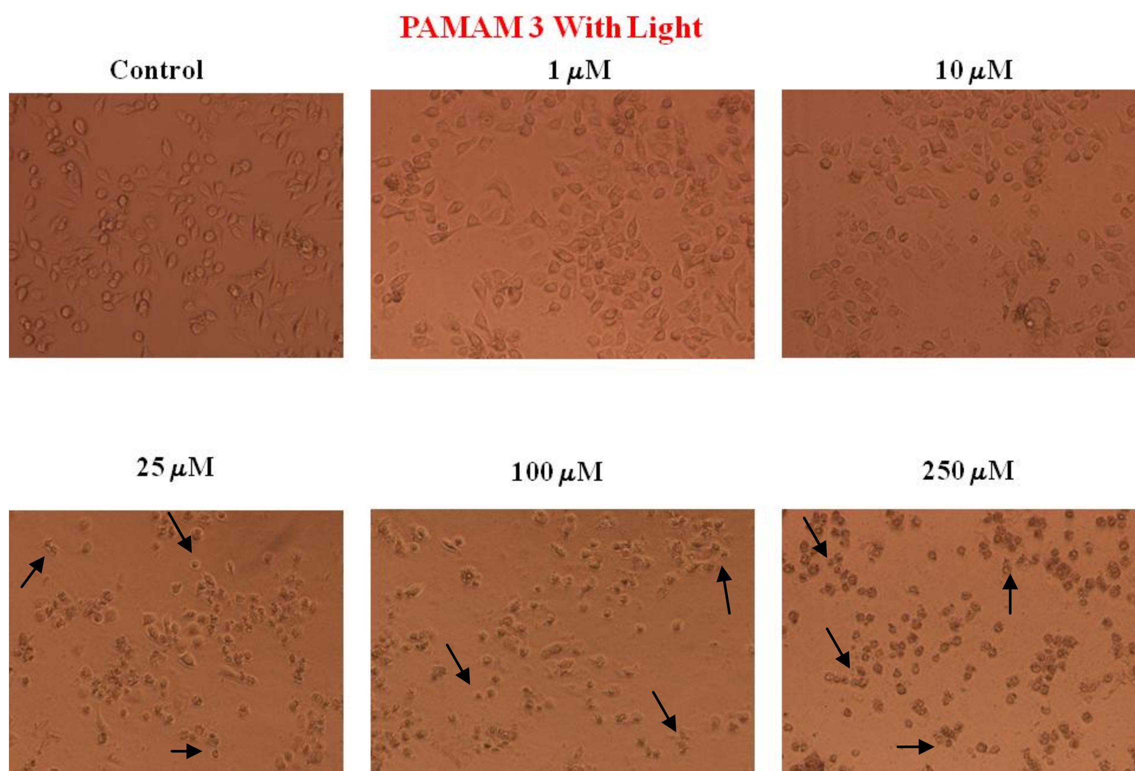

Figure 4.14 : 1 x 10^4 AGS cells seeded in 100 μl medium and let incubation with serial dilutions of the chemicals PAMAM in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_3 generation of synthesized material and incubated 24 hours than exposed to light irradiation 20 minutes and observed under an invert light microscope. Images are at 1X.

There is no change in the morphology of the cells for 1μM and 10μM concentrations. With increases concentrations and 25μM to 250μM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed (arrows indicates). As is also understood from picture 250 μM showed toxic effect. Cell death showed dose dependent manner at PAMAM3 (Figure 4.14).

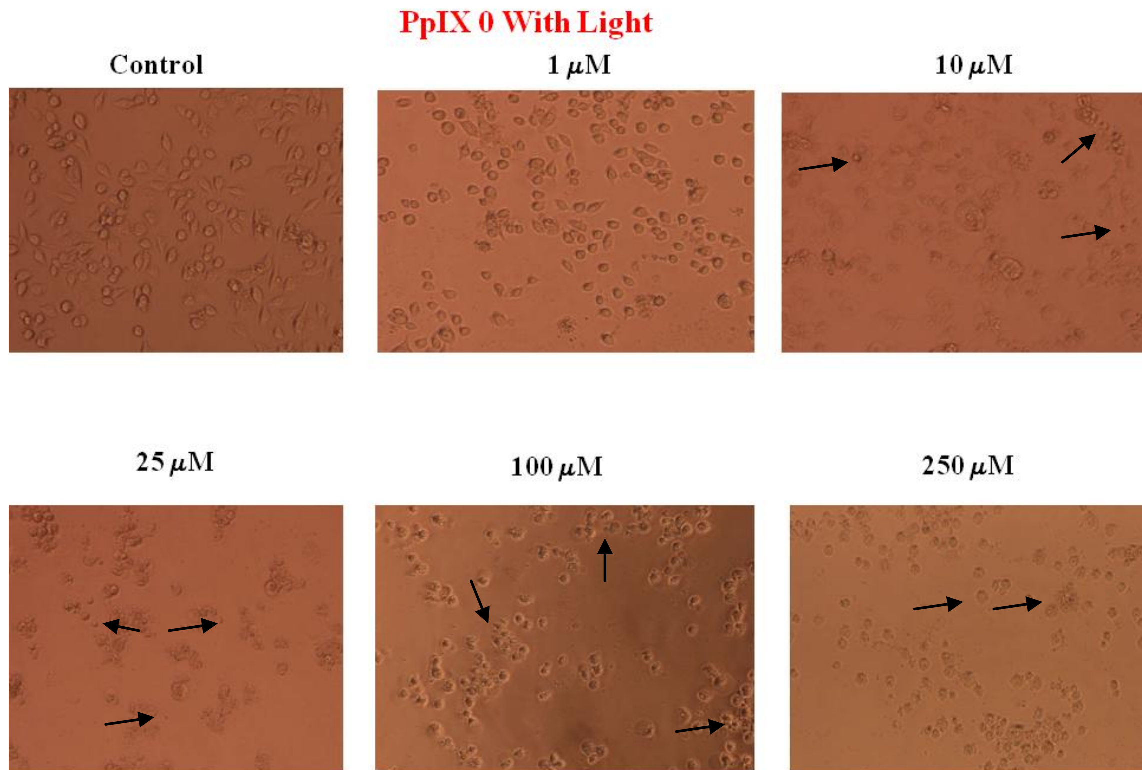

Figure 4.15 : 1 x 10^4 AGS cells seeded in 100 µl medium and let incubation with serial dilutions of the chemicals PpIX in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_0 generation of synthesized material and incubated 24 hours than exposed to light irradation 20 minutes and observed under an invert light microscope. Images are at 10X.

After 20 minutes PDT application there is no change in the morphology of the cells for 1µM concentration. With increases concentrations 10µM to 250µM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed (arrows indicates) As is also understood from picture 250µM showed toxic effect. Cell death showed dose dependent manner at unmodified PpIX (PpIX0) (Figure 4.15).

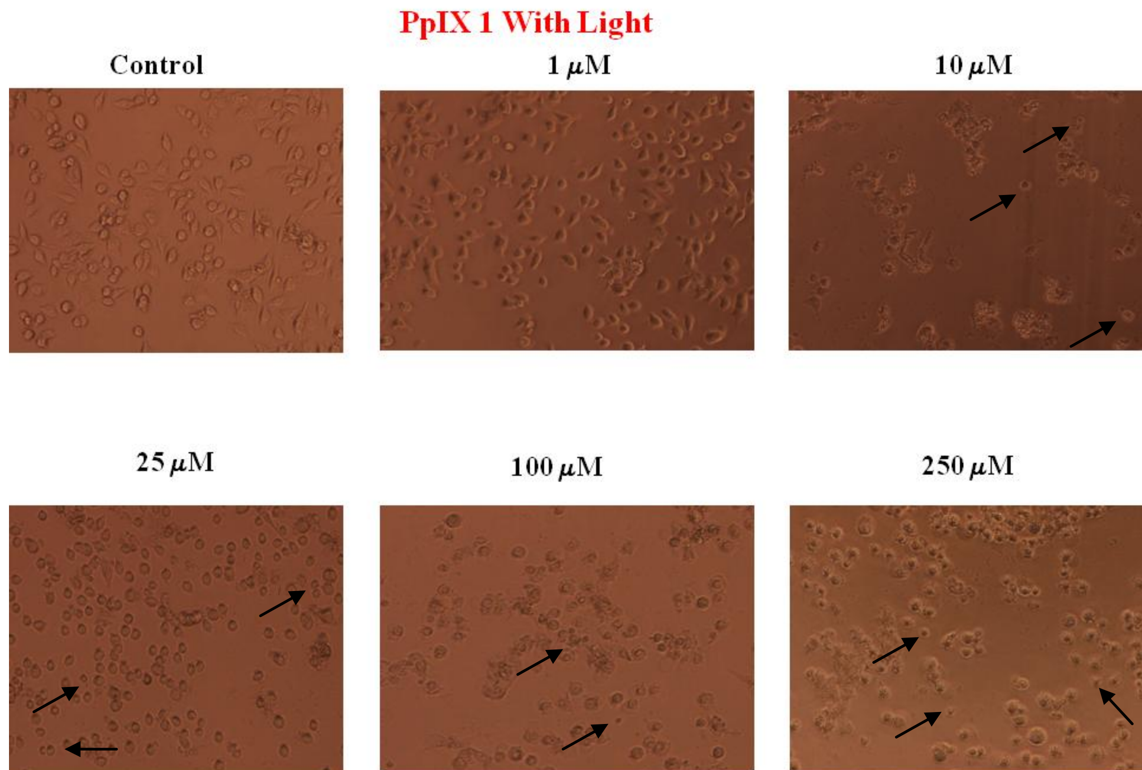

Figure 4.16 : 1 x 10^4 AGS cells seeded in 100 µl medium and let incubation with serial dilutions of the chemicals PpIX in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_1 generation of synthesized material and incubated 24 hours than exposed to light irradiation 20 minutes and observed under an invert light microscope. Images are at 10X.

After 20 minutes PDT application there is no change in the morphology of the cells for 1µM concentration. With increases concentrations 10µM to 250 µM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed also in some parts necrosis have been seen (arrow indicates). Cell death showed dose dependent manner at first generation of PpIX (PpIX1) (Figure 4.16).

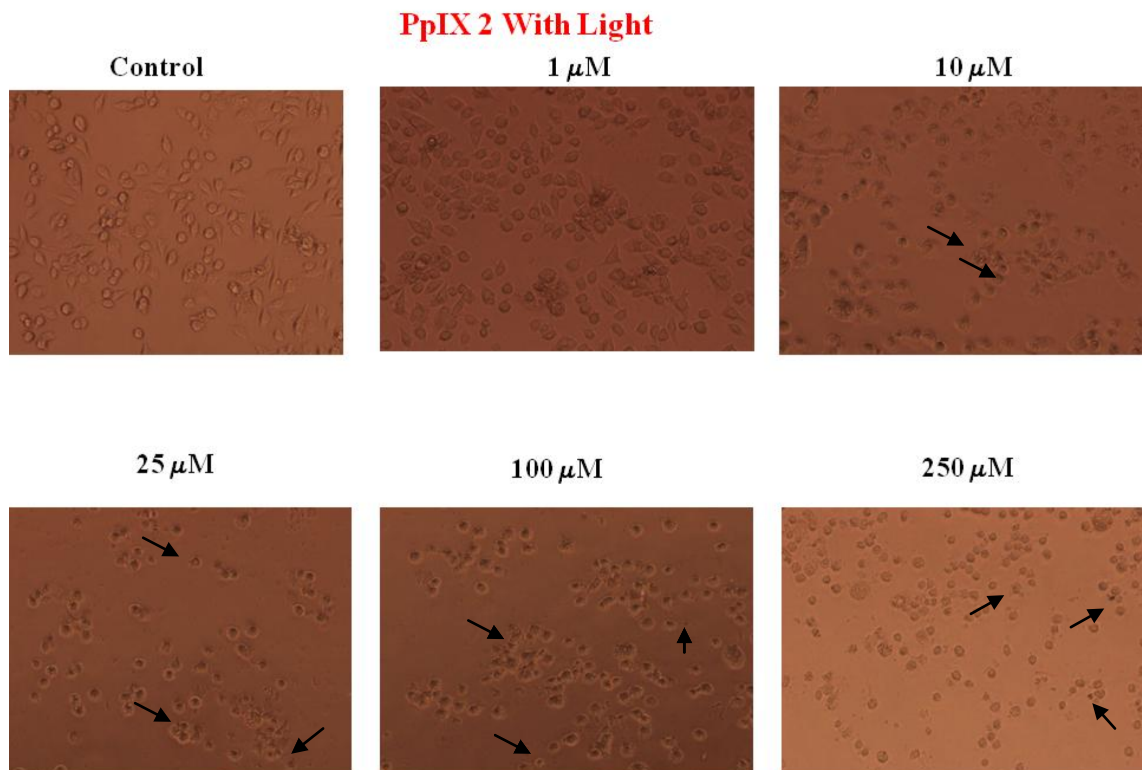

Figure 4.17 : 1 x 10^4 AGS cells seeded in 100 µl medium and let incubation with serial dilutions of the chemicals PpIX in dilution ratio of, 1:10, 1:25, 1:100 and 1:250 with P_2 generation of synthesized material and incubated 24 hours than exposed to light irradiation 20 minutes and observed under an invert light microscope. Images are at 10X.

After 20 minutes PDT application there is no change in the morphology of the cells for 1µM concentration. With increases concentrations 10µM to 250µM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed (arrows indicates). Cell death showed dose dependent manner at second generation of PpIX (PpIX2) (Figure 4.17).

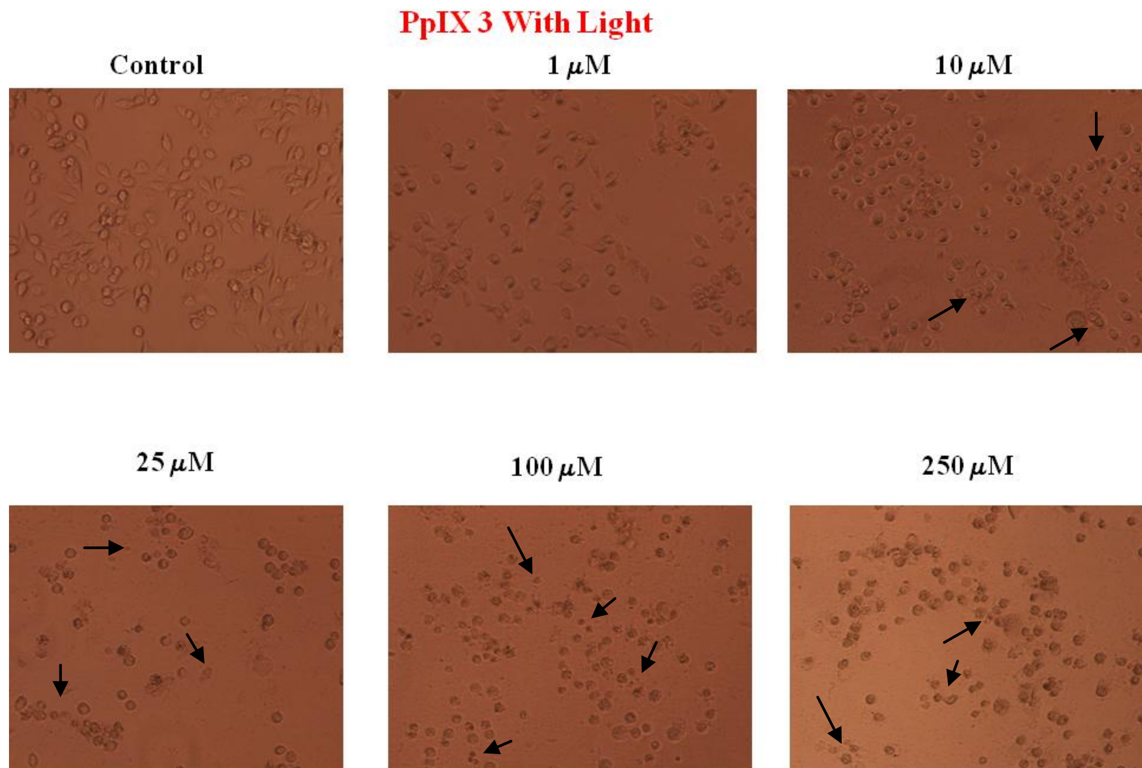

Figure 4.18 : 1 x 10^4 AGS cells seeded in 100 μl medium and let incubation with serial dilutions of the chemicals PpIX in dilution ratio of 1:10, 1:25, 1:100 and 1:250 with P_3 generation of synthesized material and incubated 24 hours than exposed to light irradation 20 minutes and observed under an invert light microscope. Images are at 1X.

Morphology and real time monitoring results supports each other. After 20 minutes PDT application there is a slight changes in the morphology of the cells for 1μM concentration. With increases concentrations 10μM to 250 μM cell shrinkage, cytoplasmic degradation took place and cell blebbing observed (arrows indicates). Cell death showed dose dependent manner at third generation of PpIX (PpIX3).

10 minutes PDT application real time results showed that 10 minutes doesn't sufficient light duration cause of this 10 minutes PDT application results were not monitorized with microscope (Figure 4.18). Also 10 minutes irradiation showed that low energy application may result with biostimulation effect on cells. It can be easily understand from 10 minutes irradiation results. After a while cells showed proliferation that proofs

that 10minutes irradiation at 465nm 21mW with even if different PS concentration lead to biostimulation on stomach cancer cells.

Morphology and real time monitoring analysis results hold up each other and confirmed that, PpIX molecules with and without modificated high concentrations (250µM - 100µM) caused of cells death cause of toxicicity. Via PAMAM modification water solubility and uptaken increased also toxicicity decreased. 20 minutes PDT application with PAMAM modified PpIX at 3 generation caused cell death via apoptosis. Cell shrinkage, nuclear fragmentation, chromatin condensation and cell blebbing observed this morphological changes indicated that cella death via apoptosis. This cell death mechanism will confirmed in further studies with other methods. 1µM, 10µM, 25µM, 100µM and 250µM concentraions were tested. Results showed that 250µM was toxic for each generation with light and without light. 100µM concentration showed less toxic effect but still toxic for cells. Best effective concentration for PAMAM modified PpIX should be more than 25µM but less than 100µM.

For clearly specifying the effective dose further studies should be done and concentration dose should be chosen between 25-100µM. Also viability should confirmed via another methods.

CONCLUSIONS AND RECOMMENDATIONS

PpIX-PDT has been reported to be effective in inducing cell death in a variety cancer cell lines [94, 104-107]. This thesis study addressed an important issue to develop better approaches to enhance PDT response in cancer treatment. The results of these study showed that PAMAM modified PpIX - mediated PDT induces apoptosis on human stomach cancer cells.

It was demonstrated that Protoporphyrin-IX and its PAMAM modified Protoporphyrin IX induces apoptosis in AGS cell lines by confirming real time monitoring of cell viability - proliferation and cell death – cytotoxicity. One of the major challenge in PDT treatment is to develop new photosensitizers that can be used at wavelengths to treat deep tissue tumors and uptaken by cell easily. The photosensitizer PpIX with PAMAM modification can penetrate deep into the tissue thanks to easily uptaken by cell.

Based on obtained results, it can be concluded that PpIX or PAMAM modified PpIX at concentrations between 25-100µM, 50 µM and 75 µM are best candidates combined with 21mW power density with 465nm can be used as photosensitizers and are effective PDT candidates in the treatment of cancer.

Even though, it has not been approved for clinical treatment of cancer in humans, it represents a promising candidate. The molecular basis of PAMAM modified PpIX - PDT mediated cell death is unclear. Additionally, light source is composed of a 12x8 LED array and every well is paired with a LED which is fixed on a perforated plate which was designed and manufactured by our group was used to photoactivate PpIX. Our results showed that PpIX when used in combination with LED light source showed an selective photo-toxic effect on human stomach cancer cells. PAMAM modified PpIX -PDT was rapid, uptaken by cells increased but not stable. After a while number of living cell have been increased. It can be due to insufficient light dose. Same total light dose, lowering light intensity increase total amount of photobleaching. 21 mW has been applied for this study and it can be cause of this undesired effect. Hence PDT couldn’t be effective as expected. Also duration time can be tolerate with increasing the power

density. We applied 21 mW it can be insufficient. For future experiments appliying light duration or / and power density should increase.

Alone PAMAM molecule (without PpIx conjugation) didn't show any photosensitizer property after appliying light there were not significant changes on morphology. Concluded that PAMAM molecule didn't increase the photosensitizing property of PpIX while PAMAM increase the ability of PpIX's water solubility and uptaken by cell.

Morphology and real time cell viabilty results are supports each other strongly.

Except 100µM and 250 µM concentrations PAMAM molecule didn't show cytotoxic effect on cells. Viabilty at 25µM, 10µM, 1µM concentration lines above the control group (untreated group). That confirmed that alone PAMAM molecule don't have toxic property on cells.

The System measures electrical impedance (Figure 3.6) across interdigitated micro-electrodes integrated on the bottom of tissue culture E-Plates (Figure 3.7). The impedance measurement yields quantitative information about the biological position of the cells, including cell number, viability, and morphology.

In order to examine the in vivo relevance of this finding, future work should focus on testing the in vivo effects of PAMAM PpIX - PDT on animal tumor models. Also concentraions between 25-100µM should be tested.

Additionally, it is important to investigate the molecular basis of PpIX-PDT mediated cell death in tumors to develop strategies to overcome possible side effects as observed for PpIX-PDT. The findings of this study grants further experimental and clinical investigation of PAMAM modified PpIX - PDT applications.

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