SENSITIVITY OF NSCLC AND LUNG FIBROBLAST CELLS TO UVC, HDR GAMMA RAYS AND PH-PDT

IN-VITRO SENSITIVITY OF NON-SMALL CELL LUNG CANCER CELL LINES TO UVC, HIGH DOSE-RATE GAMMA RAYS AND PHOTOFRIN-MEDIATED PHOTODYNAMIC THERAPY

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2007) McMaster University
(Biology) Hamilton, Ontario

TITLE: *In vitro* sensitivity of non-small cell lung cancer cell lines to UVC, high dose rate gamma rays and Photofrin-mediated photodynamic therapy.

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NUMBER OF PAGES: xvii, 153

Abstract

It has been suggested that combination treatment of high dose rate (HDR) intraluminal brachytherapy and PDT (Photodynamic therapy) in non-small cell lung cancer (NSCLC) may improve the efficacy of treatment, reduce the toxicity and improve quality of life for patients. To provide a cellular basis for this approach we have examined the *in vitro* sensitivity of normal lung fibroblasts (MRC5) and four NSCLC cell lines (SKMES-1, A549, NCIH460 and NCIH23) following, UVC treatment, HDR radiation, HDR radiation with Photofrin alone, PDT and combined HDR radiation and PDT. Cell sensitivity was measured using clonogenic survival. HDR radiation was cobalt-60 gamma rays (1.5-1.9 Gy/min). For PDT treatment, cells were exposed to 2.5 μg/ml Photofrin for 18-24 h followed by light exposure (20mW/cm^2). D_{37} values calculated from the survival curves indicated a 2-fold difference in sensitivity to UVC, 6-fold difference in HDR radiation sensitivity and an 8-fold difference in PDT sensitivity. All cell lines showed a similar Photofrin uptake per cell when measured by flow cytometry using 488nm excitation and 620-675 nm emission wavelengths. Photofrin alone at concentrations up to 10 μg/ml had no significant effect on the survival of the NSCLC cell lines, whereas 10 μg/ml of Photofrin alone reduced survival significantly in MRC5 cells. A radiosensitizing effect of Photofrin was detected in MRC5 and NCIH460 cells, but not in A549, SKMES-1 and NCI-H23 cells. For combined treatment cells were exposed to Photofrin and then either exposed to light and 15-30 minutes later exposed to HDR radiation or exposed to HDR radiation and 15-30 minutes later exposed to light. Results showed that although light followed by gamma rays resulted in a somewhat

greater tumor cell kill compared to gamma rays followed by light this difference was not significant for any of the cell lines tested. However, this difference was significant when data for all NSCLC cell lines were pooled. The combined treatment with high dose rate HDR radiation and PDT was not significantly different from an additive effect of the individual treatment modalities for *in vitro* survival of 4 NSCLC cells. In contrast the combined treatment was less than additive for the MRC5 cells suggesting that the combined treatment would have the potential advantage of doing less damage to the normal lung cells and suggests that equivalent tumour cell kill *in vivo* may be possible at reduced systemic effects to patients. In preliminary experiments we have started to examine the effects of Photofrin-mediated PDT on the extra cellular signal-activated protein kinase (ERK) signaling pathway in NSCLC cells. The use of multiple NSCLC cell lines allows for the possible identification of cell line specific changes involved in resistance to PDT and HDR radiation and this will be explored in future work.

Acknowledgements

I would like to give immense thanks to my supervisor, Dr. Andrew J. Rainbow, for providing me the opportunity to do such interesting and meaningful graduate work under his direction. This project work is an outcome of his continuous encouragement and guidance. He responded readily to all my inquisitive queries regarding the project and moreover, being an enabling and supportive person to have guided me throughout the thesis writing period. I have a great deal of respect for his knowledge and experience.

I also wish to greatly acknowledge and thank Dr. Gurmit Singh, the other member of my committee for his guidance, valuable suggestions, and for providing the necessary resources whenever required. I am very thankful to Dr. Singh and Dr. Rainbow for giving me the opportunity to do research work at Juravinski Cancer Centre, for it gave me an insight into different therapies used in cancer treatment and the various research projects going on in other labs. I would like to give appreciation to Myrna Espiritu for teaching me many techniques and all the particulars specific to working with Photofrin, along with all her technical support. Furthermore, I want to express my sincere gratitude to Dr. S. Igdoura and Dr. Xu-Dong Zhu for taking the time to serve on my defence committee.

I would like to thank all the current members and recent alumni of Dr. Rainbow's lab at McMaster. I found the collaborative teamwork environment to be very encouraging and supportive. In particular, Natalie Zacal provided valuable help during the project, and assisted by teaching me details of tissue culturing. I would also like to thank all the personnel in the research department at the Juravinski Cancer Centre.

Lastly, my sincerest feeling of appreciation goes to my family members for their encouragement and moral support during the work. I would like to thank my husband Rahul, for encouraging me to pursue my Masters degree and giving me countless rides to Juravinski Cancer Centre and McMaster so that I could take my experimental readings, often at late night hours.

Preface

This thesis is presented in 3 chapters. The first chapter is an introduction to explain and review the general concepts presented in the thesis. The second chapter presents data obtained from experiments used to determine the sensitivity of NSCLC cell lines following UVC, Photofrin mediated PDT, gamma rays, Photofrin mediated gamma ray sensitization and the combination of PDT and gamma rays. This chapter is written as a manuscript in preparation for publication. It is followed by third chapter listing a summary of all the work together with possible future initiatives for this work. Finally, appendices contain additional pertinent data and references are listed at the end of the thesis. All work presented in this thesis has been carried out by the author.

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List of Abbreviations

$^1O2$	Singlet oxygen
$^3O2$	Triplet oxygen
ALA	5-aminolevulinic acid
AlPcS4	Aluminum phthalocyanate
APAF1	Apoptosis-activating factor 1
CHO	Chinese hamster ovary
CPDs	Cyclobutane pyrimidine dimmers
D_{37}	Dose that gives a survival fraction of 0.37 (e-1)
DNA	Deoxyribonucleic acid
DSB	Double strand break
EDTA	Ethylenediamine tetra-acetic acid
EGFR	Epidermal growth factor receptor
ERK1/2	Extra cellular signal-activated protein kinase
FBS	Fœtal bovine serum
FDA	Food and Drug Administration
H_2O_2	Hydrogen peroxide
HDR	High dose rate
HDRILBT	High Dose Rate Intraluminal Brachytherapy
HPD	Hematoporphyrin derivatives.
HPde	Hematoporphyrin dimethyl ether
HSF	Heat-Shock Transcription Factor
HSPs	Heat shock proteins
IAPs	Inhibitor of apoptosis proteins
IR	Ionizing radiation
LDL	Low-density lipoprotein
LFS	Li-Fraumeni syndrome
Lu-tex	Lutetium Texaphyrin
LY-R	Mouse lymphoma cells
MAPK	Mitogen-activated protein kinase
m-THCP	Meta-tetrahydroxyphenylchlorin
NBA	Nile Blue A
NER	Nucleotide excision repair
NF-κB	Nuclear factor-κB
NHDF	Normal human diploid fibroblasts
NHF	Normal human fibroblasts
NO	Nitric oxide
NSCLC	Non small cell lung cancer
$O2^{\bullet -}$	Superoxide anion
$OH^{\bullet}$	Hydroxyl radical
PBS	Phosphate-buffered saline
Pc4	Phthalocyanine
PDT	Photodynamic Therapy

PpIX	Protoporphyrin IX
RIF-8A	Murine radiation-induced fibrosarcoma variant cells
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
$ROO^{\cdot}$	Sugar-peroxyl radical
ROS	Reactive oxygen species
SCLC	Small cell lung cancer
SE	Standard error
SnET2	Tin ethyl etiopurpurin
SSB	Single strand break
TKD	Tyrosine kinase domain
UV	Ultraviolet
VL	Visible light

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Chapter One

Introduction

Cancer: Search for New Treatments

Cancer is a disease characterized by a population of cells that grow and divide without respect to normal limits, invade and destroy adjacent tissues. Cancer cells can invade nearby tissues and can spread through the bloodstream and lymphatic system to other parts of the body through a process called *metastasis*. Cancer is usually classified according to the tissue from which the cancerous cells originate, as well as the normal cell type they most resemble. A definitive diagnosis usually requires the histologic examination of a tissue biopsy specimen. In addition, histologic grading and the presence of specific molecular markers can also be useful in establishing prognosis, as well as in determining individual treatments (Isobe *et al.*, 2005).

Lung cancer is the cancer that forms in tissues of the lung, usually in the cells lining air passages. The two main types are small cell lung cancer and non-small cell lung cancer. These types are diagnosed based on how the cells look under a microscope (as reviewed by Raz *et al*, 2006). Lung cancer is the number one cause of cancer deaths in females and has surpassed breast cancer as the leading cause of cancer deaths around the world. Only about 14% of all people who develop lung cancer survive for 5 years (Canadian cancer society statistics, 2007).

Once diagnosed, lung cancer is usually treated with a combination of surgery, chemotherapy and radiotherapy. High Dose Rate Intraluminal Brachytherapy (HDRILBT) and Photodynamic Therapy (PDT) are two treatment options that are also used for palliation of symptoms in many institutions. Endobronchial tumors are well suited to treatment with either PDT or high dose rate brachytherapy and good palliative

results have been reported with non small cell lung cancer (NSCLC) (Graham *et al.*, 2000; McCaughan *et al.*, 1996).

High Dose Rate Intraluminal Brachytherapy (HDRILBT) is a form of radiation treatment given by placing the radioactive isotope in and around a tumour. The ionizing radiation leads to rapid necrosis of tumour tissues principally by nuclear DNA damage (Bellnier and Dougherty, 1986, Hall and Giaccia 2006).

PDT is a two-step process in which patients are first given a systemic photosensitizing drug which is selectively accumulated or retained by the tumor. The tumor is then illuminated with a light source of a specific wavelength triggering a photochemical reaction that damages tumor cells and leads to tumor regression (Dougherty *et al.*, 1998). The mechanism of action is the generation of cytotoxic singlet oxygen, triggering a series of changes that lead to apoptosis and tissue necrosis (Ahmad *et al.*, 1998).

One difficulty encountered by studying PDT is that the sensitivity of cells to PDT varies between different cell types and even between cell lines that are very closely related (Tong *et al.*, 2000). It has been previously reported that murine radiation-induced fibrosarcoma variant cells (RIF-8A) have increased resistance to Photofrin-mediated PDT compared to RIF-1 cells and show increased sensitivity to ionizing radiation (Singh *et al.*, 1991; Roy, 1996). It is also reported that the radiation resistant L5178Y murine lymphoma cell line was more sensitive to chloroaluminium phtathocyanine-mediated PDT (Evans *et al.*, 1989) compared to the radiosensitive LY-S cell line derived from it. These results suggest that some radiation resistant tumor cells are sensitive to PDT and

some PDT resistant tumor cells are more sensitive to ionizing radiation. This suggests that a combined treatment of tumors with both Photofrin–mediated PDT and ionizing radiation could be superior. Although the mechanisms of action of ionizing radiation and PDT have been studied individually and described extensively, when used sequentially the interactions between the two modalities are not clearly understood.

2.0 Lung: Non small cell carcinoma

The most common symptoms of lung cancer are shortness of breath, cough (including coughing up blood), and weight loss. There are two main types of lung cancer categorized by the size and appearance of the malignant cells seen by a histopathologist under a microscope: non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). This classification, although based on simple histological criteria, has very important implications for clinical management and prognosis of the disease (Heighway *et al.*, 2004).

2.1 Subtypes of Non Small Cell Lung Cancer (NSCLC)

The types of non-small cell lung cancer are named for the kinds of cells found in the cancer and how the cells look under a microscope. The cancer cells of each type grow and spread in different ways. About 85% of all lung cancers are of the non-small cell type. There are 3 sub-types of NSCLC and the cells in these sub-types differ in size, shape, and chemical make-up. Because treatment varies greatly depending on the type and stage of lung cancer, the diagnostic workup is critical in terms of identifying the

specific type of lung cancer, the stage of the disease, and the ability of the patient to tolerate treatment (Knop, 2005; Van Cleave and Cooley, 2004).

i. Adenocarcinoma

Adenocarcinoma occurs with a frequency of 30-40% of all lung cancer cases and tends to occur in more peripheral locations arising from the smaller airways but can be found centrally in a main bronchus. It may arise from a previous scar and an eccentric pattern of calcification may be evident (Heighway *et al.,* 2004). The key diagnostic features of adenocarcinomas include gland formation - where the tumour cells are arranged around a central lumen - and/or mucin production (Raz *et al.,* 2006).

A subtype of adenocarcinoma known as bronchoalveolar cell carcinoma accounts for as many as 5% of bronchogenic carcinomas (Heighway *et al.*, 2004). The incidence of bronchoalveolar cell carcinoma is increased in patients who have underlying interstitial lung disease, parenchymal scaring, and exogenous lipoid pneumonia (Sibley, 1998). Bronchoalveolar carcinoma may spread to other sites or the other lung by means of transbronchial spread called aerogenous spread. These tumors can also demonstrate growth along the pulmonary interstitium without destroying lung architecture. This is called lepidic growth. Both types of growth are associated with a worse prognosis (Raz *et al.,* 2006).

ii. Squamous cell carcinoma

Squamous cell carcinoma occurs most frequently in the central zone of the lung and accounts for 30-35% of cases of all lung cancers (Heighway *et al,* 2004). It has a strong association with smoking. These tumours generally arise centrally within the lungs inside a large bronchus, although they may sometimes be peripheral and cells are large, flattened and stratified with a high cytoplasm to nucleus ratio. Squamous cell carcinomas grow intraluminally and are least likely to metastasize distantly. The mode of spread is direct extension to the local lymph nodes. The tumour mass generally extends into the lumen of the airway with invasion into the underlying wall (Okamoto *et al.*, 2006).

iii. Large cell carcinoma

Large cell carcinomas account for only 10-15% of bronchogenic carcinomas and are strongly associated with cigarette smoking. They tend to grow and spread quickly. The lesion occurs peripherally and grows rapidly, with early metastases and a poor outcome. A subtype of large cell carcinoma is giant cell carcinoma. This is highly malignant and associated with a poor prognosis. These tumours tend to consist of large cells with abundant cytoplasm, large nuclei and prominent nucleoli and they may occur peripherally or centrally (Heighway *et al.*, 2004).

3.0 Photodynamic Therapy

Photodynamic therapy (PDT) is rapidly becoming an accepted therapeutic modality for the treatment of some types of malignant tumors. PDT is a two-part process. First, the drug is administered to the patient and absorbed for 48 hours when it is

selectively accumulated or retained by the tumor. Secondly, a non-thermal laser is used to activate the drug from within cancer cells, effectively destroying them (Shackley *et al.*, 1999) (Figure.-1). PDT utilizes light of a specific wavelength to activate a preadministered photosensitizer to initiate a chain of photochemical reactions that result in the production of highly cytotoxic singlet oxygen or other reactive oxygen species (or both) (as reviewed by Dougherty *et al.*, 1998) (Figure. - 2). PDT may achieve localized tumor control via either direct cell killing or tissue necrosis as a result of vascular destruction (Ferrario *et al.*, 2000).

3.1 Various types of Photosensitizers

The synthesis and evaluation of new photosensitizers for the treatment of malignant and infectious disorders continues to be an active area of investigation. The most extensively studied photosensitizers are porphyrins that were identified over 150 years ago. For a long time most preclinical studies were dominated by the use of hematoporphyrin derivatives (HPD). HPD, the first generation of photosensitizers, is a mixture of porphyrins prepared from hematoporphyrin by acetylation and hydrolysis under basic conditions (as reviewed by Stewart *et al.,* 1998). Most preclinical and clinical studies have been performed so far with Photofrin II, a chemically prepared derivative of haematoporphyrin. It is a mixture of monomers, dimers and oligomers and was the first clinically approved photosensitizer (as reviewed by Pandey *et al.,* 2000). In spite of the proven efficacy of Photofrin in cancer treatment, it has certain limitations, i.e. a complex chemical composition, low extinction for tissue penetrating red light, and

tendency to accumulate in the skin. The last property results in cutaneous photosensitivity in patients for 1-2 months after a single administration. The limitations of Photofrin have created an industry in which a large number of porphyrin and porphyrin related compounds have been synthesized and studied as potential new photosensitizers for PDT. These factors have stimulated research leading to the development of second generation photosensitizers (as reviewed by Oleinick and Evans, 1998). Some of the second-generation photosensitizers that are in clinical trials include ALA (5-aminolevulinic acid, Levulan), m-THCP (meta-tetrahydroxyphenylchlorin, Foscan), Benzoporphyrin Derivative Monoacid (Verteporfin) and Lutetium Texaphyrin (Lu-tex).

ALA (5-aminolevulinic acid, Levulan) is the precursor of the photosensitizer protoporphyrin IX (PpIX) that is generated in mitochondria and can be activated with 635 nm light (Bisland *et al.*, 2004). It can be applied topically as a cream or an emulsion or in the case of internal malignancy, systemically (as reviewed by Stewart *et al.,* 1998). Both photo detection and PDT are based on the selective accumulation of PpIX in the neoplastic tissue. The introduction of exogeneous ALA to a cellular system can override negative feedback controls, leading to the over production of PpIX, which is then accumulated in cells because of a slow conversion rate into heme (as reviewed by Oleinick and Evans, 1998). The time course of PpIX production and retention is a function of the ALA diffusion rate, heme production rate, and tissue metabolism of PpIX. The time of the typical ALA application is usually 3-8 hours to allow penetration of ALA into the lesion and synthesis of PpIX, followed by treatment with light at 630-635 nm. (as reviewed by Oleinick and Evans, 1998, Stewart *et al.*, 1998).

m-THCP(meta-tetrahydroxyphenylchlorin, Foscan), a reduced porphyrin of the chlorine class, is an extremely potent photosensitizer that requires various low drug doses (typically less than 0.1 mg per kg) and low fluence rates (10 J/cm^2) for clinical treatment. Uptake of m-THCP in tumor tissue relative to skin and muscle appears to be somewhat better than for Photofrin (as reviewed by Stewart *et al.*, 1998).

Benzoporphyrin Derivative Monoacid (Verteporfin).This chlorine type compound is a mixture of two isomers with essentially equivalent photosensitizing properties, absorbs 690 nm light and is formulated in liposomes (as reviewed by Gomer *et al.*, 1991). Benzoporphyrin derivative has recently been approved by the United States Food and Drug Administration for the treatment of macular degeneration (as reviewed by Pandey *et al.,* 2000). One of the disadvantages of using this drug is difficult separation of the A and B ring Diels-Alder product from the mixture, and only the ring A isomer is effective. Both isomers from the mixture showed photo toxicity and some dark photo toxicity (Fowler *et al.*, 1990).

Lutetium Texaphyrin (Lu-tex) Results show that this drug is highly selective for tumors with very deep tissue penetration and limited skin photo toxicity. The drug shows strong absorption at 732nm and is an effective photosensitizer (as reviewed by Pandey *et al.*, 2000). Lu-tex induces only mild transient skin photo toxicity and exerts its effect mainly by direct tumor cell toxicity with little evidence for vascular-mediated damage and it is one of the few photosensitizers which is reported to give a selective tumor necrosis (as reviewed by Stewart *et al.,* 1998).

Table-1 includes a list of other photosensitizers; for example, chlorine derivatives, phthalocyanines, texapyrins, and less well characterized porphycens, and antracens, chlorophyll derivatives, purpurins, hypocrellins and hypericin.

3.2 Mechanism of action of photosensitizers

Following absorption of a photon of light of specific wavelength, the photosensitizer is activated to an excited and short lived singlet state (Moan *et al.*, 1991). The photosensitizer returns to the ground state by emitting a photon (fluorescence) or by internal conversion with energy loss as heat, enabling identification of tumor tissue (Figure-1.3). It is also possible that the molecule may convert to the triplet state via intersystem crossing which involves the change in the spin of an electron. The triplet state photosensitizer has lower energy than the singlet state, but has a longer life time and this increases the probability of energy transfer to other molecules (as reviewed by Dougherty *et al.*, 1998). There are two mechanisms by which the triplet state photosensitizer can react with biomolecules; these are known as the Type I and Type II reactions.

i. Type I reaction mechanism

Type I photochemistry involves electron/hydrogen transfer between the photosensitizer triplet and a nearby molecule e.g. a membrane lipid. After this oxidation-reduction reaction, the resultant substrate radical may react with oxygen to generate free radicals which will undergo typical radical chain reactions (Foote *et al.*, 1991). These radicals then react rapidly, usually with oxygen, resulting in the production of highly

reactive oxygen species (e.g. the superoxide and the peroxide anions). These radicals then attack cellular targets (as reviewed by Dougherty *et al.*,1998).

ii. Type II reaction mechanism

Type II reactions produce the electronically excited and highly reactive state of oxygen known as singlet oxygen. (as reviewed by Dougherty *et al.,* 1998; Jones *et al.*, 1994). Direct interaction of the excited triplet state photosensitiser with molecular oxygen results in the photosensitiser returning to its singlet ground state and the formation of singlet oxygen. Both singlet oxygen and many of the radicals can produce damage to cellular structures (Foote *et al.,* 1991). Singlet oxygen has a lifetime of the order of 0.01μs in cells, and can react with number of biological molecules, including membrane lipids, proteins, and nucleic acids. The radius of action of singlet oxygen in cells is of the order of only 0.01μm (Moan *et al.*, 1991). Thus, a study of intracellular and intratumoral targets is closely linked to study of sensitizer localization.

In PDT, it is difficult to distinguish between the two reaction mechanisms. There is probably a contribution from both Type I and II processes indicating the mechanism of damage is dependent on oxygen tension and photosensitiser concentration (as reviewed by Dougherty *et al.*,1998; Jones *et al.,* 1994).

iii. Acute vs. Metronomic

Metronomic PDT is a treatment in which both the photosensitizer and light are administered during an extended period, whereas acute PDT treatment involves delivery of single dose of photosensitizer and light at a single time. *In vitro* studies shows that

metronomic PDT with ALA induces a higher incidence of apototic cells as compared with an acute, high dose ALA-PDT (Bisland *et al.,* 2004). Comparing metronomic and acute ALA-PDT *in vitro* reveals an almost two-fold enhanced incidence of apoptosis in 9L cells after metronomic PDT compared to acute PDT (Bisland *et al.*, 2004). Madsen *et al.* have shown a significant decrease in glioma cell survival *in vitro* after treatment with multiple drug-light fractions at intervals of 2 days as compared with the same total doses administered as an acute, single treatment (Madsen *et al.*, 2000). Fractionated PDT is not a new concept. For example, repeated drug and light administration has been shown to be effective in basal cell carcinoma (Henderson *et al.*, 2000). Secondly the light can be fractionated after a single photosensitizer dose, with the time intervals varied to achieve maximal photodynamic effect. Several metronomic PDT studies using ALA have reported improved therapeutic results with light fractionation compared with single, acute treatments for both clinical and experimental cancers (Muller *et al.,* 1998). It will be of interest in future studies to determine whether metronomic PDT using Photofrin can improve therapeutic results in the clinic.

iv Apoptosis vs. Necrosis

Apoptosis is characterized by cell shrinkage, plasma membrane blebbing (loss of asymmetry), protease and endonuclease activation, and internucleosomal DNA fragmentation. In contrast, necrosis (non-programmed death) is characterized by random DNA fragmentation, cell swelling, lysis, and elicitation of an inflammatory response. Apoptosis has been found to be a prominent form of cell death in response to PDT for many cells in culture. There are by now numerous examples in which the ability of PDT

exposed cells to initiate the apoptotic process differs depending on the cell line, the photosensitizer and its subcellular location, the overall dose and other conditions. For example, in an early study, He *et al.* exposed three carcinoma cell lines to equitoxic doses of PDT with Photofrin and found apoptosis in two of the cell lines but not in the third (He *et al.*,1994).

The subcellular location of a photosensitizer has a strong influence on whether and to what extent cells undergo apoptosis in response to photoactivation. Dellinger *et al.* found apoptosis in normal green monkey kidney fibroblast CV-1 cells, if they were photoirradiated 24 hours after introduction of photofrin, when the photosensitizer was internalized, whereas after only one hour when photofrin was primarily in the plasma membrane, necrosis was the predominant form of cell death (Dellinger *et al.*, 1996). With Rose Bengal , a photosensitizer that is distributed in cellular membranes, Kochevar *et al* observed extensive apoptosis in HL-60 cells, if the dye was photoactivated by visible light, producing predominately singlet oxygen; in contrast if the dye was activated by UVA radiation, producing Rose Bengal- derived radicals in addition to singlet oxygen, there was no further increase in the yield of apoptosis, inspite of much greater yield of lipid peroxidation products. These results demonstrated that different reactive species produced at the same sites of photosensitizer location can have markedly different cellular effects (Kochevar *et al.*, 2000). Kessel and Luo studied a series of photosensitizers in L1210 murine leukemia and other cells and demonstrated that photosensitizers that bind to mitochondria induce apoptosis upon photoirradiation, whereas those that bind to the plasma membrane or lysosomes, but not to mitochondria,

kill cells less efficiently and by a non apoptotic mechanism (Kessel *et al.,* 1998). In a comparison of PDT- induced killing of Madin-Darby canine kidney (MDCK-II) cells and WiDr human colon adenocarcinoma cells, with the lipophilic photosensitizer 3THPP, the degree of the bystander effect was greater when the normal cells died by necrosis than by apoptosis and greater for normal cells than for the cancer cells (Dahle *et al.,* 2000). These studies show that although PDT can produce apoptosis or necrosis, or evoke a combination of the two outcomes, in many cases PDT is highly efficient in inducing apoptosis.

3.3 Biologic mechanisms and damage to subcellular targets

The particular mode of cell death in response to PDT depends on experimental conditions, such as the dose of PDT (Tsvetan *et al.,* 1994), and the subcellular localization of the photosensitizer. Many factors determine the cellular targets of photosensitisers. The incubation parameters and mode of delivery as well as the chemical nature of the drug all influence subcellular localisation, creating a number of potential targets for photodamage (as reviewed by Moor *et al.,* 2000). In cell culture studies with porphyrin based photosensitisers, short incubation times (up to 1 h) prior to illumination leads primarily to membrane damage whereas extended incubation periods followed by light exposure results in damage to cellular organelles and macromolecules (Kessel *et al.,* 1986). The diffusion distance of singlet oxygen is relatively short (about 0.1 micron), therefore the photosensitiser must associate intimately with the substrate for efficient photosensitisation to occur. Although the Type II process is considered the more relevant

reaction mechanism in PDT, cytotoxic species generated by the Type I reaction process can also act in a site-specific manner (Moan *et al.*, 1990)

PDT produces cytotoxic effects through photodamage to subcellular organelles and biomolecules. Hydrophobic (lipophilic) compounds preferentially bind membranes and will target structures such as the plasma membrane, mitochondria, lysosomes, endoplasmic reticulum and the nucleus (as reviewed by Dougherty *et al.*, 1998).

i Mitochondrial damage

Much work has focused on photosensitisation of mitochondria because these organelles perform vital functions in the cell. ATP is synthesised by oxidative phosphorylation in the mitochondria and is required for energy requiring processes such as replication, protein synthesis, DNA synthesis and transport (Gomer *et al.*, 1988). The mitochondria have been shown to be critical targets in PDT. Lipophilic porphyrins have demonstrated intimate intracellular association with mitochondrial membranes (Kessel *et al.*, 1997). Kessel and Luo have shown that photosensitizers that bind to mitochondria induce apoptosis upon photoirradiation, whereas those that bind to the plasma membrane or lysosomes, but not to mitichondria , kill cells less efficiently and by a mechanism not involving apoptosis (Kessel and Luo, 1999). The mitochondria are critical sites for release of factors, especially cytochrome c, that triggers the final stages of apoptosis . It has been proposed that cytochrome c, in combination with the cytoplasmic protein, apoptosis-activating factor 1 (APAF1), directly activates the cascade of caspases that carry out the final stages of apoptosis (as reviewed by Moor *et al.*, 2000). Expression of BCL-2, a protein of the mitochondrial outer membrane, prevents the release of the

mitichondrial release factors, particularly protects cells from PDT-induced apoptosis, and endows cells with a partial resistance to PDT (Oleinick *et al.,* 2002).

Mitochondrial photosensitisation may cause the uncoupling of respiration and phosphorylation resulting in the impairment of ATP synthesis and subsequent loss of cellular function. At the molecular level several mitochondrial enzymes and carriers involved in ATP synthesis have displayed sensitivity to mitochondrial photosensitisation (as reviewed by Hilf *et al.,* 2007). In addition, a rapid loss of mitochondrial membrane potential could be observed upon PDT which might be due to the opening of a large channel, called the mitochondrial permeability transition pore. This leads to Ca^{2+} release and might play a role in the observed cytochrome c loss (as reviewed by Moor et al., 2000). Following PDT the loss of mitochondrial integrity has been observed to occur before the loss of plasma membrane integrity, underlining the importance of the mitochondria as targets for PDT.

ii. Membrane damage

Oxidative degradation of membrane lipids can cause the loss of membrane integrity, resulting in impaired membrane transport mechanisms and increased permeability and rupturing of membranes. Cross-linking of membrane associated polypeptides may result in the inactivation of enzymes, receptors and ion channels (as reviewed by Moor et al., 2000). Photoperoxidation of membrane cholesterol and other unsaturated phospholipid leads to changes in membrane permeability, loss of fluidity, cross-linking of aminolipids and polypeptides, and inactivation of membrane associated

enzyme systems and receptors This membrane damage in the form of lipid peroxidation has shown to play a major role in photosensitizer mediated cytotoxicity (Thomas and Girotti, 1989).

Dellinger *et al.* showed that CV- 1 cells irradiated after a short incubation with photofrin showed accumulation of the photosensitizer primarily in the plasma membrane (Dellinger *et al.*,1996). Kessel *et al.* demonstrated significant effects of photoactivated porphyrins on the cell surface at PDT doses which markedly reduced cell viability but which did not affect internal aspects of metabolism e.g the incorporation of precursors into nucleic acids (Kessel *et al.,* 1997). Specht and Rodgers showed that plasma membrane depolarization takes place within a very few minutes after PDT treatment (Specht and Rodgers, 1991). Leunig *et al.* studied cell swelling after PDT which might be attributed to the influx or formation of additional osmotic substances within the cells, such as lactic acid, or calcium following depolarisation within the plasma membrane (Leunig *et al.*,1994). Alternatively, influx or uptake of electrolytes into the cells after damage of the plasma membrane might equally contribute to cell swelling (Specht and Rodgers, 1991). Plasma membrane damage is evidenced by bleb formation and reduction of active transport after treatment of the cell with PDT (Moan *et al.,* 1983).

iii Lysosomal damage

Lysosomal localisation has been observed for a number of photosensitisers. Several photosensitizers localise in lysosomes and upon illumination they can cause cell death via two different routes: via the release of lysosomal enzymes in the cytosol, or via relocalisation of the photosensitizer after illumination to other, non-lysosomal targets (as

reviewed by Berg *et al.*, 1997). In some cases apoptosis induction mediated by photosensitizers localised in the lysosomes has been described, but it is a much slower process than that induced by mitochondrial based photosensitizers (Noodt *et al.*, 1999). Recent studies have demonstrated that photosensitisers are redistributed from the lysosomes to other cellular sites upon light exposure (Berg and Moan., 1997). Lin and colleagues have reported that the degree of lysosomal damage caused by PDT was not correlated with the photocytotoxicity. Some cells survive partial lysosomal disruption, probably because lysosomal enzymes are inactivated by the treatment (Lin *et al.,* 1993; Berg and Moan., 1994). Initially it was thought that cell death was due to the release of enzymes following lysosomal membrane photodamage, however cell survival has since been observed following photodamage to 80% of cellular lysosomes (as reviewed by Moor *et al.*, 2000).

iv Nuclear DNA damage

The lipophilic PDT photosensitizers generally localize in membranes, including the nuclear membrane. *In vitro* studies have shown that PDT can produce both single and double strand breaks and sister chromatid exchanges in Chinese hamster ovary (CHO) cells and NHIK cells treated with hematoporphyrin and red light (Moan et al.,1980). Although DNA, RNA and protein synthesis are affected following PDT, recovery occurs suggesting that such damage may not necessarily be lethal (as reviewed by Moore *et al.,* 1997). In two closely related radiation resistant L5178Y murine lymphoma cell line (LY) cell lines, the cytotoxicity of PDT did not correspond to the induction or rejoining of single strand breaks, but did correlate with the number of DNA-protein crosslinks and

with the extent of DNA degradation caused by the treatment (as reviewed by Nowis *et al.*, 2005).

3.4 Photofrin Drug

Photofrin is a commercially available, partially purified hematoporphyrin derivative that has received intensive interest in recent years. Photofrin is the first-generation photosensitizer that has been approved by the U.S. Food and Drug Administration (FDA) for superficial and obstructing non–small cell lung cancer and obstructing esophageal cancer in 1995. Three years later Photofrin-PDT was approved for the treatment of lung cancer (Dougherty *et al.,* 1998). Photofrin is now used in the treatment of a number of cancers throughout parts of North America, Europe and Japan. Photofrin belongs to the group of medicines known as antineoplastics and is also known by the names Porfimer sodium or dihematoporphyrin ether.

i. Structure

The first photosensitizer tested in the clinic was haematoporphyrin derivative (HpD), a complex mixture of porphyrins. This was subsequently purified to give an enriched fraction of the active material, Photofrin. Photofrin is a lipophilic heterogeneous compound (as reviewed by Wang *et al.*, 2002). It is a mixture of haematoporphyrin monomers, dimers and oligomers and their dehydration products, with both ether and ester linkages (Dougherty and Marcus, 1992). The oligomers range in size from two to eight porphyrin units, although the major portion appears to be trimeric. The active component is believed to be either the di-haematoporphyrin ether or di-haematoporphyrin

ester (as reviewed by Wang *et al.*, 2002). Figure-1.4 shows chemical structure of Photofrin (Dougherty and Marcus, 1992).

ii. Photofrin uptake

For efficacy of photodynamic therapy, selective uptake and retention of photoactive substances has been postulated. Photofrin is a photosensitizer, which is preferentially retained by tumor cells and becomes active only upon light exposure (Jori, 1996). Poor lymphatic circulation in the tumor vasculature, leading to the aggregation of sensitizer molecules, is thought to contribute to preferential Photofrin accumulation (Dougherty and Marcus, 1992). In an *in vitro* study, enhanced Photofrin photosensitivity was observed in bovine aortic endothelial cells in comparison to mouse fibroblasts and amelanotic hamster melanoma cells. It was suggested that this might be because of the higher number of low-density lipoprotein (LDL) receptors in endothelial cells in comparison to other cell lines. The LDL carrier system is involved in delivery of porphyrin to tissue and it has been suggested that higher LDL receptor activity is responsible for high drug uptake in endothelial tissues (Leunig *et al.*, 1994). It has also been reported that tumor-associated macrophages in animal tumors take up large amounts of Photofrin (Korbelik *et al.*, 1991).

Drug uptake has been shown to be dependent on time, dose, temperature, pH , serum and cell volume (Moore *et al.*, 1997). The intracellular localization of Photofrin has also been determined. Following short incubations, Photofrin has shown to localize in plasma membranes, cytoplasm, nuclear membranes and nucleoli (Kessel *et al.*,1986). Following longer incubations (>16 hours), intracellular fluorescence is pronounced and

the binding is stronger in nuclear membranes and surrounding areas, mitochondria and other cellular organelles (Wilson and Singh., 1997). Photosensitizers that are negatively charged, such as Photofrin, have been found to accumulate in mitochondria and are thought to bind to various mitochondrial constituents (as reviewed by Oleinick *et al.*, 2001). The increased affinity of mitochondria for porphyrins has been suggested to be due to their lipophilicity. Depending on the target organelle, both necrotic and apoptotic cell death can be induced by Photofrin-mediated PDT. As discussed earlier Photofrin that binds to plasma membrane may lead to necrosis, whereas mitochondrial localized Photofrin could trigger the apoptotic cascade (Tong *et al.*, 2000).

iii. Light Source and Oxygen presence

The response of PDT depends on a combination of photosensitizer, light and oxygen. Photofrin absorbs in both the visible (500-700nm) and UV (360-400nm) region of the spectrum. However it's absorption at the higher wavelengths (>600nm) is relatively poor and it is these wavelengths in the red region of the spectrum that are most effective due to their ability to penetrate tissue (Gomer *et al.*, 1989). Clinically, light of a specific wavelength (630 nm) is used to activate intravenously preadministered Photofrin (as reviewed by Dougherty *et al*, 1998). While any light source that has sufficient power within the photo activation region can be used. These days diode lasers which are usually used are more reliable and usually cheaper, than other types of laser such as pumped-dye lasers or frequency-doubled or quadrupled lasers. If fiber optic delivery of the light is unnecessary, different types of lamps like xenon arc lamps or quartz halogen lamps can be used in combination with suitable filter systems (as reviewed by Moan *et al.*, 1998).

Experimental studies demonstrate that the availability of molecular oxygen during Photofrin-PDT has a profound effect on the treatment outcome. For Photofrin photosensitization of cells *in vitro*, full effects are observed at about 5% O_2 levels and no photosensitization can be observed in the absence of measurable oxygen (as reviewed by Henderson and Dougherty, 1992). Similar to ionizing radiation effects, where hypoxic cells are less sensitive than well-oxygenated ones, tissues oxygenation levels plays a significant role in influencing PDT treatment with Photofrin. Zheng showed that when a PDT treatment is combined with hyper oxygenation it could be more effective in controlling hypoxic tumors (Zheng, 2003). Oxygen is stable and normally found as triplet oxygen ($^3O_2$). When it interacts with the activated photosensitizer, this new singlet oxygen product ($^1O_2$) is believed to be the principle mediator of PDT cytotoxicity through subsequent interactions with cellular targets (as reviewed by Wang *et al.,* 2002). This $^1O_2$ has a short half-life of approximately 0.6 μs in cells and a limited diffusion distance of about 0.1 μm. As a result, oxygen must be present at the site of activation and will damage those structures close to it (Afonso *et al.,* 1999).

iv. Mechanism of cell killing

The reason that singlet oxygen is generated in cells following Photofrin-mediated PDT can be explained by simple photo physics. Provided that the Photofrin possesses an absorption maximum at a wavelength corresponding with that of the incident laser light, shining light on a highly coloured Photofrin causes excitation to the singlet excited state. The singlet excited Photofrin can decay back to the ground state with release of energy in the form of fluorescence. The photosensitiser returns to the ground state by emitting a

photon (fluorescence) or by internal conversion with energy loss as heat. It is also possible that the molecule may convert to the triplet state via intersystem crossing which involves a change in the spin of an electron. The triplet sensitizer can then be involved in a type I or type II photochemical reaction to produce damaging reactive oxygen species (ROS)(Pass., 1993) which is believed to be responsible for the cytotoxicity effects of Photofrin-mediated PDT. The triplet state can react with molecular oxygen, generating singlet oxygen ($^1O_2$) (Type II reaction) or leading to free radical formation (Type I reaction). In the biological system $^1O_2$ is the predominant form generated *via* the energy transfer of the excited triplet state of Photofrin to the ground state of oxygen (as reviewed by Wang *et al.*, 2002). In addition, reactive nitrogen species (RNS) and nitric oxide (NO), has also been found to participate in the events associated with Photofrin- PDT- mediated tumor destruction, particularly in the vascular response (Korbelik *et al.*, 2000).

A large portion of Photofrin-mediated PDT damage to tumors *in vivo* has been shown to be related to vascular damage. Photofrin mediated PDT causes a reduction in blood flow in tumors and normal tissue that is associated with vaso-constriction of arterioles and thrombosis of venules, as well as erythrocyte aggregation with decrease in blood flow (Engbrecht *et al.*, 1999) Studies evaluating the mode of cell death after PDT *in vivo* have shown evidence of both apoptosis and necrosis. It has been reported that with increasing time after tumor illumination, endothelial cell and tumor cell apoptosis was demonstrated in tissue sections (as reviewed by Dougherty *et al.*, 1998). One potential explanation for this is that blood vessel occlusion and subsequent endothelial damage lead to tumor hypoxia. Hypoxia has been shown to lead to apoptosis through a

variety of mechanisms, including change in p53 protein level and caspase activation associated with cytochrome c release from mitochondria. It is postulated that hypoxia induced by PDT leads to apoptosis of the surrounding tumor and contributes substantially to the tumor response (as reviewed by Dougherty *et al.,* 1998*).*

3.5 Cellular sensitivity to PDT

Numerous studies have been performed to identify subcellular PDT targets and to examine site-specific responses to the therapy (Kessel *et al.*, 1997; Kessel and Luo, 1999; Thomas and Girotti., 1989). These efforts have successfully identified mitochondria, lysosomes and various cytoplasmic membranes as among the primary targets of PDT-mediated damage, depending on the particular photosensitizer. PDT also activates several signal transduction pathways, which play an important role in cellular sensitivity to PDT.

i) The role of heat shock proteins (HSPs) in cellular sensitivity to PDT

PDT-mediated oxidative stress can initiate several different cell-signaling pathways, leading to either gene and stress protein expression or cell death (as reviewed by Moor, 2000). A number of stress proteins, including heat shock proteins (Gomer *et al.,* 1996), glucose regulated protein 78 (Xue *et al.,* 1995) and heme oxygenase-34 (Gomer *et al.,* 1991), can be induced by PDT in a photosensitizer-dependent manner. Heat shock proteins are categorized into several families that are named on the basis of their approximate molecular weight, for example, the 60 kDa Hsp60 family and belong to a large family of protein chaperones involved in assisting protein folding and unfolding in cells (as reviewed by Pockley, 2003). They are inducible in response to a variety of

stressful conditions and are thus commonly referred to in the literature as stress proteins. In addition to induction by hyperthermia, HSPs are activated by a wide range of physical, chemical and biological agents, including anticancer drugs, transition heavy metals, amino acid analogues, etc (as reviewed by Pockley, 2003). These stress proteins are involved in the rescue responses of cells following PDT. They play an important role in the cellular defense against photo oxidative damage. Although HSPs were assumed to localize exclusively at various intracellular sites (cytoplasm, mitochondria, endoplasmic reticulum, and nucleus), it is now clear that these molecules can be also expressed on outer cellular membranes and even released from damaged and viable cells (Kleinjung *et al.*, 2003; as reviewed by Pockley, 2003). The induction of cell surface expression and release of HSPs by PDT may represent an important event in the response of tumors to this treatment modality with a critical role in the induced inflammatory and immune responses that contribute to the therapeutic outcome (Korbelik *et al.*, 2005). Among the stress proteins, the HSP-70 family is the most abundant and conserved and has frequently been proposed potential biomarker of cellular toxicity. A study showed that HSP-70 was expressed constitutively in human lymphoma cells (Raji) but not in human leukemia cells (HL60). Heat treatment of HL60 cells induced expression of HSP-70 and resulted in significant reduction of PDT-mediated apoptosis. From the results of this experiment, it is suggestive that HSP-70 contributes to inhibition of apoptosis mediated by PDT (Nonaka *et al.,* 2004). Almost instantaneously after the treatment of mouse SCCVII tumor cells and human umbilical vein endothelial cells with Photofrin-based photodynamic therapy (PDT), a fraction (15-25%) of total cellular heat shock protein 70 (HSP70) became

exposed at the cell surface. The level of this surface-expressed HSP70 then remained unchanged for the next 6 hours and persisted at lower levels even at 18 hours after PDT. The same analysis for several other HSPs revealed the induced surface expression of HSP60 and GRP94, but not GRP78, on PDT-treated SCCVII cells. A fraction of total HSP70 existing in SCCVII cells at the time of PDT treatment was promptly (within 1 hour) released from cells after high treatment doses, whereas even lower PDT doses induced a substantial HSP70 release at later time intervals (Korbelik *et al.*, 2005). The results presented in this report suggest that PDT has a profound effect on HSPs in treated cells, which could have a significant bearing on the therapeutic outcome. Curry and Levy reported increased induction of heat shock proteins HSP47, HSP70, HSP90 and HSP110 after benzoporphyrin derivative (BPD)-PDT in vitro (Curry and Levy, 1993). Hanlon *et al.* used flow cytometry to analyze the induction of HSP60 expression in two different cell lines, RIF and HT29, using a fluorescent antibody targeted to HSP60 and found enhanced stress protein levels induced not only by Photofrin-PDT but also by Photofrin incubation alone. Heat shock protein 60 has been found to be greater in the PDT-resistant HT29-P14 cell line, suggesting that stress proteins might be involved in the cellular response to PDT induced damage alone (Hanlon *et al.*, 2001). Gomer *et al.* reported increased heat-shock transcription factor (HSF) binding in response to SnET2 incubation, although minimal HSF binding and no HSP-70 induction was observed after Photofrin incubation. These varied results thus indicate that stress responses to drug incubation are cell specific and strongly sensitizer dependent. Elevated expression of heat shock protein 27 (Hsp27), a member of the small heat shock protein family has been shown to protect

cells from death induced by hyperthermia, inflammatory cytokines and oxidative stress (Mehlen *et al.,* 1997). Wang *et al* showed that the Hsp27 gene was up-regulated 20-fold in Photofrin-mediated PDT resistant cell line HT29-P14. This study also showed that overexpression of Hsp27 alone in HT29 cells resulted in a cell line, H13, which displayed increased cell survival in response to Photofrin-mediated PDT. In addition, rapid phosphorylation of Hsp27 upon Photofrin photo oxidation in both HT29-P14 and H13 cells was detected suggesting that the increased expression of Hsp27 and the phosphorylation of Hsp27 after PDT play a critical role in cellular resistance to PDT. (Wang *et al.,* 2002).

ii) Signaling pathways involved in cellular sensitivity to PDT

PDT-mediated oxidative stress can initiate several different cell-signaling pathways in the treated cells, leading to either gene and stress protein expression or cell death (as reviewed by Moor, 2000). The interaction between various signaling pathways may play an important role in the efficiency of PDT (Oleinick *et al.*, 1998). The role of extra cellular signal-activated protein kinases (ERKs) in cell survival after PDT has been studied by Tong *et al.* (Tong *et al.*, 2002). They examined the response of ERK1/2 in PF-PDT-resistant (LFS087) and PDT-sensitive (GM38A) cells. ERK1/2 activity was induced rapidly in both cell types after PDT but was transient in GM38A cells and by 3 h had returned to a level significant lower than basal levels, whereas the induction of ERK1/2 was sustained in LFS087 cells and lasted for at least 11 h. In addition, the activation of ERK1/2 by Photofrin-mediated PDT is Raf-1 independent (Tong *et al.*, 2002). Western blot analysis performed on the proteins of LY-R (mouse lymphoma) cells and CHO

(Chinese hamster ovary) cells at various times following lethal (90—99% cell kill) doses of phthalocyanine Pc4-PDT showed that, p46 and p54 SAPK/JNKs were activated. However, PDT did not affect ERK and p38/HOG activation in LY-R cells. In contrast for CHO cells, ERK2 was slightly activated at 5 min post-PDT, and then declined, and p38/HOG was strongly activated from 5 to 60 min post-PDT. This study suggests that PDT can stimulate SAPK and p38/HOG cascades and that the latter participates in both rapid and slow PDT-induced apoptosis (Xue *et al.*, 1999). Another study reported that PDT with hypericin induced a strong and persistent activation of the JNK and p38 MAPK signaling pathways while inhibiting ERK2 activity. There was a protective role for the JNK/p38 MAPK pathways during PDT-induced apoptosis (Assefa *et al.*, 1999). Comparison of the gene expression profiles between the HT29 PDT-resistant cell line and its parental cell line showed that resistance to Photofrin-mediated PDT correlated to an increased expression/phosphorylation level of HSP27, a downstream target of the p38 MAPK signal, through phosphorylation and activation of the HSP27 kinase or MK2. Overexpression of HSP27 in the parental HT29 cell line increased survival in response to Photofrin-mediated PDT, suggesting a role for this signal in the PDT-mediated adaptive responses (Wang *et al.,* 2002). These results indicate that the phosphorylation of Hsp27 by Photofrin-PDT is catalyzed by signaling pathways other than p38 MAPK. It is also reported that Photofrin-mediated PDT increased the activity of JNK1 and p38 stress activated protein kinases within 30 minutes in Li-Fraumeni syndrome (LFS) and normal human fibroblasts after PDT treatment (Tong *et al.*, 2003). The epidermal growth factor receptor (EGFR) is a tyrosine kinase involved in the initiation and progression of various

cancers especially their proliferative, angiogenic, invasive, and metastatic aspects (as reviewed by Castano *et al.*, 2005). Wong *et al.* used ALA and Photofrin-PDT on human cancer cell lines: hypopharyngeal carcinoma FaDu; cervical adenocarcinoma HeLa; and hepatocellular carcinoma HepG2, and studied the cells response to cytokines, IL-6 and EGF, after PDT. PDT-induced the complete loss of EGFR on the cell membrane (Wong *et al.*, 2003). Luna et al. studied the PDT-mediated induction of the early response genes, c-fos, c-jun, c-myc, and egr-1, in murine RIF-1 cells. Incubation of exponentially growing cells with porphyrin based PSs in the dark also induced an increase in mRNA levels of early response genes (Luna *et al.*, 1994). PDT with Photofrin also increased transiently c-jun, c-myc and egr-1 mRNA in human adenocarcinoma HeLa cells (Kick *et al.*, 1996). It is known that signaling pathways involved in drug-induced apoptotic responses are often defective leading to resistance to chemotherapy. Since PDT can bypass defective signaling routes and activate the late stages of the apoptotic program this may explain the success of this therapy for the eradication of malignant cells resistant to conventional chemotherapy. Research advances in understanding cellular mechanisms of PDT will also shed light on the myriad of possible combination treatments.

iii) The role of p53 in cellular sensitivity to PDT

The p53 tumor suppressor protein, also called 'the guardian of the genome', is constitutively present in healthy cells and functions as a detector of DNA damage induced by different kinds of stress (as reviewed by Nowis *et al.*, 2005). Thus, in response to DNA damage, cells with wild-type p53 either become delayed in progression

through the cell cycle to undergo repair of the damage or are directed into apoptosis. Cells with mutant or no p53 are deficient in these responses (as reviewed by Oleinick *et al.*, 2002). The significance of p53 expression as a predictor of treatment outcome continues to be examined by many investigators. It is known that PDT functions by generating reactive oxygen species, and this treatment can induce both a rapid form of apoptosis and cell cycle arrest. In addition, many of the types of clinical tumors treated with PDT have a high frequency of p53 mutation. Alterations in the p53 gene have been detected in about 45–50% of Non-small cell lung carcinoma (NSCLC) patients (Takahashi *et al.* 1989). Alterations in the tumor suppressor gene p53 are frequent events in non-small cell lung cancers (NSCLC) with a point mutation in one allele and complete loss of the second allele (Ebina *et al.,* 2001). It is therefore of both basic mechanistic and clinical interest to evaluate the significance of the p53 phenotype in tumor cells exposed to PDT-mediated oxidative stress. Loss of p53 or p53 mutations has been reported to increase the resistance of some tumor cells to chemotherapy and radiation therapy, both *in-vitro* and *in-vivo*, and reintroduction of wild-type of p53 into some types of tumor cells with mutant p53 has been reported to result in an increased chemo sensitivity (as reviewed by Oleinick *et al.,* 2001). It is reported that Li-Fraumeni syndrome (LFS) cells expressing only mutant p53 are more resistant to Photofrin-mediated PDT compared to normal human fibroblasts that express wild-type p53. It was found that transient expression of wild-type p53 using a recombinant adenovirus increased the sensitivity of LFS cells to PDT. These results suggest a role for the p53 tumor suppressor gene in the cellular sensitivity of some human cells to Photofrin-mediated PDT (Tong *et al,* 2000).

Similar results were observed in a study by Fisher et al, where the efficacy of tin ethyl etiopurpurin (SnET2)-PDT, or Photofrin PDT, was investigated in human promyelocyte leukemia (HL60) cells exhibiting wild-type p53, mutated p53, or deleted p53 expression. This study demonstrated that HL60 cells expressing wild-type p53 were more sensitive to Photofrin and SnET2-mediated photosensitization, as well as to UVC irradiation, when compared to HL60 cells exhibiting deleted or mutated p53 phenotypes. Results of this study indicate that photosensitivity is increased in HL60 cells expressing wild-type p53 and that photosensitizer-mediated oxidative stress can induce apoptosis through a p53-independent mechanism in HL60 cells (Fisher *et al.,* 1997). Similarly a human colon carcinoma cell line (LS513) expressing wild-type p53 was more sensitive to PDT than another colon carcinoma cell line (LS1034) with mutated p53 (Fisher *et al.,* 1998). Although these studies provided evidence that p53 can sensitize cells to PDT- induced apoptosis, introduction of the viral oncoprotein E6 to abrogate wild-type p53 function of the LS513 cells or of breast carcinoma cells (MCF-7) did not alter their PDT sensitivity to loss of clonogenicity or induction of apoptosis, leading to the conclusion that PDT sensitivity is not p53-dependent (Fisher *et al.,* 1999). Thus, results aiming to determine the role of p53 in sensitivity of cells to PDT are conflicting. In another study the photosensitivity of two osteosarcoma cell-lines (U2OS and U2OS+p53DD) that are isogenic except that the latter expresses dominant negative p53 using hypericin as the photosensitizer was investigated (Lee *et al.,* 2006). Hypericin uptake was observed to be equivalent in both cell lines and there were no significant differences in cell killing between these cell-lines in clonogenic assays following PDT. p53 expression did not

increase up to 24 h after PDT treatment and there were also no significant differences in the cell-cycle arrest profiles and timing of onset of apoptosis (Lee *et al.,* 2006). These results suggest that for some tumor cell types the status of p53 may not be important in PDT-mediated cell killing or induction of apoptosis and these results imply that PDT may be used with equal efficacy for the treatment of some p53-positive and-negative tumors.

3.6 Alterations in PDT-resistant cells

A fruitful approach in cancer research is to obtain PDT-resistant cell variants that could be used for the molecular, biochemical, and cellular characterization of PDT cytotoxicity. Isolation of drug-resistant cell lines has produced models for subsequent biochemical and molecular studies related to drug action, drug resistance, and cross-resistance. Many investigators obtained PDT-resistant cells that could be used in evaluating cellular mechanisms of action and target sites associated with PDT photosensitization (Luna *et al.,* 1991; Singh *et al.*, 2001). Resistant cell lines have been shown to be a good model system in studying the mechanisms of anticancer treatment (Luna *et al.,* 1991). Singh *et al.* previously reported the isolation of three PDT resistant HT29 human colon adenocarcinoma cell lines that were generated by repeated exposure to three different photosensitizers. HT29.A11 was generated by 11 cycles PDT in the presence of aluminum phthalocyanate (AlPcS4), HT29. N8 by eight cycles using Nile Blue A (NBA), and HT29.P14 by 14 cycles using Photofrin. (Singh *et al.*, 2001). It was found that HT29-P14 cells were more resistant to Photofrin-mediated PDT compared with HT29 cells, as measured by the clonogenic survival assay. It was found that there was no difference in cellular uptake of Photofrin between the resistant cells and the

parental cells (Singh *et al.,* 2001). These results suggest that the difference in the sensitivity may result from a difference in gene expression profile of HT29-P14 cells compared with the HT29 cells. Further study by Yun Shen *et al.* suggested that the increased BNIP3, Hsp27 and Bcl-2 and decreased Bax and mutant p53 protein levels were found in the PDT-resistant variants (Yun Shen *et al.*, 2005). More recent studies showed that the PDT-resistant HT29 cell variants are differentially sensitized to UVA compared to UVC due, in part at least, through the altered expression levels of BNip3, Hsp27 and mutant p53 (Zacal and Rainbow, 2007). In an earlier study, a PDT resistant cell line, Rif-8A, was also isolated by repeated exposure of the Rif-1 mouse fibrosarcoma cell line to Photofrin-mediated PDT and found to be cross-resistant to cisplatin compared with the parental Rif-1 cell line (Moorehead *et al.,* 1994). In addition, the resistant variant had decreased plasma and mitochondrial membrane potentials showing that alterations in the plasma and/or mitochondrial membrane potentials may provide cells with a survival advantage when challenged with either photodynamic therapy or Cisplatin *in vitro.* Using the viral capacity assay, DiProspero *et al* showed cross-resistance of the Rif-8A cells to UV light suggesting some overlap in the type(s) of cellular damage induced by UV and PDT and / or an overlap in the pathways for the repair of UV and PDT damage in Rif cells (DiProspero *et al.*, 1997). These findings have shown that some of the possible mechanisms responsible for the altered sensitivity in these newly generated cell lines include altered drug uptake, or intercellular distribution, increase levels of scavenger molecules and enhanced repair activity. By comparing different cell lines in both their inherent sensitivity as well as their ability to become resistant it is hoped that

general principles may be extracted concerning the mechanisms and degrees of possible induced resistance. The use of multiple photosensitizers, combined with multiple cell lines in various studies, will allow for the identification of cell line specific or sensitizer-specific changes involved in resistance.

4.0 Brachytherapy

4.1 Procedure

Brachytherapy is a form of radiotherapy where a radioactive source is placed inside or next to the area requiring treatment. The use of machines to focus radiation on a cancer site is called external beam radiotherapy. In high dose rate brachytherapy (HDR), thin catheters are first placed in the tumor which is then connected to an HDR after loader. This machine contains a single highly radioactive pellet at the end of a wire, which is pushed into each of the catheters one by one under computer control. The computer controls how long the pellet stays in each catheter (Sur *et al.,* 1995). With a few well placed catheters in the tumour, HDR brachytherapy can provide a very precise treatment that takes only a few minutes. After a series of treatments, the catheters are removed, and there are no radioactive seeds left in the body.

4.2 Gamma rays

Gamma rays have the highest frequency and energy and shortest wavelength within the electromagnetic spectrum, i.e. high energy photons. Due to their high energy content, they are able to cause serious damage when absorbed by living cells (Hall and

Giaccia, 2006). Gamma-rays are produced spontaneously as certain elements (such as radium, uranium, and cobalt 60) release radiation as they decompose, or decay. Each element decays at a specific rate and gives off energy in the form of gamma rays and other particles (Watters *et al.,* 1999). X-rays and gamma rays have the same effect on cancer cells (Hall and Giaccia, 2006). Gamma rays are physically the same as x-rays. The only difference is their origin and they do not differ in nature or in properties. Gamma rays come from the decay of the nucleus of a radioactive atom and x-rays come from transitions of the electron orbits of an atom. Gamma rays and x-rays of the same energy have exactly the same penetrating power and can pass through the human body. Thick barriers of concrete, lead or water are used as protection from them (Durovic *et al.,* 2004). The energy from ionizing radiations is not deposited uniformly in the absorbing medium but is located along the tracks of the charged particles set in motion - electrons in case of x- or gamma rays, protons and α - particles in the case of neutrons. The biologic effect of radiation is determined not by the amount of energy absorbed but by the photon size, or packet size, of the energy. In their biologic effects, electromagnetic radiations are usually considered ionizing if they have photon energy in excess of 124eV, which corresponds to a wavelength shorter than about 10^{-6} cm (Hall and Giaccia, 2006).

4.3 Mechanism of cell killing

Ionizing radiation has the ability to excite electrons from atoms and molecules such that the electrons are ejected, producing free electrons, and free radicals that damage cellular DNA (Hall and Giaccia, 2006). In the case of x-rays, it is estimated that 2/3 of DNA damage in mammalian cells occurs through the indirect action of

radiation. In the indirect action, a secondary electron resulting from absorption of an x-ray photon interacts with water for example, to produce a hydroxyl radical. The hydroxyl radical then produces damage to DNA by causing a single strand break. Double strand breaks are formed when two single strand breaks are opposite one another or separated by only a few base pairs. (Hall and Giaccia, 2006). When exposed to gamma rays, a single electron is ejected from a molecule of water, leaving behind a positively charged free radical, $H2O^{\cdot+}$ This radical rapidly loses a proton to give the hydroxyl radical, $^{\cdot}OH$ (Figure-4). There are two ways in which a hydroxyl radical can induce DNA damage; the hydroxyl radical can abstract hydrogen from the deoxyribose sugar units, or, it can add to the Π-bonds of the nucleotide bases. Each of these results in specific DNA damage (Zaider *et al.,* 1994). Hydrogen abstraction from a deoxyribose sugar can occur at any of the carbon atoms of the sugar, resulting in the formation of water, as well as carbon centered radical. The resulting carbon-centered radicals then react with oxygen to form a sugar-peroxyl radical ($ROO^{\cdot}$) that leads to the cleavage of the sugar. This sugar cleavage results in a break in the sugar phosphate backbone of the DNA molecule and is known as single strand break (SSB). When multiple SSB's occur on both strands, near to each other, a double strand break (DSB) can occur (Breen *et al,* 1995; Hall and Giaccia, 2006). Secondly these carbon centered radicals have the ability to react with oxygen to produce their corresponding base peroxyl radicals which have the ability to react with water, hydrogen ions, free electrons, other organic molecules (including DNA) and oxygen to produce a wide variety of modified bases that no longer have the ability to bond to their original

corresponding base, or, that develop the ability to bond with an incorrect base (Breen *et al*, 1995; Hall and Giaccia, 2006) (Figure-5). Radiation-induced breakage and incorrect rejoining in pre-replication (G_1) and post-replication (late S or G_2) chromosomes can lead to chromosomes aberrations. Lethal aberrations include dicentrics, rings and anaphase bridges. Dicentrics are unstable aberrations; they are lethal to cell and are not passed on to progeny and its incidence declines slowly with time after exposure (Hall and Giaccia, 2006). The prevailing hypothesis on the nature of the lethal damage produced by ionizing radiation identifies heterologous double strand breaks in the DNA as the most common type of lesions that lead to mammalian cell death. Whereas mammalian cells are proficient in the capacity to repair most DNA double strand breaks (Bradford, 1991; Steele *et al.,* 1989), not all such lesions are repairable (Fuks *et al.*, 1994). Residual unrepaired DNA lesions are known to lead to post-mitotic cell death, associated with chromosomal aberrations and DNA dysfunction (Bradford, 1991). Both the interphase and postmitotic mechanisms contribute to cell killing after exposure of mammalian cells to ionizing irradiation. The relative contribution from each mode of cell death may differ with dose and from one cell type to another, relative to their inherent and inducible capacities to overcome each of these types of lethal radiation damage (Fuks *et al.*, 1994).

4.4 Cellular sensitivity of cells to ionizing radiation

Ionizing radiation activates not only signaling pathways in the nucleus as a result of DNA damage, but also signaling pathways initiated at the level of the plasma membrane (as reviewed by Watters, 1999). At high doses, ionizing radiation has plasma

membrane-direct effects such as increase of membrane intrinsic velocity, rigidity of lipid bilayer and lamellar structures. Radiation indirectly affects the plasma membrane through radiolysis, resulting in production of free radicals. The membranes of the cells and cellular organelles are the main targets for free radicals attack and could initiate lipid peroxidation and destruction of cell surface (Zaider *et al*, 1994). Low doses of ionizing radiation could also initiate biochemical reactions at the cell surface, resulting in the production of the second messengers as ceramide, which triggers apoptosis, or play a role in cell adaptive response to irradiation (Durovic *et al*, 2004). Radiation has been shown to activate multiple signaling pathways within cells that can alter cell survival or proliferation depending upon the radiation dose, the cell type and the culture conditions (Chmura *et al.,* 1997). It has been reported that ionizing radiation induces rapid sphingomyelin, a type of lipid found in animal cell membranes which surrounds some nerve cell axons, hydrolysis to ceramide and apoptosis in bovine aortic endothelial cells. This report provided the first evidence that apoptotic signaling can be generated by the interaction of ionizing radiation with cellular membranes and suggest an alternative to the hypothesis that direct DNA damage mediates radiation-induced cell kill (Friedman *et al.,* 1994). It has been reported that ionizing radiation caused activation of the MAPK pathway in RT2 glioblastoma cells derived from Fisher 344 rats that weakly enhanced the ability of radiation to reduce RT2 cell growth in clonogenic growth assays. These findings argue that inhibition of MAPK signaling reduces proliferation and enhances cell killing by ionizing radiation in transformed astrocytes (Park *et al.,* 2001). Apoptosis is the major mechanism by which ionizing radiation causes tumor cell death, and normal

p53 function is required to induce apoptosis by radiation. Ekedahl and coworkers showed that small cell lung cancer (SCLC) and NSCLC cells both initiate apoptotic signaling, resulting in caspase activation, after treatment with anti- cancer agents. However, in contrast to SCLC cells, NSCLC cells do not fully execute apoptosis. The apoptotic process in NSCLC cells seems to be blocked down stream of caspase activation, thus the failure of NSCLC cells to execute apoptosis could result from inhibition of active caspase by inhibitor of apoptosis proteins (IAPs) (Ekedahl *et al.*, 2002). In several cancers, including non–small cell lung carcinoma (NSCLC), the epidermal growth factor receptor (EGFR) is an important determinant of radio response (Das *et al.,* 2006, Das *et al.*, 2007). Thus, it is of great importance to learn how EGFR mediates tumor responses to ionizing radiation (IR). There is evidence that IR-induced activation of EGFR increases tumor cell proliferation through the activation of the EGFR/RAS/mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, which is thought to result in rapid repopulation after radiation exposure (Putz *et al.,* 1999). NSCLC cell lines harbouring somatic, activating mutations in the tyrosine kinase domain (TKD) of the EGFR exhibit significant delays in the repair of DNA double-strand breaks (DSB) and poor clonogenic survival in response to radiation (Das *et al.*, 2007). The effect of p53 mutations on the radio sensitivity of cells is controversial. The p53 gene product, which blocks cell entry into the S-phase in response to DNA damage, is associated mainly with regulation of the cell cycle (as reviewed by Watters, 1999). Cells show increased resistance to ionizing radiation due to the loss of growth arrest and/or apoptosis in the absence of p53 function (Matsuzoe *et al.* 1999). Other studies have shown that loss of p53 function has no effect

on radio sensitivity or that correlation between p53 and radio sensitivity may be a tissue-specific phenomenon (Kawabe *et al.*, 2001).

4.5 Intrinsic sensitivity of cells to gamma rays

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The predictive factors which contributes to the radiation therapy outcome of NSCLC lines are i) tumor related factors example tumor stage, tumor size, presence of regional lymph node metastasis, ii) host related factors (clinical factors), iii) technical factors in terms of radiation dose fractionation schedule, dose intensity and total dose which should be high enough to provide local tumor control in the majority of patients, and finally iv) radiobiological, molecular and metabolic markers which may have potential for monitoring tumor response and optimizing radiation therapy (Choi *et al.,* 2000). In a clinical study, Choi *et al.* showed that there is a dose-response relationship between radiation dose and local tumour control, and also between local tumor control and survival in stage III NSCLC (Choi *et al*, 2000). Studies done previously on lung cancer cell lines showed that SCLC cell lines were more radiosensitive than NSCLC cell lines (Krarup *et al.,* 1997; Carney *et al.,* 1983). In an *in-vitro* study by Carmichael *et al.* the radiation sensitivity of 17 human lung cancer cell lines was examined. The study showed that in comparison to small cell lung cancer cell lines (SCLC), NSCLC cell lines were generally less sensitive to radiation. A great variation in the sensitivity of NSCLC cell lines was found showing that mesothelioma cell lines (NCIH-290 and JMN) were remarkably sensitive to radiation and both squamous (NCI-H226 and NCI-H520) and all three adeno-squamous cell lines (NCI-H322, NCI-H595 and NCI-H647) were relatively

resistant to radiotherapy, as were variant SCLC cell lines (NCI-H526 and NCI-H841). In contrast, there was wide intra-group variation between the adenocarcinoma (NCI-H23, A549, NCI-H358, NCI-H522) and large cell carcinoma lines (NCI-H157, NCI-H460, NCI-H661), with a small group relatively sensitive to radiation (NCI-H157, NCIH23). Finally, the classic SCLC cell line (NCI-H69) was found to be most sensitive of all groups tested (Carmichael *et al.*, 1989).

Another study demonstrated that infection of cells with Ad5/CMV/p53, a recombinant adenovirus expressing wild-type p53, induces cell death in NSCLC cells and also increases the radiation sensitization of human tumour cells in experimental models that have endogenous wild-type p53 expression. These *in vitro* clonogenic survival results indicate that increased expression of wild-type p53, due to infection with Ad5/CMV/p53 adenovirus, can enhance the radio-sensitivity of NSCLC cells. A549 and H322 cells infected with Ad5/CMV/p53 adenovirus both showed increased radio-sensitivity in spite of their differences in p53 status of wild-type and mutant respectively. Possible explanations for the differences between the response of normal and NSCLC cells were investigated by comparing the ability of Ad5/CMV/p53 adenovirus to enhance the expression of p21 and Bax, two genes known to be regulated by p53 and implicated in radio sensitivity. In all the 4 cell lines examined (A549, H322, MRC-9 and CCD-16), p21 protein levels were examined by the combination therapy, but Bax protein was enhanced only in NSCLC cell lines which suggests that Bax may be playing an important role in adenovirus mediated radio sensitization by enhancing or restoring apoptotic properties (Kawabe *et al.*, 2001). Results from another study indicated that the irradiation

of NSCLC (A549) tumour cells significantly increased their adhesive interaction with endothelial cells. In contrast, when endothelial cells were irradiated, rather than tumour cells, adhesive interaction decreased with an increase in the radiation dose from 0, 5, 10, 20 Gy respectively. These findings may have important implications for the metastatic ability of irradiated tumour cells (Kiani *et al.,* 2004). The radiation sensitivity of the radiation induced H1299-IR NSCLC cell line was apparently the same as the parental NSCLC cell line H1299. Compared with the parental cell line H1299 and H1299-IR were both more radio tolerant than the A549 cell line. However, H1299-IR became significantly more sensitive to cisplatin, an anti tumour agent (Tsutsumi *et al.*, 2006).

4.6 Photofrin as a radiation sensitizer

Scientists are looking for ways to increase the effectiveness of radiation therapy. Various types of investigational drugs are being studied for their effectiveness in sensitizing tissue to radiation. These radio sensitizers make the tumor cells more likely to be damaged from the effects of radiation. It is suggested that radio sensitization is dependent on the dose of the photosensitizer, the type of sensitizer, and the dose rate at which radiation is administered (Berg *et al* 1995). Kostron *et al.* found an interaction of HPD with light and ionizing radiation in a rat glioma tumour model (Kostron *et al.*1986). Roy, 1996 showed that a drug concentration of 20 μg/ml of Photofrin results in a significant increase in the sensitivity of RIF-1 murine fibrosarcoma cells to gamma rays (Roy, 1996). In another study Schaffer *et al.* examined whether Photofrin could act as an efficient tumor radio sensitizer. To test this possibility, he injected mice with different porphyrin-type photosensitizing agents, including Photofrin, 5-aminolevulinic acid,

chlorine e, haematoporphyrin, protoporphyrin, Zn-tetrasulphophtalocyanine, and irradiated with 5 and 15 Gy using a Siemens X-ray device. Even though all the porphyrins accumulated in significant amounts in the neoplastic lesion, only Photofrin significantly improved the response of the tumor to irradiation by increasing the doubling time of the tumor volume from 6.2 days in the untreated control group to 10.9 days in the 5 and 15 Gy-irradiated groups (Schaffer *et al.,* 2002). A more recent study by Kulka *et al* showed that pretreatment of a human bladder cancer cell line (RT4) and a glioblastoma cell line (U-373 MG) with Photofrin prior to radiation, increased cell sensitivity compared to cells not pretreated with Photofrin, but irradiated under identical conditions. In contrast, for colon adenocarcinoma cells (HT-29), pretreatment of cells with Photofrin II did not increase cell sensitivity to radiation (Kulka *et al.*, 2003). It has been suggested that the quantity of the Photofrin accumulated in the cells may determine the efficiency of radio sensitization. Experimental data obtained from the not-aggressive murine hepatoma MH-22A tumor model, where extremely low HPde (hematoporphyrin dimethyl ether) and Photofrin intracellular concentrations were accumulated, showed no significant radio sensitization (Luksiene *et al.*,2005).

So far investigations on radio sensitization by Photofrin are very controversial. A detailed understanding of the mechanism involved in the radio sensitization of tumors by Photofrin is hampered by the highly heterogeneous chemical composition of such porphyrins. It has been proposed that radio-sensitizing effect of Photofrin seems to be due to some oligomeric constituents that could specifically react with radio generated-radicals thereby amplifying the effect of the radiation. (Pass *et al.,* 1996; Allison *et al.*, 1994). It is

also possible that in the presence of Photofrin the repair process of sub-lethal cell damage, after ionizing irradiation, is inhibited, thus helping in the tumor control. Further investigations have to be carried out to understand the mechanisms of the process, leading to radio sensitization effect of Photofrin.

5.0 Combination Therapy

The rationale of cancer treatment with a combination of different therapeutic modalities is to obtain improved tumor control with minimal damage to normal tissues. The potential exists for combinational therapies involving PDT. The basic requirements of PDT namely sensitizer, light and oxygen are easily integrated into many other anticancer therapies such as ionizing radiation or hyperthermia. Luminal disease causing obstruction is a common symptom of advanced lung cancer that has failed all other forms of curative therapy. Ionizing radiation (IR) and Photodynamic Therapy (PDT) of cancer are frequently used in order to improve tumor control with other treatments. There is increasing evidence from various studies that a combined treatment with IR and PDT would be more effective than either IR or PDT alone. The mechanism of cell killing due to IR and Photofrin-mediated PDT are different and when used in combination, the effect could be, at minimum, additive or perhaps synergistic (Bellnier and Dougherty 1986).

Results investigating PDT and radiation combinational therapy have been conflicting. Concurrent delivery of the two modalities as well as PDT following radiation after various times have resulted in synergistic reduction in survival for some mammalian cell lines but not all mammalian cells (Kostron *et al.*, 1988, Kostron *et al.*,

1986, Ben-Hur *et al.*,1988). In an *in-vitro* study a synergistic interaction between photodynamic treatment and ionizing radiation was observed with L929 fibroblasts, whereas these treatments were additive with Chinese hamster ovary and T24 cells. (Prinsze *et al.,* 1992). These results indicate that the most effective combination of treatment modalities is different in different cell types. It has been suggested that these conflicting results may be due to cell line differences in the sensitivities to PDT induced inhibition of DNA repair caused by radiation damage (Prinsze *et al.*, 1992).

6.0 Project Introduction

It has been previously reported that PDT resistant murine fibrosarcoma cell line RIF-8A (Singh *et al.,* 1991) showed increased sensitivity to ionizing radiation (Roy, 1996). It is also reported that radiation resistant L5178Y murine lymphoma cell line was more sensitive to chloroaluminium phtathocyanine-mediated PDT (Evans *et al.,* 1989). These results suggest that some radiation resistant tumour cells are sensitive to PDT and some PDT resistant tumour cells are more sensitive to ionizing radiation. It has been suggested that combination treatment of high dose rate (HDR) intraluminal brachytherapy and PDT in non-small cell lung cancer (NSCLC) may improve the efficacy of treatment, reduce the toxicity and improve quality of life for patients. Using a panel of 4 different non-small cell lung cancer (NSCLC) (A549, NCIH460, NCIH23 and SKMESI) cell lines and a normal fibroblast cell line (MRC5), we have examined their sensitivity to UVC, ionizing radiation alone, radiation with Photofrin but no light, Photofrin-mediated PDT alone and combination of radiation and PDT using colony forming assay. The main component of this research work was to establish an *in vitro*

protocol, which could be followed for the clinical trial setting using the results of the colony survival assays. Photofrin uptake experiments were done to know the Photofrin uptake per cell for all the cell lines. By using conventional colony forming assay, we have examined the sensitivity to UVC and Cobalt-60 gamma rays of the four different NSCLC cell lines and the normal lung fibroblast strain MRC5. The conventional colony forming protocol was altered to more closely mimic the cellular physiology of tumour cells during irradiation. Using a high cell density seeding protocol, experiments were done to examine the *in vitro* sensitivity of NSCLC cell lines to Photofrin-mediated PDT and combination of Photofrin and gamma rays. The colony survival experiments following combined PDT and gamma rays were also carried out using a similar "high cell density" protocol in which we have examined the effects of varying the time between treatments as well as the effect of the order in which treatment is given. Findings from such studies would be relevant for treatment decisions in the clinic and establish the molecular basis of a combined modality treatment involving PDT and Brachytherapy.

Table-1 Various types of photosensitizers

Photosensitizer	Trade name	Wavelength for PDT	Time before illumination	Comments
Porfimer sodium	Photofrin	630 nm	24–48 h	Most frequently used photosensitizer, approved for clinical use in treatment of many cancers
Benzoporphyrin derivative monoacid ring A, BPD-MA,verteporfin	Visudyne	690–692 nm	1–2.5 h	Sufficient tumor concentration can be achieved after 30 min, rapid clearance, low skin photosensitivity, approved for treatment of age-related macular degeneration
m-THPC, temeporfin	Foscan	652 nm	72–96 h	Skin photosensitivity for only 1–2 weeks, extremely high phototoxicity requiring very low drug and light doses
5-ALA	Levulan	635 nm	4–12 h	Precursor of photosensitizer converted in mitochondria into protoporphyrin IX
Methyl ester ALA	Metvix	635 nm	4–12 h	ALA ester with improved skin penetration
Benzyl esther ALA	Benzvix	635 nm	4–12 h	ALA ester with improved skin penetration
Hexyl ester ALA	Hexvix	635 nm	4–12 h	ALA ester with improved skin penetration
Tin ethyl etiopurpurin, $SnET_2$	Purlytin	660–665 nm	24 h	Increased cutaneous photosensitivity for up to 1 month
Hypericin	Hypericin	595 nm	24 h	Shallow light penetration, used mainly in psoriasis and superficial skin cancers
Silicon-based phthalocyanines, Pc4, Pc10, Pc12, Pc18	CGP55847, Photosense	670 nm	3 h	Deeper tissue penetration, low dark toxicity, fast accumulation in tumors, high tumor selectivity
Chloro-aluminum sulfonated phthalocyanine, CASPc		670–675	3 h	Deeper tissue penetration, low dark toxicity, fast accumulation in tumors, high tumor selectivity
Lutetium texaphyrin, motexafin lutetium, lutex	Lutrin, Antrin	720–760	2–4 h	Increased stability, very deep tissue penetration, advanced clinical trials in cardiology (treatment of atheromatous plaques), virtually no skin photosensitivity
N-Aspartyl-chlorin e6,	Npe_6	660–665	4 h	Rapid accumulation in tumor, short skin photosensitivity

(Adapted from Nowis D., Makowski M., Stoklosa T., Legat M., Issat T., and Golab J. Direct tumor damage mechanisms of photodynamic therapy. *Acta Biochimica Polonica* 2005: Vol.52 No.2 339-352.)

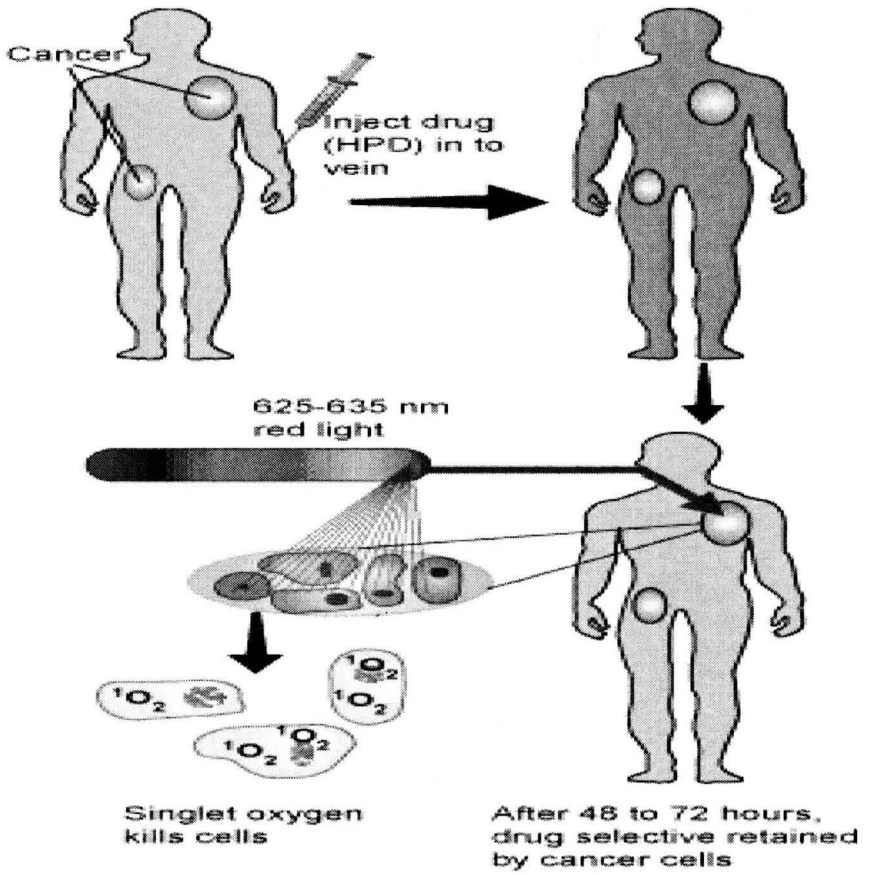


Figure 1.1. The process of photodynamic therapy.

A drug is given to the patient (1) which accumulates in the diseased tissue (2). when it is excited by light (3) it produces singlet oxygen which is highly toxic (4) and kills the cell.
(Adapted from Shackley and Moore, *J R Soc Med* (999); 92:562-565)

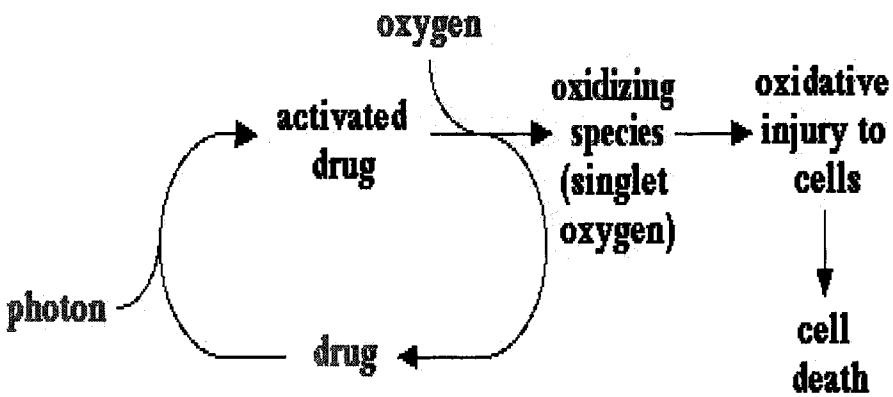


Figure-1.2 Photochemical Reaction (PDT)

A photon is absorbed by a photosensitive drug, which moves the drug into an excited state. The excited drug can then pass its energy to oxygen to create "singlet oxygen". Singlet oxygen attacks cellular structures by oxidation. When the accumulation of oxidative damage exceeds a threshold level, the cell begins to die.

(Adapted from Shackley and Moore, *J R Soc Med* (999); 92:562-565)

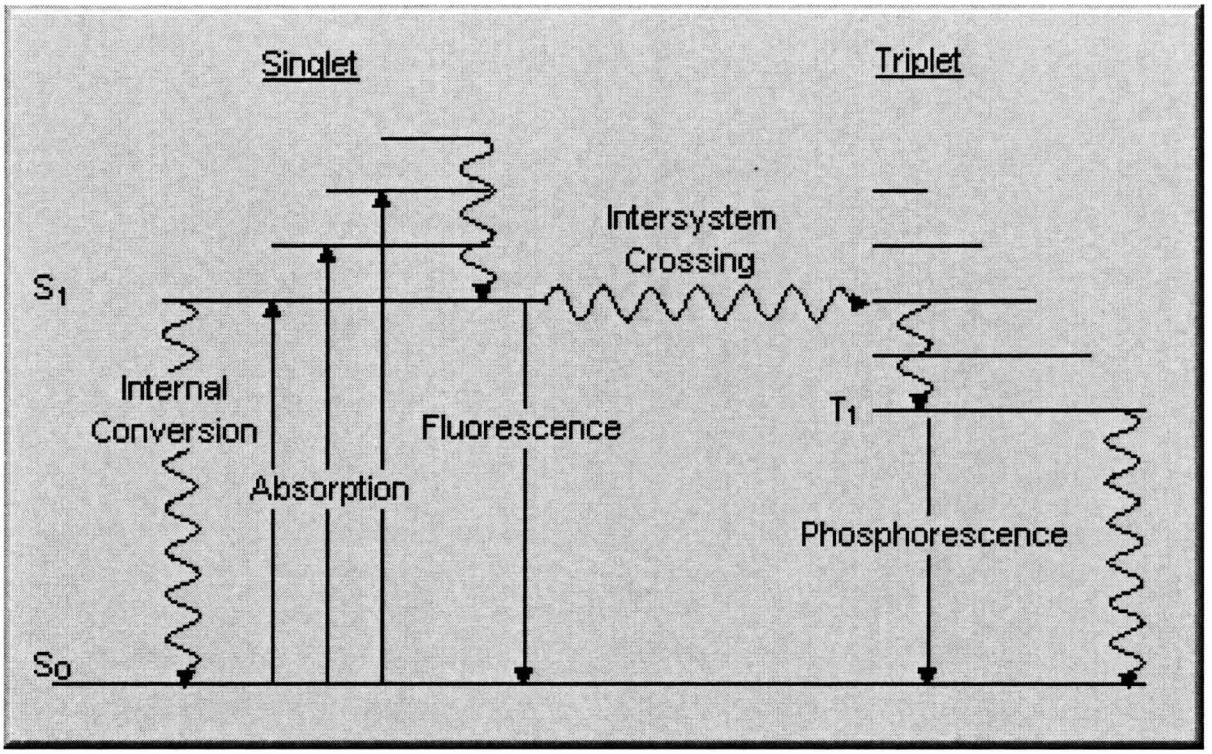


Figure 1.3. Photosensitiser excitation

Following absorption of a photon of light of specific wavelength, a molecule is promoted to an excited singlet state S_1. The photosensitiser returns to the ground state S_0 by emitting a photon (fluorescence). It is also possible that the molecule may convert to the triplet state via intersystem crossing. The triplet state photosensitiser has lower energy than the singlet state, but has a longer lifetime, and this increases the probability of energy transfer to other molecules.

(Adapted from Moan. 1998. The Biophysical Foundations of Photodynamic Therapy; *Endoscopy.* 30:387-391)

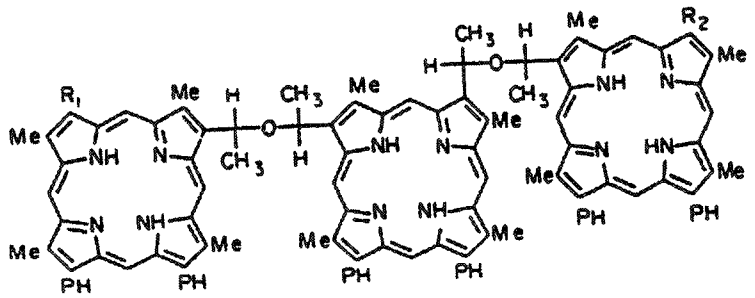


Figure 1.4 Structure of Photofrin photosensitizer

The major component of Photofrin is the porphyrin trimer. The R_1 group represents CH (OH) CH_3 and the R_2 group represents $CH=CH_2$. PH groups represent $(CH_2)2COOH$.

(Adapted from Dougherty and Marcus 1992).

$$H_2O \xrightarrow{h\nu} H_2O^{\bullet+} + e^-$$

$$H_2O^{\bullet+} + H_2O \xrightarrow[\text{rapid}]{} H_3O^+ + {}^{\bullet}OH$$

Figure 1.5 Generation of free electrons and the hydroxyl radical from the interaction of ionizing radiation and water

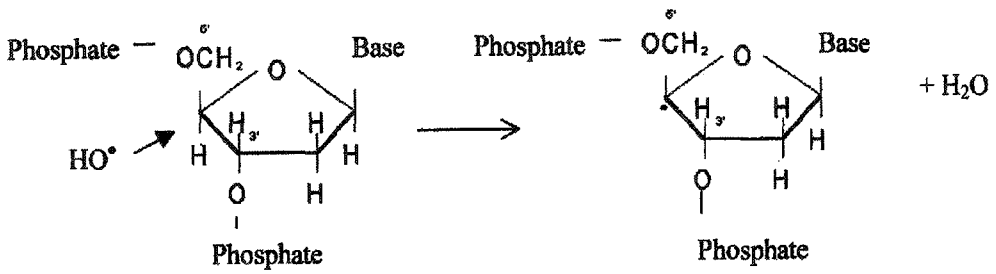


Figure 1.6 The formation of a carbon centered radical and water resulting from hydrogen abstraction by a hydroxyl radical.

CHAPTER-2

In vitro sensitivity of non-small cell lung cancer cell lines to UVC, high dose-rate gamma rays and Photofrin-mediated photodynamic therapy

1.0 Abstract

It has been suggested that combination treatment of high dose rate (HDR) intraluminal brachytherapy and PDT in non-small cell lung cancer (NSCLC) may improve the efficacy of treatment, reduce the toxicity and improve quality of life for patients. To provide a cellular basis for this approach we have examined the *in vitro* sensitivity of normal lung fibroblasts (MRC5) and four NSCLC cell lines (SKMES-1, A549, NCIH460 and NCIH23) following, UVC treatment, HDR radiation, PDT and combined HDR radiation and PDT. Cell sensitivity was measured using clonogenic survival. HDR radiation was cobalt-60 gamma rays (1.5-1.9 Gy/min). For PDT treatment, cells were exposed to 2.5 μg/ml Photofrin for 18-24 h followed by light exposure ($20mW/cm^2$). For combined treatment cells were exposed to Photofrin and then either exposed to light and 15-30 minutes later exposed to HDR radiation or exposed to HDR radiation and 15-30 minutes later exposed to light. Cellular Photofrin concentrations were measured by flow cytometry using 488nm excitation and 620-675 nm emission wavelengths. D_{37} values calculated from the survival curves indicated a 2-fold difference in sensitivity to UVC, 6-fold difference in HDR radiation sensitivity and an 8-fold difference in PDT sensitivity. All cell lines showed a similar Photofrin uptake per cell and 2.5 μg/ml Photofrin alone had no significant effect on colony survival. Photofrin alone at concentrations up to 10 μg/ml had no significant effect on the survival of the NSCLC cell lines, whereas 10 μg/ml of Photofrin alone reduced survival significantly in MRC5 cells. A radiosensitizing effect of Photofrin was detected in MRC5 and NCIH460 cells, but not in A549, SKMES-1 and NCI-H23 cells. Results shows that although light

followed by gamma rays resulted in a somewhat greater tumor cell kill compared to gamma rays followed by light, this difference was not significant for any of the cell lines tested. However, this difference was significant (as determined by a one sample one tailed t-test) when data for all NSCLC cell lines were pooled. The combined treatment with high dose rate HDR radiation and PDT was not significantly different from an additive effect of the individual treatment modalities for *in vitro* survival of 4 NSCLC cells. In contrast the combined treatment was less than additive for the MRC5 cells suggesting that the combined treatment would have the potential advantage of doing less damage to the normal lung cells and suggests that equivalent tumour cell kill *in vivo* may be possible at reduced systemic effects to patients.

2.0 Introduction

Cancer is characterized by uncontrolled growth and spread of abnormal cells in some part of the body. Lung cancer is the cancer that forms in tissues of the lung, usually in the cells lining air passages. The two main types are small cell lung cancer and non-small cell lung cancers, which are diagnosed, based on how the cells look under a microscope (as reviewed by Raz, 2006). It is estimated that 23,300 new cases of lung cancer will be diagnosed in Canada in 2007. Non-small cell lung cancer continues to be a major oncologic problem, with approximately 3-month increase in median survival per decade since the 1970s (Canadian cancer society statistics, 2007). Thus, newer strategies are needed to improve outcomes in non-small cell lung cancer

Lung cancer once diagnosed, is usually treated with a combination of surgery, chemotherapy and radiotherapy. Resistance of tumor cells to treatment often accounts for the failure of traditional forms of anti-cancer therapy. It is well known that tumours from the same histological group and stage of development are highly heterogeneous in their sensitivity to therapy (Freitag *et al.*, 2005). Among the factors that can influence tumor sensitivity are DNA repair capacity, distribution of cells throughout the cell cycle and proliferation potential (Oleinick and Evans, 1998). High Dose Rate Intraluminal Brachytherapy (HDRILBT) and Photodynamic Therapy (PDT) are two treatment options that are also used for palliation of symptoms in many institutions. Endobronchial tumors are well suited to treatment with either PDT or high dose rate brachytherapy and good

palliative results have been reported with non-small cell lung cancer (NSCLC) (Graham *et al.*, 2000, McCaughan *et al.*, 1996).

Photodynamic therapy (PDT) is a novel treatment that involves the use of a photosensitizer, such as Photofrin, the application of visible light of the wavelength specific for the photosensitizer, and the presence of oxygen leading to reactive oxygen species-mediated cytotoxicity to the treated cell (Engbrecht *et al.,* 1999). Photodynamic therapy kills tumour cells via apoptosis and/or necrosis both *in vivo* and *in vitro.* The particular mode of cell death in response to PDT depends on experimental conditions, such as the dose of PDT and the sub cellular localization of photosensitizer (Oleinick and Evans, 1998). Adams *et al.* showed *in vivo* resistance to Photofrin-mediated photodynamic therapy in radiation-induced fibrosarcoma cells RIF-8A resistant to *in vitro* Photofrin-mediated photodynamic therapy (Adams *et al.*, 1999). Photofrin, the photosensitizer used in this study, is a partially purified derivative of hematoporphyrin that is activated by light at 630 nm. Photofrin is the only approved photosensitizer by the U.S. Food and Drug Administration (FDA). Clinical recommended dose for Photofrin is 2 mg/kg given 24-48 hours before planned treatment. The retention of Photofrin in some solid tumors could be caused by poor lymph drainage and fragile vessels within the tumor.

High Dose Rate Intraluminal Brachytherapy (HDRILBT) is a form of radiation treatment given by placing the radioactive isotope in and around a tumor. The ionizing radiation leads to rapid necrosis of tumour tissues principally by nuclear DNA damage (Bellnier and Dougherty, 1986, Hall and Giaccia 2006). It is suggested that the epidermal

growth factor receptor (EGFR) is an important determinant of radiation response, whose elevated expression and activity frequently correlates with radio resistance in several cancers, including non-small cell lung carcinoma (NSCLC) (Das *et al.,* 2006). Das *et al.* reported recently that NSCLC cell lines harbouring somatic, activating mutations in the tyrosine kinase domain (TKD) of the EGFR exhibit significant delays in the repair of DNA double-strand breaks (DSB) and poor clonogenic survival in response to radiation (Das *et al* .,2007).

One difficulty encountered by studying PDT is that the sensitivity of cells to PDT varies between different cell types and even between cell lines that are very closely related (Tong *et al.*2000). It has been previously reported that PDT resistant murine fibrosarcoma cell line RIF-8A (Singh et al., 1991) showed increased sensitivity to ionizing radiation compared to PDT sensitive RIF-1 cells (Roy, 1996) It has also been reported that the radiation resistant L5178Y murine lymphoma cell line was more sensitive to chloroaluminium phtathocyanine-mediated PDT (Evans et al., 1989). compared to the radiosensitive LY-S cell line derived from it. These results indicate that some radiation resistant tumour cells are sensitive to PDT and some PDT resistant tumour cells are more sensitive to ionizing radiation. This suggests that a combined treatment of tumours with both Photofrin–mediated PDT and ionizing radiation could be superior to the use of the single modalities of PDT and ionizing radiation alone.

In the present study we have examined the sensitivity of 4 different non-small cell lung cancer (NSCLC) cell lines A549, NCIH460, NCIH23 and SKMES1 and a normal lung fibroblast cell line (MRC5) to UVC, ionizing radiation alone, ionizing radiation with

Photofrin but no light, Photofrin alone, Photofrin-mediated PDT alone and combination of ionizing radiation and PDT using a colony forming assay. We report that all the cell lines showed a similar Photofrin uptake per cell and observed that as the Photofrin concentration increases, its uptake by the cancer cell also increases. D_{37} values calculated from the survival curves indicated a 2-fold difference in sensitivity to UVC, 6-fold difference in radiation sensitivity and an 8-fold difference in PDT sensitivity of different non-small cell lung cancer (NSCLC) cell lines and a normal lung fibroblast. Photofrin alone at concentrations up to 10 μg/ml had no significant effect on the survival of the NSCLC cell lines, whereas 10 μg/ml of Photofrin alone reduced survival significantly in MRC5 cells. A radiosensitizing effect of Photofrin was detected in MRC5 and NCIH460 cells, but not in A549, SKMES-1 and NCI-H23 cells.

Results show that, although light followed by gamma rays resulted in a somewhat greater tumor cell kill compared to gamma rays followed by light this difference was not significant for any of the cell lines tested. However, this difference was significant (as determined by a one sample one tailed t-test) when data for all NSCLC cell lines were pooled. The combined treatment with high dose rate HDR radiation and PDT was not significantly different from an additive effect of the individual treatment modalities for *in vitro* survival of 4 NSCLC cells. In contrast the combined treatment was less than additive for the MRC5 cells suggesting that the combined treatment would have the potential advantage of doing less damage to the normal lung cells and suggests that equivalent tumour cell kill *in vivo* may be possible at reduced systemic effects to patients.

Findings from such studies would be relevant for treatment decisions in the clinic and establish the molecular basis of a combined modality treatment involving PDT and Brachytherapy.

3.0 Materials

3.1 Cell lines

The NSCLC cell lines employed in the colony survival assays include A549, SKMES-1, NCIH460, NCIH23 and normal human fibroblast strain MRC5. These cell lines were obtained from ATCC (Rockville, Maryland, U.S.A). **A549** is human adenocarcinoma and was initiated from a human alveolar cell carcinoma. This cell line has a wild-type p53 gene (Magrini *et al.*, 2007). **NCIH460** is human large cell carcinoma cell line and has a wild-type p53 gene (Andriani *et al.*, 2006). **NCIH23** is a human adeno carcinoma cell line and has a mutated p53 gene (Pellizzaro *et al.*, 2001). **SKMES1** is a human lung squamous cell carcinoma line and has a mutated p53 gene (Magrini *et al.*, 2007). **MRC5** is normal lung fibroblast tissue derived from 14-week old male foetus and has a wild-type p53 gene (Arima *et al.*, 2005).

All cell cultures were grown as monolayer in GIBCO RPMI medium 1640 modified with L-glutamine, ribonucleosides and deoxyribonucleosides supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic-antimycotic (100 μg/mL penicillin G sodium, 100 μg/mL streptomycin sulphate and 250 ng/mL amphotericin B in 0.85% saline) obtained from Gibco-BRL. Cultures were maintained in 37°C-humidified air containing 5% CO_2 at 90% humidity. For PDT, Photofrin + gamma, and combination

experiments RPMI medium 1640 was supplemented with 2% antibiotic-antimycotic to avoid contamination since experiments were performed under minimal light conditions.

3.2 Photosensitizer

Photofrin® was obtained from Axcan Pharma Inc, Quebec, Canada. It was reconstituted in 5% dextrose to a concentration of 2.5 mg/ml. 1mL and 0.5 mL aliquots of Photofrin were then prepared in cryovials and stored covered in aluminium foil in a freezer at -20°C in the dark. Storage, dilution steps, and incubation period were performed under experimental conditions avoiding the exposure of Photofrin to light.

3.3 Irradiation sources

The light source was a LED (Light Emitting Diode) that emits red light in the visible region of the spectrum at a wavelength range of 620-640 nm. The power output of the source was 20mJ/cm^2/sec.

The UVC source was from a G8T5 (General Electric) germicidal bulb predominantly producing light at 254 nm at a fluence rate of 1 J/m^2/s.

The Cobalt-60 gamma ray source in use in colony forming assays is a radiation unit at JCC with a half-life of 5 yrs. It emits gamma rays at a dose rate of 140-190 cGy/min.

4.0 Methods

4.1 Colony Forming Assay: Treatment with UVC or Gamma rays

Cells were seeded at low density and irradiated with either UVC or gamma rays. Confluent 75 cm^2 flasks of cells were trypsinised, cells were counted using a haemocytometer and the desired number (300 – 1000 cells per well) was seeded in a 6-well plate in 2 mL of supplemented RPMI medium 1640. Following a 24-hour incubation period, the media was aspirated from each well and replaced with 1 mL of warmed PBS. Plates were then exposed to different fluences of UVC (for UVC experiments) or different doses of gamma rays (for gamma radiation experiments). Cells were irradiated for varying times with UVC (254 nm) from a G8T5 (General Electric) germicidal bulb predominantly producing light at 254 nm at a fluence rate of 1 J m^{-2} $s^{-1.}$ Cells were irradiated for varying times of 5, 10, and 15sec (for UVC experiments) and doses of 2, 4, 6 and 8 Gy were given for the gamma radiation experiments. The controls in these experiments were cells without any radiation exposure. After exposure of cells (UVC or gamma rays), PBS was replaced by 2 mL of supplemented RPMI medium 1640 and plates were placed in humidified air at 37°C and 5% CO_2 for an incubation period of 10-12 days.

Afterwards, the plates were removed from incubation and the media was aspirated from each well. Approximately 1 mL of crystal violet solution (63% absolute ethanol, 27% H_2O, 10% methanol, 5 g/L crystal violet) was added to each well to stain colonies over a period of 30 minutes. Plates were then submerged in water to remove any excess crystal violet solution. Colonies were subsequently counted.

In additional UVC survival experiments the protocol was altered to more closely mimic the cellular physiology of tumor cells during irradiation. In these experiments, 10^5 cells were seeded into 12 or 24 well plates. After 18-24hr of incubation, media was replaced with 1 mL of PBS and cell monolayers were treated with UVC. After irradiation of cells with UVC, PBS was replaced again with 2 mL of fresh medium and 3 hr after irradiation the high-density cell monolayers were trypsinised, diluted in growth medium and an appropriate number of cells were seeded into 6 well plates at low cell density. Colonies were stained and counted after incubation of 8-10 days.

4.2 Flowcytometry: Photofrin Uptake

Before starting the colony survival experiments with PDT, it was necessary to know the uptake of Photofrin by the different cell lines. As a result, uptake experiments was designed to see how much Photofrin was absorbed by a cell for each cell line. Cells were counted on a haemocytometer and seeded for confluence (1×10^6 cells per well) in a 6-well plate in 2 mL of supplemented RPMI and allowed to incubate in humidified air for 6 hours at 37°C and 5% CO_2. Subsequent to the 6-hour incubation period, the media was aspirated from each well and replaced with 3 mL of supplemented RPMI containing the desired concentration of Photofrin (the incubation period allowed the cells to adhere to the surface of the plate). Photofrin concentrations were prepared with a set volume of supplemented RPMI medium 1640 and variable volume of Photofrin to obtain the desired concentration. Plates were incubated again, this time for an overnight period (20 - 24 hours) and kept under aluminum foil to minimize the effect of ambient lighting. Following the 20-24 hour incubation period, the media was aspirated from each well and

the cells were washed with 1 mL of warmed PBS. The PBS was also aspirated from the wells and replaced with 1ml of 2×trypsin-EDTA (0.5% trypsin, 5.3 mM EDTA • 4Na). Cells were trypsinised, 5 mL of supplemented RPMI medium 1640 was added and the solution was mixed several times with a micropipette. The contents of the well were removed to a 15-mL Falcon tube and centrifuged. The pellet obtained was resuspended in one mL of PBS. The Photofrin concentration of the cell was measured by flow cytometry using an excitation wavelength of 488nm and emission measurements at 620-675nm. Fluorescence per cell was plotted against Photofrin concentration.

4.3 Colony Forming Assays: Photofrin-Light (PDT) / Photofrin-Gamma rays

4.3 i) Photofrin and light (PDT)

a) Low cell density seeding protocol:

Confluent 75 cm^2 flasks of cells were trypsinised, cells were counted using a haemocytometer and the desired number (300–1000 cells per well) was seeded in a 6-well plate in 2 mL of supplemented RPMI medium 1640. Cells were left to adhere for a minimum of 6 hours before treating with Photofrin. After the 6 hr adhering period, the media was aspirated from 6-well plate, 2ml of the appropriate Photofrin dilution (2.5 μg/ml) was overlayed on each well, and plates were incubated again for an overnight period (18 - 24 hours) and kept under aluminum foil to minimize the effect of ambient lighting. After 18-24 hrs of incubation, medium containing Photofrin was aspirated and 2 mL of fresh RPMI 1640 medium without Photofrin was added to each well. Photofrin treated plates were exposed to visible light for different times using the LCD light source.

Following illumination of cells with visible light, plates were placed in an incubator containing humidified air at 37°C and 5% CO_2. Controls used in this protocol were no drug no light (NDNL), drug no light (DNL) and no drug light (NDL). Following an incubation period of 8-10 days, the plates were removed from incubation and the media was aspirated from each well. Approximately 1 mL of crystal violet solution (63% absolute ethanol, 27% H_2O, 10% methanol, 5 g/L crystal violet) was added to each well to stain colonies over a period of 30 minutes. Plates were then submerged in water to remove any excess crystal violet solution. Colonies were subsequently counted.

b) High cell density seeding protocol

The sensitivity of cells to PDT was examined using Photofrin concentration of 2.5μg/ml with varying light exposure in order to more closely simulate the clinical conditions of PDT. Conventional colony forming protocol was altered in order to closely mimic the tumour physiology for which confluent 75 cm^2 flasks of cells were trypsinised, counted with a haemocytometer, and $1x10^5$ cells/well (high cell density) were plated in a 24-well tissue culture plate. Cells were left to adhere for a minimum of 6 hours before treating with Photofrin. After the 6 hr adhering period, the media was aspirated from 6-well plate, 2ml of the appropriate Photofrin dilution (2.5 μg/ml) was overlayed on each well, and plates were incubated again for an overnight period (18-24 hours) and kept under aluminium foil to minimize ambient lighting. After 18-24 hrs of incubation, Photofrin-containing medium was aspirated and 2 mL of fresh RPMI 1640 medium without Photofrin was added to each well. Photofrin treated plates were exposed to various doses of visible light using a LCD light source. 3 hrs after irradiation plates were

trypsinised diluted and an appropriate number of cells were seeded in 6 well plates and incubated. After 6-10 days of incubation media was aspirated and colonies were stained with crystal violet (0.5% in 70% ethanol and 10% methanol). Colonies containing at least 32 cells were counted as surviving colonies.

4.3 ii) Photofrin and gamma rays

In second set of colony survival experiments, confluent 75 cm^2 flasks of cells were trypsinised, counted using a haemocytometer and $1x10^5$ cells/well (high cell density) were plated in a 24-well tissue culture plate. Cells were left to adhere for a minimum of 6 hours before treating with Photofrin. After the 6 hr adhering period, the media was aspirated from 6-well plate, 2ml of appropriate dilutions of Photofrin (made in complete growth medium) were then overlayed on each plate. After adding photofrin at different concentrations (2.5, 5, 10 μg/ml) the plates were incubated again for an overnight period (18 - 24 hours) and kept under aluminium foil to minimise ambient lighting. After 18-24 hrs of incubation, photofrin-containing medium was aspirated and 2 mL of fresh RPMI 1640 medium without Photofrin was added to each well. Photofrin treated plates were exposed to 7 Gy (single dose of gamma rays) with a Cobalt-60 source. Three hours after irradiation, plates were trypsinised, diluted and an appropriate number of cells was seeded into 6 well plates and incubated. After 6-10 days of incubation media was aspirated and colonies were stained with crystal violet (0.5% in 70% ethanol and 10% methanol). Colonies containing at least 32 cells were counted as surviving colonies.

4.4 Colony Forming Assay : Combined Treatment with Photofrin mediated PDT and ionising radiation

Combined treatment consisted of 2.5 μg/ml Photofrin (all NSCLC cell lines), light exposure 400 sec and 7 Gy gamma rays (SKMES1, A549 and NCIH460), light exposure 100 sec and 2 Gy gamma-rays (NCIH23) or light exposure 100sec and 7 Gy gamma rays (MRC5). Confluent 75 cm^2 flasks of cells were trypsinised, counted by using a haemocytometer and $1x10^6$ cells/well (high cell density) were plated in a 12-well tissue culture plate. Cells were left to adhere for a minimum of 6 hours before treating with Photofrin. After the 6 hr adhering period, the media was aspirated from 6-well plate, 2ml of appropriate dilutions of Photofrin (made in complete growth medium) were then overlayed on each plate. Plates were incubated again for an overnight period (18 - 24 hours) and kept under aluminium foil to minimise ambient lighting. After 18-24 hrs of incubation, photofrin containing medium was aspirated and 2 mL of fresh RPMI 1640 medium without Photofrin was added to each well. In order to determine if the order of combined treatment influences tumour cell kill, we used the following experimental protocol. After adding fresh RPMI 1640 medium without Photofrin, cells were either (a) first exposed to light and within 15-30 minutes exposed to gamma rays or (b) exposed to gamma rays and within 15-30 minutes exposed to light. Cells were then incubated for 3 hr covered in aluminium foil in the dark at 37°C-humidified air containing 5% CO2 at 90% humidity. After the 3 hr incubation, the cells were trypsinised, diluted and an appropriate number of cells was seeded in 6 well plates. After 6-10 days of incubation

media was aspirated and colonies were stained with crystal violet (0.5% in 70% ethanol and 10% methanol). Colonies containing at least 32 cells were counted as surviving colonies.

Experimental sets used in the combination experiments included:

1 NDNL (No Drug No Light)

2 DNL (Drug alone)

3 NDL (Light alone)

4 PDT

5 Gamma Ray

6 Photofrin + light + Gamma rays

7 Photofrin + Gamma rays + Light

5.0 Results and Discussion

5.1 Sensitivity of non-small cell lung cancer cell lines to UVC

Colony survival was examined in selected NSCLC cell lines following exposure of cells to UVC. [Table-3.1 in appendix-1, shows raw data obtained in a typical experiment for a single cell line using a low cell density protocol]. Figure-2.1 shows pooled data obtained from colony survival assays using UVC. We examined the sensitivity to UVC of the four different NSCLC cell lines A549, NCIH23, NCIH460 and SKMES-1 and the normal lung fibroblast strain MRC5. Table 2.1 shows D_{37} values (the dose that result in 37% surviving colonies) calculated from the survival curves, which indicate a 2-fold difference in sensitivity to UVC for the 4 NSCLC cell lines. It is seen

from the table that A549 and NCIH460 are the most resistant cell lines and NCIH23 and SKMES1 are the most sensitive to treatment with the MRC5 cell line not significantly different compared to NCIH23, showing that the normal lung fibroblast line, MRC5, is also one of the most sensitive cells compared to the tumour cell lines. Plating efficiency (the percentage of untreated cells seeded that grow into macroscopic colonies) of the cell lines were calculated as seen in Table-2.2.

The DNA damaging effects of UVC irradiation are known to induce the formation of cyclobutane pyrimidine dimers (CPDs) and the (6-4) photoproduct (Friedberg *et al.*, 1995). These types of bulky DNA lesions are both repaired by nucleotide excision repair (NER) (Mitchell, 1988). Fisher *et al.* reported that human promyelocytic leukemia HL60 cells expressing WT-p53 were more sensitive to UVC irradiation when compared to HL60 cells exhibiting deleted or mutated p53 phenotypes, suggesting that wild-type p53 status confers increased cellular sensitivity to UVC (Fisher *et al.*, 1997). In contrast we report here that NCIH460 and A549 cells (expressing wild-type p53) were most resistant to UVC, whereas SKMES1 and NCIH23 (mutated-p53) cells were most sensitive to UVC. Taken together, this indicates that alterations in tumour cells, other than changes in p53, can affect the sensitivity of cells to UVC. Thus no correlation was observed between mutant p53 protein expression levels and UVC sensitivity, it is possible that the increased UVC sensitivity in the NCIH23 and SKMES1 cell lines may be mediated through means other than through the alteration in these protein expression levels, perhaps through alterations in the expression of proteins involved in the removal of CPDs, 6-4PPs and other bulky DNA adducts, which are repaired by the NER pathway.

Other factors that might influence the sensitivity of cells to UVC are the expression levels of BNip3, Hsp27 and mutant p53. It has been reported that increased expression of Hsp27 and BNip3 in a panel of HT29 human colon carcinoma cell variants correlated with increased sensitivity to UVC, whereas increased expression of mutant p53 showed no significant correlation with sensitivity to UVC (Zacal and Rainbow, 2007). BNip3 functions in the mitochondria and its over-expression has been shown to induce apoptosis in various cell types (Ray *et al.*, 2000). The study by Zacal and Rainbow showed that overexpression of BNip3 alone does not result in increased sensitivity of HT29 human colon adenocarcinoma cells to UVC, and suggests that increased BNip3 expression together with the altered expression of other proteins may be responsible for the increased sensitivity of HT29 cells to UVC (Zacal and Rainbow, 2007).

In additional experiments the protocol to determine UVC colony survival was altered to more closely mimic the cellular physiology of tumor cells during irradiation. Figure-2.2 shows the comparison of UVC survival for conventional and "high cell density" protocol for the SKMES-1 cell line. It is seen from the results that the SKMES-1 cell line showed an increased survival when determined using the high cell density protocol compared to using the conventional protocol where cells are seeded at low cell density. The increased survival of the SKMES-1 cell line might be because cells when seeded at high cell density are nearer to each other and repair their damage better during 3 hr of incubation after irradiation compared to cells seeded at low density. This 3 hrs of liquid holding was not followed in the conventional protocol where cells were seeded at low cell density. These results are consistent with previous reports showing that confluent

cultures of normal human diploid fibroblasts (NHDF) exposed to UV light exhibited a time-dependent increase in survival when subculture was delayed up to 24 h after irradiation. (Grosovky and Little, 1985). Castro and coworkers examined the relationship among cell density, cell phenotype and cell survival using the human A549, H596 and H520 non-small cell lung carcinoma lines. Cells from monolayers, aggregated and suspended cultures at different densities were exposed to UV-radiation and both the density and the phenotype of the cells induce shifts in cellular growth rate whereas in suspended cultures, they observed a UV-sensitivity closely related to the proliferative status of the cells (Castro *et al.,* 1999). In contrast to A549 and NCI-H596, irradiated NCI-H520 cells presented lower DNA fragmentation and an aggregated cell culture phenotype even prior to confluence, suggesting that a contact-effect mechanism provides further protection against UV radiation (Castro *et al.,* 2001). Thus there might be differences among the cell lines which are related to the extent of intercellular contact among cells.

5.2 Sensitivity of non-small cell lung cancer cell lines to gamma rays

Colony survival was examined in selected NSCLC cell lines following gamma rays. Cells were seeded in a 6-well plate and exposed to different doses of gamma rays. [Table-3.2 in appendix-1 shows result of a typical experiment for a single cell line using a low cell density protocol]. We examined the sensitivity to gamma rays of the four different NSCLC cell lines A549, NCIH23, NCIH460 and SKMES-1 and the normal lung fibroblast strain MRC5. Figure-2.3 shows pooled data obtained from colony survival assays using Cobalt-60 gamma rays. D_{37} values for colony survival following gamma ray

treatment were calculated from the survival curves and are shown in Table-2.1. D_{37} values indicated a 6-fold difference in sensitivity to gamma rays with A549 being the most resistant cell line and NCIH23 being the most sensitive to ionizing radiation and all other cell lines NCIH460, SKMES-1 and MRC5 showed intermediate sensitivities. D_{37} values were significantly greater in the NSCLC cell lines A549, NCIH460 and SKMES1 compared to normal MRC5 lung fibroblasts, whereas NCIH23 cells showed a significantly reduced D_{37} value compared to MRC5 cells. These results are similar to the results showed by Carmichael *et al.,* showing that at a X-ray dose of 2 Gy, A549 is the most resistant cell line and NCIH23 the most sensitive with NCIH460 having intermediate sensitivity (Carmichael *et al.,* 1985).

In lung cancer cell lines, the predictive role of p53 in radiosensitivity has been evaluated in a number of studies with contradictory results (as reviewed by Viktorsson *et al.*2005). There are many reports of investigations of the relationship between p53 mutations and radiosensitivity *in vitro* and in animal studies for example the relationship between p53 gene status and response to radiation in NSCLC has been studied *in vivo* by Matsuzoe *et al.* The results of this study support the *in vitro* observations that cells have increased resistance to ionizing radiation in the absence of p53 function (Matsuzoe *et al.* 1999). Kawabe *et al.* compared the effects of adenovirus-mediated expression of wild type p53 plus radiation on NSCLC cell lines A549 and NCI-H322 with mutant and wild-type p53 conformation and on normal human lung fibroblasts MRC-9 and CCD-16. Adenovirus-mediated expression of wild type p53 enhanced the radiosensitivity of NSCLC cells irrespective of their p53 status. However, p53 overexpression had no

sensitizing effect on the normal human lung fibroblasts, suggesting that this gene therapy strategy provides a therapeutic advantage for the treatment of lung cancer (Kawabe *et al.*, 2001). By implanting NSCLC cells with a p53 mutation in the core domain or NSCLC cells expressing wild-type p53 in nude mice, it was observed that tumours formed by NSCLC cells with a mutated p53 responded less well to radiation as well as to the chemotherapy agent cisplatin compared to tumors formed by cells expressing wild-type p53. Furthermore, reduced apoptosis was observed upon radiation or cisplatin treatment within tumors formed by p53-mutated NSCLC cells (Perdomo *et al.*, 1998). These results are in contrast to the results of the present study in which an NSCLC cell line having wild type p53 (A549) was most resistant to the treatment with gamma rays and an NSCLC cell line having a mutant p53 gene (NCIH23) was most sensitive suggesting that the mechanism of cell killing in these cell lines is independent of p53 status. It has also been reported that NSCLC cell lines with non-functional p53 (H661 and H520) have a higher fraction of radiation-induced apoptosis than cell lines with functional p53 (H460, A549) and that apoptosis follows after release from G2/M arrest (Stuschke *et al.*, 2002). When p53 responsive pathways were blocked in NSCLC cells, radiation-induced G2 arrest was decreased and radiation-induced apoptosis was increased in these cells (Sak *et al.* 2003). This shows the importance of p53 in the regulation of these events. The influence of p53 mutations on radiosensitivity as measured by clonogenic survival assays has also been reported to be p53-exon specific (Bergqvist *et al.*, 2003). Bergqvist and co-workers reported that in NSCLC cell lines, mutations within exon 7 correlated with increased radiosensitivity compared to mutations located in other exons, i.e., 8, 5, 4 or 10. It is

therefore possible that the reported discrepancy of p53 status on radiosensitivity in NSCLC cell lines might be due to different exon localization of the p53 mutation. It is likely that some of the contradictory results obtained in lung cancer cell lines on the role of p53 status as a predictor of radiation sensitivity reflect differences in the assessment of death. More studies are therefore warranted. Results from some experimental studies suggest that oxygen deprivation results in an increase in the radiation sensitivity of p53 mutants, but not cells expressing wild-type p53. Independent of p53 status, energy depletion could cause a reduced ability to repair radiation damage. The general breakdown of the cellular energy metabolism during chronic hypoxia could bring along a delay in DNA replication and repair, making cells more sensitive to radiation damage (as reviewed by Wouters *et al.,* 2007). This seems to be true for NCIH23 (mutated-p53) cell line that showed highest sensitivity to gamma radiation. It is possible that the sensitivity of NCIH23 cells results from a reduced repair of radiation-induced DNA damage due to a depletion of oxygen levels in the medium of NCIH23 cells.

Recent reports have shown that the epidermal growth factor receptor (EGFR) is an important determinant of radio response, whose elevated expression and activity frequently correlates with radio resistance in several cancers, including non-small cell lung cells (Das *et al.,* 2006, Das *et al.,* 2007). NSCLC cell lines harbouring somatic, activating mutations in the tyrosine kinase domain (TKD) of the EGFR exhibit significant delays in the repair of DNA double-strand breaks (DSB) and poor clonogenic survival in response to radiation (Das *et al.,* 2007). Das *et al.* also examined the effect of ionizing radiation (IR) on clonogenic survival in two wild type WT EGFR NSCLCs, H1299 and

A549, and in two mutant EGFR NSCLCs, HCC827 and H820 (Das *et al.,* 2007). The WT EGFR cell lines A549 and H1299 showed a significant tolerance to radiation and modest loss of colony-forming ability at 8 Gy. In contrast, the mutant EGFR-expressing NSCLC cell line H820 exhibited high radio sensitivity compared with unirradiated controls. In comparison, HCC827, which also harbors the mutant EGFR, was also moderately sensitive to radiation. Consistent with the results of Das *et al.* we show in the present work that WT EGFR cell lines A549, NCIH460 are more resistant compared to NCIH23 and SKMES1, the other two NSCLC cell lines tested.

5.3 Uptake of Photofrin by non-small cell lung cancer cell lines

Because drug uptake is a potential factor influencing cellular sensitivity to PDT, we examined the uptake of Photofrin in non–small cell lung cancer cell lines and the normal lung fibroblast strain MRC-5. Photofrin uptake experiments were designed to see how much Photofrin was absorbed per cell by each cell line. The cells were allotted 20 - 24 hours to absorb Photofrin, then they were trypsinised and the resulting Photofrin concentrations of cells were measured by flow cytometry using an excitation wavelength of 488nm and emission measurements at 620-675nm. Fluorescence per cell was plotted against Photofrin concentration. Results are shown in Figure-2.4. It can be seen that all the cell lines showed a similar Photofrin uptake per cell over the range of Photofrin concentrations employed.

In vitro cellular uptake and retention of Photofrin has been found to be a passive process not involving energy expenditure. pH and temperature of the incubation media have been found to profoundly influence these processes, while a complex relationship

exists between physiological state of the cell and accumulation of the photosensitizer (Khanum and Jain, 1997). Consistent with the results of the present study, Perry et al. also showed no significant difference in Photofrin uptake between A549 and NCI-H460 (Perry *et al.,* 1990). Although some studies have reported that malignant cells take up more drug in comparison to normal cell lines (as reviewed by Oleinick *et al.,* 2001), other studies have reported that normal human fibroblast cells show greater uptake compared to immortalized Li-Fraumeni syndrome (LFS) cells (Tong *et al.,* 1999). In contrast, the results of the current work show no difference in Photofrin uptake per cell between the MRC5 normal lung fibroblasts and the 4 NSCLC cell lines.

5.4 Sensitivity of non-small cell lung cancer cell lines to Photofrin alone

The effect of different doses of photofrin alone (without light) were examined in order to determine if any of the NSCLC cell lines were sensitive to the drug alone. [Table–3.3 in appendix-1 shows the result of a typical colony forming experiment used to determine cell sensitivity to Photofrin alone using a high cell density protocol]. Figure-2.5 shows the influence of different Photofrin concentrations without light exposure on cell survival, for normal lung fibroblasts MRC5 and 4 NSCLC cell lines (A549, SKMES1, NCIH23, and NCIH460). It is seen that MRC5 cells showed increased sensitivity compared to the other NSCLC cell lines following treatment with 10 μg/ml of Photofrin alone, whereas 2.5 and 5 μg/ml of Photofrin alone had no significant effect on colony survival for MRC5 or any of the NSCLC cell lines. In addition it was seen that 10 μg/ml of Photofrin reduced survival significantly in MRC5 cells when compared to

control (no drug no light, NDNL) plates. For the given incubation time 18-24 hrs with increasing photofrin concentration from 2.5 to 5 μg/ml medium, there was no significant difference in the survival among the cell lines but at Photofrin concentration of 10μg/ml medium, MRC5 cell line showed decreased survival which is significantly less than NCIH23 and NCIH460 cell line (by two sample independent one tailed t-test) and cell lines A549 and SKMES1 shows p values ($p=0.06$) close to significance, suggesting that PDT with high drug concentrations might be toxic for normal cells than tumour cells. Fibroblasts do not necessarily represent the most critical normal cell type for lung damage following radiation and it would be of interest to also examine the response of normal lung epithelial cells. Perry *et al.* showed that there was no effect on survival of 6 NSCLCs (A549, NCIH23, NCIH841, NCIH460, JMN, NCIH520) and normal lung fibroblast CCL-210 when cells were incubated at 25μg/ml of photofrin alone for 2 hr (Perry *et al.,* 1990). Matthews *et al.* showed that there was no effect on survival of A549 on varying Photofrin concentrations (2.5, 25 or 50 μg/ml) for different periods of time (2, 4, or 6 hr). There were minimal changes in cellular fluorescence recorded at the lowest Photofrin level, and this was not influenced by prolonging the incubation time. At higher drug concentrations, there was a rapid accumulation of sensitizer in the cells over the first two hr, without a significant change in this level up to 6 hr (Matthews *et al.,* 1988) suggesting a saturation effect of photofrin in cells.

5.5 Sensitivity of non-small cell lung cancer cell lines to Photofrin-mediated PDT

After determining Photofrin uptake per cell for all the cell lines, the sensitivity of cells to PDT was examined using the high cell density protocol and a Photofrin concentration of 2.5 μg/ml with varying light exposure in order to more closely simulate the clinical conditions of PDT. [Table–3.4 in appendix-1 shows the result of a typical colony forming experiment using high cell density protocol for single NSCLC cell line]. Figure-2.6 shows the effect of PDT on the survival of 4 NSCLC cell lines (A549, NCIH23, NCIH460, SKMES-1) and normal lung fibroblast MRC5, at a Photofrin dose of 2.5 μg/ml and variable light exposure at a power output of 20mW/cm^2/sec. D_{37} values calculated from the survival curves indicated a 8-fold difference in sensitivity to PDT with A549 (WT-p53) being the most resistant cell lines and NCIH23 (mutated-p53) being the most sensitive to PDT treatment and all other cell lines (NCIH460 WT-p53), SKMES-1(mutated-p53) and MRC5 (WT-p53)) showed intermediate sensitivities (Table 2.3(A)). Our results demonstrate differences among human lung cancer lines and their sensitivity to PDT under controlled identical conditions. These results are consistent with the results of Perry et al. showing that NCIH23 was more sensitive to PDT than A549 (Perry *et al.,* 1990). In the current work, there were no significant differences in sensitizer uptake among cell lines yet there were significant differences among cell lines to PDT-mediated cell killing. Therefore, PDT sensitivity did not correlate well with sensitizer uptake. Although total cellular fluorescence per cell may be the same, differences in cellular organelle sensitizer uptake may account for survival differences. Intracellular localization may differ between cells or even sub strains of the same cells (Kessel and

Woodburn., 1995) suggesting that differences in sensitivity to PDT of the 4 NSCLC cell lines and MRC5 cell line might be because of differences in cellular organelle uptake by cell lines. Bohmer and Morstyn have shown that different cell lines under identical conditions took up different amounts of sensitizer. These differences were dependent upon the cell size, with larger cells taking up more sensitizer than small cells (Bohmer and Morstyn, 1985). The same Photofrin uptake per cell in all the cell lines in our experiments under identical experimental conditions indirectly shows that this might be because of the same size of cancer cell lines. It should be noted that sensitizer uptake measurements were made using the entire population of cells, while the PDT sensitivity assessments represent only the proportion of cells that actually form colonies. Ideally, the appropriate way to correlate sensitizer uptake with PDT sensitivity would be to use only cell lines with 100% plating efficiencies, a rare characteristic of most human lung cancer cell lines.

Tong *et al.* reported that immortalized Li-Fraumeni syndrome (LFS) cells having mutant p53 gene are more resistant to Photofrin-mediated PDT compared to normal human fibroblasts (NHF) having wild type p53, at equivalent cellular Photofrin levels (Tong *et al.,* 2001). These results are in contrast to the results of the present study in which NSCLC cell lines having wild type p53 were most resistant to the PDT treatment and NSCLC cell lines having a mutant p53 gene were most sensitive to treatment suggesting that mechanism of cell killing in these cell lines is independent of p53 status. Similarly a human colon carcinoma cell line (LS513) expressing wild-type p53 was more sensitive to PDT than another colon carcinoma cell line (LS1034) with mutated p53

(Fisher *et al.,* 1998). As discussed earlier, although these studies provided evidence that p53 can sensitize cells to PDT-induced apoptosis, introduction of the viral oncoprotein E6 to abrogate wild-type p53 function of the LS513 cells or of breast carcinoma cells (MCF-7) did not alter their PDT sensitivity to loss of clonogenicity or induction of apoptosis, leading to the conclusion that PDT sensitivity is not p53-dependent (Fisher *et al.,* 1999). Thus, results aiming to determine the role of p53 in sensitivity of cells to PDT are conflicting. However, the normal MRC5 (WT p53) cells were generally sensitive to PDT. It is possible that differences in the phase of the cell cycle affect PDT efficacy. Moan and colleagues showed that, for human NIHK 3025 cells, S phase cells were more sensitive than cells in G1 to Photofrin-mediated PDT (Moan *et al.,* 1979). This suggests the possibility that a different distribution of cells in the cell cycle influences PDT sensitivity. It has been reported that low-density lipoprotein receptors have specific affinity with Photofrin (as reviewed by Moore *et al.,* 1997). From our results it suggests that NSCLCs and normal lung fibroblasts have a high as well as same number of low-density lipoprotein receptors on them.

In another set of PDT experiments, the sensitivity of cells to PDT was examined using low cell density protocols and a photofrin concentration of 2.5μg/ml with varying light exposure. The aim of these experiments was to compare the survival curves obtained by using both high and low cell density protocols. Figure-2.7 shows the effect of PDT on the survival of the 4 NSCLC cell lines and the normal lung fibroblast, MRC5, when seeded at low cell density on 6-well plates. D_{37} values calculated from the survival curves indicated a 5-fold difference in sensitivity to PDT with A549 (WT-p53) being the

most resistant and MRC5 (WT-p53) being the most sensitive to PDT treatment, and all other cell lines (NCIH460 WT-p53), SKMES-1(mutated-p53) and NCIH23 (mutated-p53)) showing intermediate sensitivities (Table-2.3 (B)). Higher D_{37} values obtained in the low cell density experiments in comparison to high cell density PDT experiments showed that cells are more sensitive to killing when seeded at high cell density. This difference was significant for the NCIH23 and NCIH460 cell lines (Table-2.4). Cell lines were seeded for confluency in the high cell density plates and were more dispersed in the low cell density plate. It could be argued that since a higher surface area is available for photofrin absorption in the low cell density plate, cells would be more sensitive to killing under the conditions of low cell density seeding. However, this was not the case since cells seeded at low cell density were more resistant to killing by PDT than when seeded at high cell density. This suggests that mechanisms other than a differential Photofrin uptake are responsible for the increased resistance to PDT for low density compared to high density cell seeding.

Another difference in the two protocols is the use of trypsin after irradiation of cells in the high cell density protocol and this might have contributed to lower colony survival in high cell density protocol than in low cell density protocols. However, this seems unlikely since the controls for these experiments (no drug, no light, drug no light and no drug light) were also given the same trypsin treatment. A more likely explanation is that the 3 hr liquid holding period following incubation results in an enhanced bystander effect in the high cell density seeding protocol. Bystander effects have been demonstrated in several cell systems to result from a transferable factor(s) in exposed

cells causing radiobiological effects in unexposed cells to radiation (Seymour and Mothersill, 2000). The bystander effect is greater when cells die by necrosis, possibly because necrotic cells have a better chance of leaking toxic substances through their damaged membranes. For example, in a PDT-induced killing of WiDr human colon adinocarcinoma cells with the lipophilic photosensitizer 3THPP, the degree of bystander effect was greater when the normal cells died by necrosis compared to apoptosis. The bystander effect was also greater for normal cells than for the cancer cells; and confluent cultures with greater possibility for cell-to-cell interactions were more sensitive than sub-confluent ones (as reviewed by Oleinick et al., 2001).

5.6 Radio sensitization of non-small cell lung cancer cell lines by Photofrin

There are some reports suggesting that photosensitizers in combination with ionizing radiation treatment can act as radiosensitizers under aerobic conditions (Luksiene *et al.*1994; Berg *et al.*1995). To explore whether photofrin sensitizes NSCLC cell lines to gamma rays, another set of colony survival experiment was performed in which the exposure to gamma rays remained constant (7 Gy), but the Photofrin concentration varied (2, 5, 10 μg/ml). It was decided that control plates of the effect of different doses of Photofrin alone (without gamma rays) should also be observed for any toxicity it may have on cells (as discussed in section 5.4 before). [Table–3.5 in appendix-1 shows the result of a typical colony forming experiment using the high cell density protocol]. It was seen that drug concentrations up to 10 μg/ml of Photofrin alone had no significant effect on survival of NSCLC cell lines. Figure 2.8-2.12 shows that Photofrin concentrations up to 10 μg/ml had no significant effect on the survival of the NSCLC cell

lines following 7 Gy of gamma rays when compared to respective controls (no drug no light) by two sample independent two tailed t-test. In contrast, it was seen that 10 μg/ml of Photofrin reduced survival significantly in MRC5 cells for both Photofrin alone and Photofrin plus gamma rays. In addition, there is no significant difference in survival of A549, SKMES1 and NCIH23 cell lines when treated with Photofrin followed by gamma rays in comparison to the survival seen with Photofrin alone, whereas this difference is significant at 10μg/ml of Photofrin in the MRC5 cell line (by two sample independent one tailed t-test). At 2.5 μg/ml of Photofrin there is significant difference in survival of NCIH460 cell line when treated with Photofrin followed by gamma rays in comparison to the survival seen with Photofrin alone. This difference is significant in a two sample independent one tailed t-test but not in a two sample independent two tailed t-test. These results suggest a radosensitizing effect of Photofrin for MRC5 and NCIH460 cells. In contrast, the interaction of photofrin and gamma rays does not show a radiosensitizing effect for the NSCLC cell lines A549, SKMES1 and NCIH23. Roy, 1996 reported that a drug concentration of 20 μg/ml of Photofrin results in a significant increase in the sensitivity of RIF-1 murine fibro sarcoma cells to gamma rays (Roy, 1996). It is suggested from previous studies that the combined action of Photofrin and ionizing radiation is a saturable process, and no measurable enhancement of the radio sensitizing effect takes place upon increasing the radiation dose from 5 to 15 Gy and the Photofrin concentration above 7.5 mg/kg (Schaffer *et al.,* 2002). In addition, no residual radio sensitizing activity could be detected by using 2.5 mg/kg Photofrin. Schaffer *et al.* also showed that Photofrin at a concentration of 5 mg/kg proved to be a chemical modifier of

ionizing radiation, by delaying tumour growth and reducing the overall tumour volume by about 50% after six days (Schaffer *et al.,* 2001). *In vivo* experiments with and without Photofrin during irradiation showed an increase in tumour doubling time using the combination modality of radiation plus Photofrin (Kulka *et al.*, 2003). This suggests that the sensitizing effects of Photofrin to ionizing radiation may be different *in vivo* compared to *in vitro.* These differences may result from effects on surrounding tissue and vasculature in the *in vivo* situation.

5.7 Sensitivity of non-small cell lung cancer cell lines to combined Photofrin mediated PDT and ionizing radiation

The rationale of cancer treatment with a combination of different therapeutic modalities is to obtain improved tumour control with minimal damage to normal tissues. Such studies indicated that improvement of combined treatment protocols relies largely on the elucidation of the underlying mechanisms of cell killing by the separate as well as by the combined treatment modalities. Luminal disease causing obstruction is a common symptom of advanced lung cancer that has failed all other forms of curative therapy. High Dose Rate Intraluminal Brachytherapy (HDRILBT) and Photodynamic Therapy (PDT) of cancer are frequently used in order to improve tumor control with other treatments. It has been suggested that the cellular damage and the mechanisms of cell killing are different following PDT compared to ionizing radiation (Bellnier and Dougherty 1986) such that when used in combination, the effect could be, at minimum, additive or perhaps synergistic.

In our experiments for combined treatment, cells were exposed to Photofrin and then either exposed to light and 15-30 minutes later exposed to gamma radiation or exposed to gamma radiation and 15-30 minutes later exposed to light. The two major questions addressed in this section of the project were (a) Is there any effect of order of combined treatment on NSCLC cell lines and normal lung fibroblast MRC5? (b) Is combined treatment of HDR ionizing radiation and PDT more effective than individual treatment?

Table-3.6 in the appendix-1 shows the raw data obtained from a typical colony survival experiment for A549 cell line representive of NSCLCs. Cells were seeded in triplicates for all the experimental sets (please refer to section 4.4 in methods) included in the study in each experiment. Experiments were done in triplicate for each cell line. Survival of cells in all the experimental sets was calculated relative to the control set (no drug no light, NDNL). The fraction of cells surviving a given dose is determined by counting the number of macroscopic colonies as a fraction of the number of cells seeded. Allowance is made for the plating efficiency (PE) in each calculation. Cell survival was calculated as

$$\text{Surviving Fraction} = \frac{\text{Number of colonies counted}}{\text{Numbers of cells seeded X (PE/100)}}$$

Table-3.7 in the appendix-1 shows the spreadsheet of surviving fractions obtained in all the colony survival experiments for all the 5 cell lines. Surviving fractions are calculated for all the experimental sets in each experiment (please refer to section 4.4 in

methods). Survival of cells in all the experimental sets was calculated relative to the control set (no drug no light, NDNL).

Table-2.5 shows the effect of order of combined treatment on NSCLC cell lines. Mean surviving fractions ± SE (calculated from all experiments) of NSCLC cells and normal lung fibroblast MRC5 are reported when cells were exposed to Photofrin and then either exposed to light and 15-30 minutes later exposed to gamma radiation (a) or exposed to gamma radiation and 15-30 minutes later exposed to light (b). Ratio of (b)/(a) for all the experiments is also calculated as mean values ± SE. Results shows that although light followed by gamma rays resulted in a somewhat greater tumor cell kill compared to gamma rays followed by light this difference was not significant for any of the cell lines tested. However, this difference was significant (as determined by a one sample one tailed t-test) when data for all NSCLC cell lines were pooled.

Table-2.6 shows the effect of combined treatment in comparison to predicted survival. The survival of NSCLC cells following combined treatments (a) & (b) (as discussed above) was compared to predicted survival values based on survival following PDT and gamma-rays alone (c) (PDT X gamma-rays). Since P+L then gamma (a) was not significantly different from P+G then light (b) for each of the cell lines, we were able to pool the data from both the treatments to compare with the expected survival.

Survival of cells following the combined treatment was greater than that expected based on an additive effect for SKMES1, NCIH460, NCIH23 and MRC5. This less than additive effect of the combined treatment was significant in a two sample independent *t*-test for the MRC5 line, only significant in a 1 tailed t-test for the SKMES1 and NCIH23

cells and not significant in either test for the NCIH460 cells. In contrast, the survival of A549 following combined treatment was less than that predicted based on an additive effect, suggesting a synergistic effect of combined treatment in A549 cells. This synergistic effect in A549 cells was only significant in one sample one tailed *t*-test, but not by one sample two tailed *t*-test or a 2 sample independent *t*-test. Thus, the combined treatment with high dose rate HDR radiation and PDT was not significantly different from an additive effect of the individual treatment modalities for *in vitro* survival of the 4 NSCLC cells. Interestingly, A549 cells were the most resistant to PDT and gamma rays and the only NSCLC cells that we tested which showed a possibly synergistic effect following the combination treatment, based on one sample one tailed *t*-test. In contrast the combined treatment was less than additive for the MRC5 cells. This suggests that the combined treatment would have the potential advantage of doing less damage to the normal lung cells and suggests that equivalent tumour cell kill *in vivo* may be possible at reduced systemic effects to patients.

Bellnier and Dougherty presented data suggesting that PDT and ionizing radiation act by independent mechanisms, i.e., the PDT preceding gamma irradiation does not directly influence the radio sensitivity of PDT surviving cells (Bellnier and Dougherty 1986). Consistent with this hypothesis, Henderson and Fingar showed that when a single PDT treatment was immediately followed by treatment with graded doses of gamma irradiation in a RIF mouse tumor cell model, the resulting cell survival curves showed similar survival in comparison to cultures which were treated with Photofrin and graded doses of gamma irradiation alone, indicating that the damaging effects of gamma

irradiation were not further reduced by preceding PDT treatment (Henderson and Fingar, 1987). In contrast an *in vitro* study reported a synergistic interaction between PDT and ionizing radiation in mouse fibroblast cells (L929) and an additive effect in Chinese hamster ovary cells and human bladder transitional cancer cells (T24) (Prinsze *et al.*, 1992). These results, together with the results of the current work indicate that the outcome of combined PDT and ionizing radiation treatment can be additive, less than additive or synergistic depending on cell type and the conditions of the combined treatment.

In an *in vivo* clinical study, Freitag *et al.* showed that the combination of PDT and brachytherapy for treating patients with lung cancer and extensive endobronchial tumor is safe and had excellent therapeutic efficacy. Biopsy specimens were taken from the treated sites during bronchoscopy 5-6 weeks after PDT and high dose brachytherapy with iridium-192 was administered. It was found that the combined treatment had a complete histological response rate of 97 % (Freitag *et al.* 2005). In our study both PDT and high dose brachytherapy were given over a period of not more than 30 minutes because it would be more convenient for the patient if both treatments are done using the same endoscopy procedure, otherwise the patient would have to have 2 visits and 2 endoscopies.

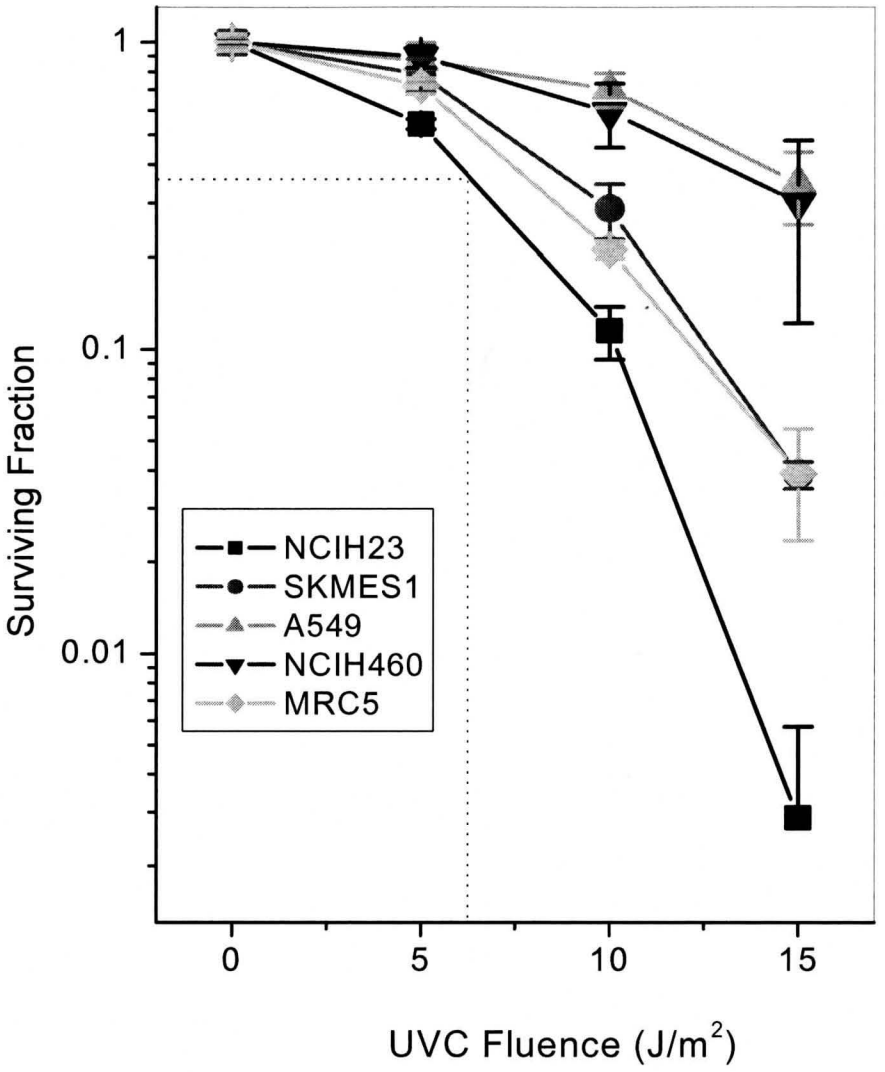


Figure-2.1 Clonogenic survival of selected non small cell lung cancer cell lines and a normal lung fibroblast following treatment with varying doses of UV-C. Cells were seeded at low density, treated with varying doses of UVC and assayed for clonogenic survival 8-12 days later. Data points are mean values from 2 to 7 independent experiments each performed in triplicate. Cell lines observed were A549 (▲), NCIH23 (■), NCIH460 (▼) and SKMES - 1 (●) and MRC5 (◆).

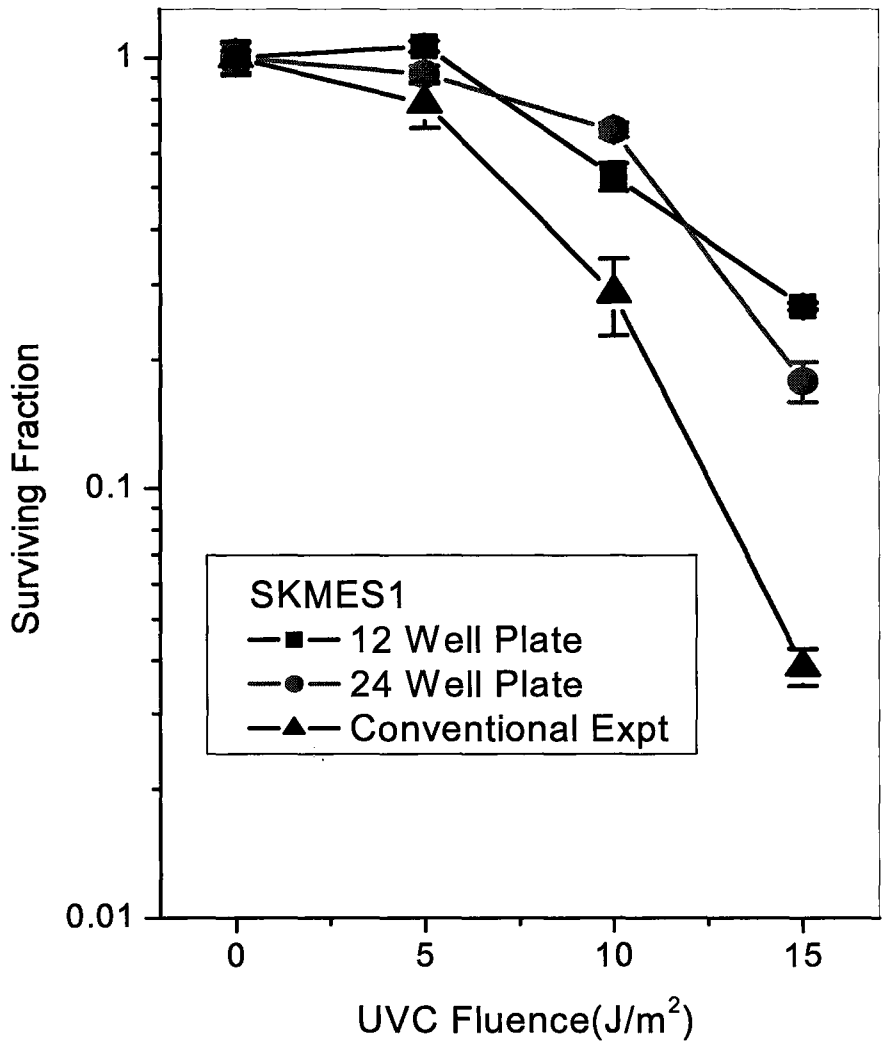


Figure-2.2 Clonogenic survival of the non small cell lung cancer cell line SKMES1 following treatment with varying doses of UVC using a high cell density protocol. Results of a colony survival assay where the NSCLC cell line SKMES 1 was exposed to different fluences of UVC. Cells were seeded into 12 (■) and 24 (●) well plates at high cell density and into 6 well plate at low cell density (▲) (conventional experiments). Colonies were counted 6-8 days after treatment.

Table- 2.1 D_{37} values obtained from colony survival assays for exposure to UVC or gamma rays in NSCLC cells and normal lung fibroblasts, MRC5. The average $D_{37} \pm$ SE, are reported, in addition to the number of experiments (N) used to determine the values. Results that are significantly different from the MRC5 fibroblasts by the two sample independent *t*-test ($P < 0.05$) are indicated.

D_{37} UV Fluence				
Serial #	**Cell Lines**	**N**	**Absolute D_{37} /SE ±**	**Pvalue**
1	MRC5	2	7.3 ± 0.3	
2	NCIH23	7	6.25 ± 0.6	0.37
3	A549	3	12.9 ± 0.8	0.01
4	NCIH460	2	14.19 ± 3.44	0.18
5	SKMES1	5	8.32 ± 0.58	0.19

Cobalt 60 (gamma rays)				
Serial #	**Cell Lines**	**N**	**Absolute D_{37} /SE ±**	**Pvalue**
1	MRC5	4	1.42 ± 0.16	
2	NCIH23	3	0.85 ± 0.05	0.04
3	A549	4	5.46 ± 0.44	0.00013
4	NCIH460	2	4.89 ± 0.11	0.00020
5	SKMES1	2	3.72± 0.15	0.001

Table-2.2 Average plating efficiency ± SE of NSCLC cell lines and normal lung fibroblasts MRC5. Results are from 4-9 experiments.

Serial #	Cell Line	# of Exps	Average Plating Efficiency ± SE
1	NCIH23	9	10.42 ± 0.94
2	MRC5	5	7.46 ± 2.50
3	SKMES-1	6	21.91 ± 7.18
4	A549	6	46.22 ± 11.29
5	NCIH460	4	55.91± 17.11

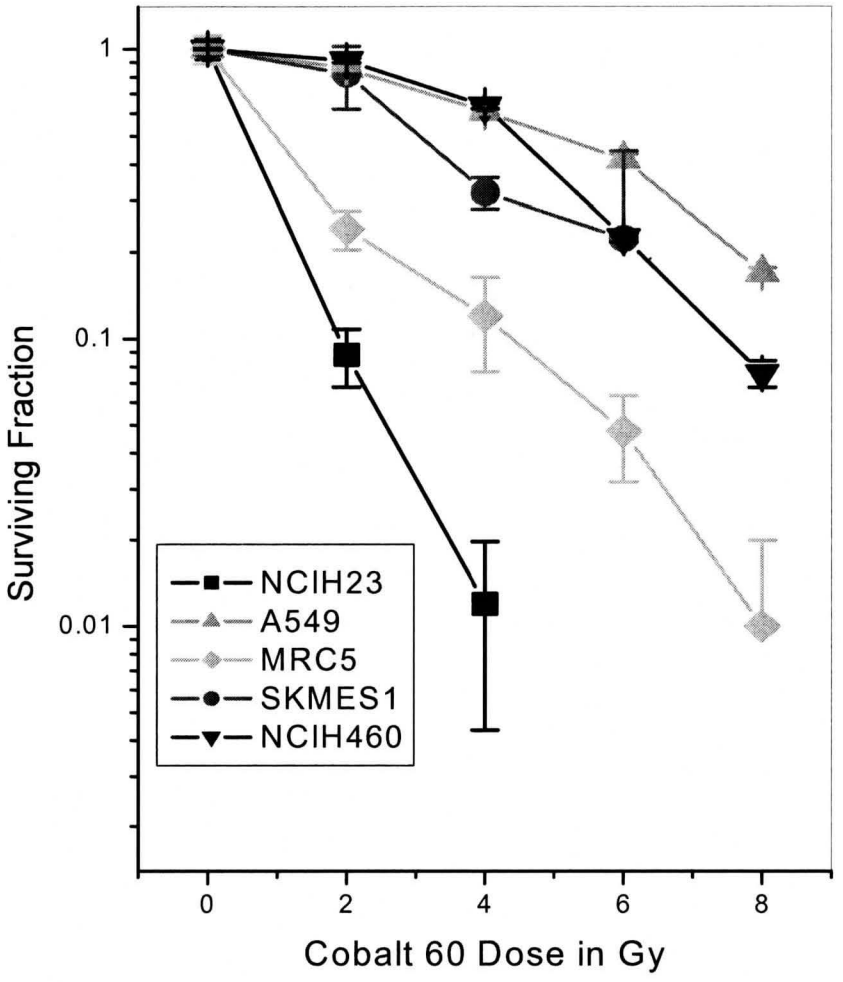


Figure-2.3 Clonogenic survival of selected non small cell lung cancer cell lines and a normal lung fibroblast following treatment with varying doses of gamma rays. Cells were seeded at low density, treated with varying doses of gamma rays and assayed for clonogenic survival 8-12 days later. Data points are mean values from 2 to 4 independent experiments each performed in triplicate. The cell lines examined were A549 (▲), NCIH23 (■), NCIH460 (▼) and SKMES - 1 (●) and MRC5 (◆).

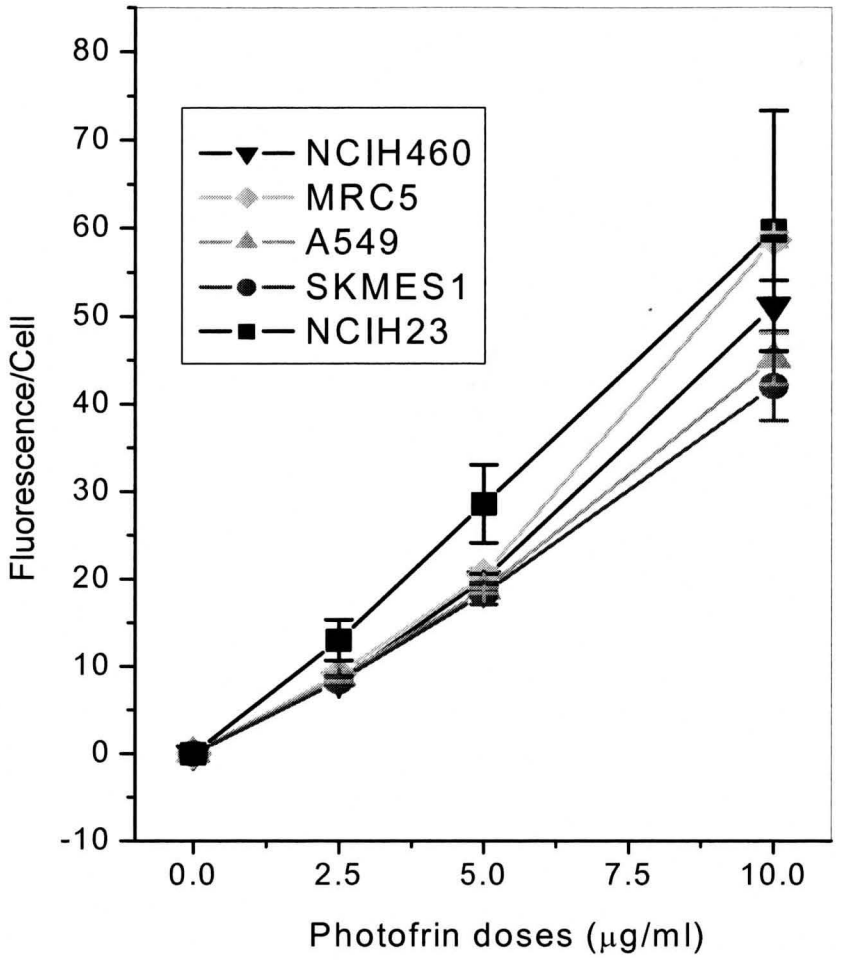


Figure-2.4 — Uptake of Photofrin per cell for the NSCLC cell lines A549 (▲), NCIH23 (■), NCIH460 (▼) and SKMES - 1 (●) and normal lung fibroblasts MRC5 (◆).

Cells were incubated in humidified air at 37°C and 5% CO_2 with varying concentrations of Photofrin overnight (20-24 hours). The Photofrin concentration of cells was measured by flow cytometry using an excitation wavelength of 488nm and emission measurements at 620-675nm. Fluorescence per cell was plotted against Photofrin concentration. Data points are mean values ± standard error from 2-3 independent experiments each performed in duplicate.

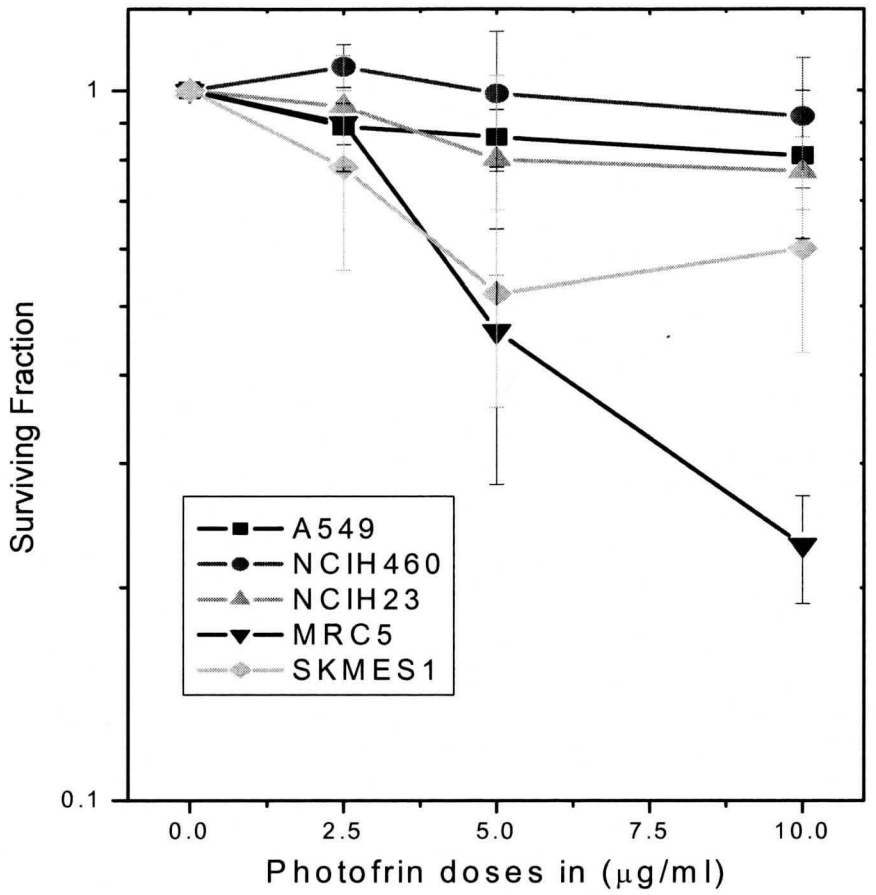


Figure-2.5 Effect of Photofrin alone on the survival of NSCLC cell lines and normal lung fibroblasts MRC5

The graph shows the influence of different Photofrin concentrations on colony survival using a high cell density protocol. Confluent monolayers of cells were overlayed with different concentrations of Photofrin (2.5, 5 and 10 μg/ml) and after 18-24 hr fresh medium without Photofrin was changed in all the plates. 3hr later the monolayers were trypsinised and the cells diluted and plated on 6 well plates. Colonies were stained and counted after 7 days. Results are from three experiments conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

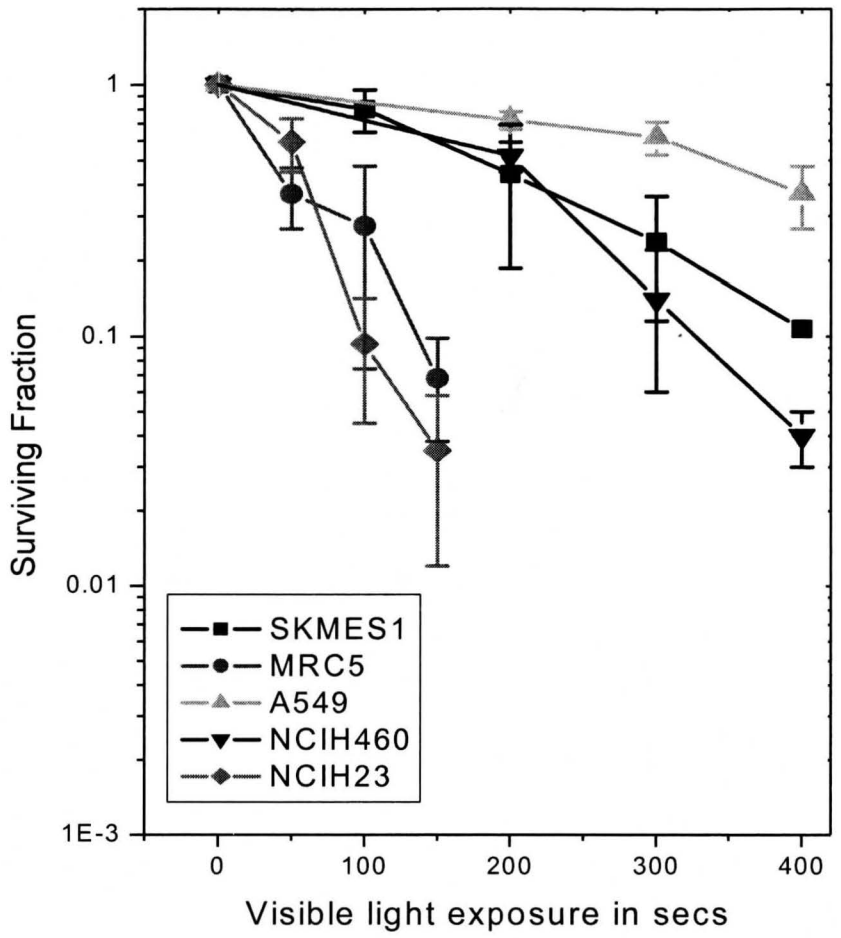


Figure-2.6 Effect of Photofrin and light on the survival of NSCLC cell lines and normal lung fibroblasts MRC-5 (High cell density protocol)

Photofrin concentrations of 2.5 μg/ml with varying light exposure at a power output of 20mJ/cm^2/sec were given to cells using a high cell density protocol. The cells were pre-incubated with Photofrin for 18-24 hr and subsequently exposed to visible light. 3hr after exposure to light, cell monolayers were trypsinised diluted and plated on 6 well plates. Colonies were stained and counted after 7 days.

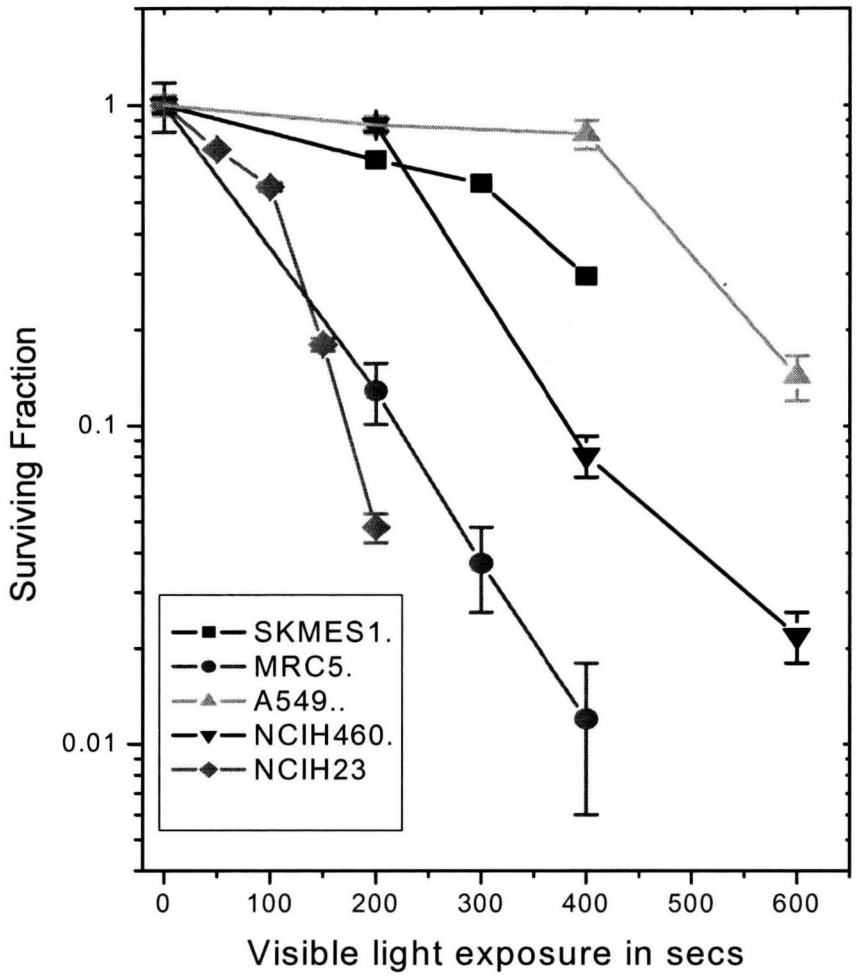


Figure-2.7 Effect of Photofrin and light on the survival of NSCLC cell lines and normal lung fibroblasts MRC5 (Low cell density protocol)

Photofrin concentrations of 2.5 μg/ml with varying light exposure at a power output of 20mJ/cm^2/sec were given to cells using a low cell density protocol. The cells were plated at low dilution on 6 well plates, pre-incubated with Photofrin for 18-24 hr and subsequently exposed to visible light. Colonies were stained and counted after 7 days.

Table-2. 3 D_{37} values obtained from colony survival assays of Photofrin and visible light treatment to NSCLC cells and normal lung fibroblasts MRC5. In Table 2.3(A) the average $D_{37} \pm$ SE are reported, in addition to the number of experiments (N) used to determine the values by using a high cell density protocol. Results that are significantly different from the normal lung fibroblasts MRC5 by the two sample independent *t*-test ($P < 0.05$) are also indicated. Table 2.3 (B) shows absolute D_{37} values obtained from colony survival assays of Photofrin and visible light treatment to NSCLC cell lines and normal lung fibroblasts MRC5 using a low cell density protocol.

Table 2.3 (A)

D_{37} PDT (High cell seeding protocol)				
Serial #	**Cell Lines**	**N**	**Average $D_{37} \pm$ SE (light exposure in secs)**	***P value***
1	**MRC5**	**4**	**64.4 ± 23.4**	
2	**NCIH23**	**3**	**61.8 ± 7.8**	**0.931**
3	**A549**	**4**	**478.1 ± 80.6**	**0.002**
4	**NCIH460**	**4**	**222.6 ± 13**	**0.001**
5	**SKMES1**	**3**	**221.81 ± 64**	**0.047**

Table 2.3 (B)

D_{37} PDT (Low cell seeding protocol)			
Serial #	**Cell Lines**	**N**	**Absolute D_{37} (light exposure in secs)**
1	**MRC5**	**1**	**96.3**
2	**NCIH23**	**1**	**116.41**
3	**A549**	**1**	**487.36**
4	**NCIH460**	**1**	**270.6**
5	**SKMES1**	**1**	**346.45**

Table- 2.4 Comparison of D_{37} values obtained from colony survival assays of Photofrin and visible light treatment to NSCLC cell lines and normal lung fibroblasts MRC5. The average $D_{37} \pm$ SE are reported for high density seeding experiments and absolute D_{37} values are reported for low cell density seeding experiments, in addition to the number of experiments (N) used to determine the values. NCIH23 and NCIH460 shows significant difference in survival when cells seeded by using high cell density and low cell density protocols. Results that are significantly different by the two sample independent *t*-test ($P < 0.05$) are also indicated.

Cell lines	D_{37} values (secs) (Low density exps)	N	$D_{37} \pm$ SE (secs) (High density exps)	N	P-Value
MRC5	96.3	1	64.42 ± 23.46	4	0.26
SKMES1	346.45	1	221.81 ± 64.01	3	0.19
NCIH23	116.41	1	61.85 ± 7.85	3	0.02
A549	487.36	1	478.1 ± 80.60	4	0.91
NCIH460	270.6	1	222.63 ± 13.07	4	0.04

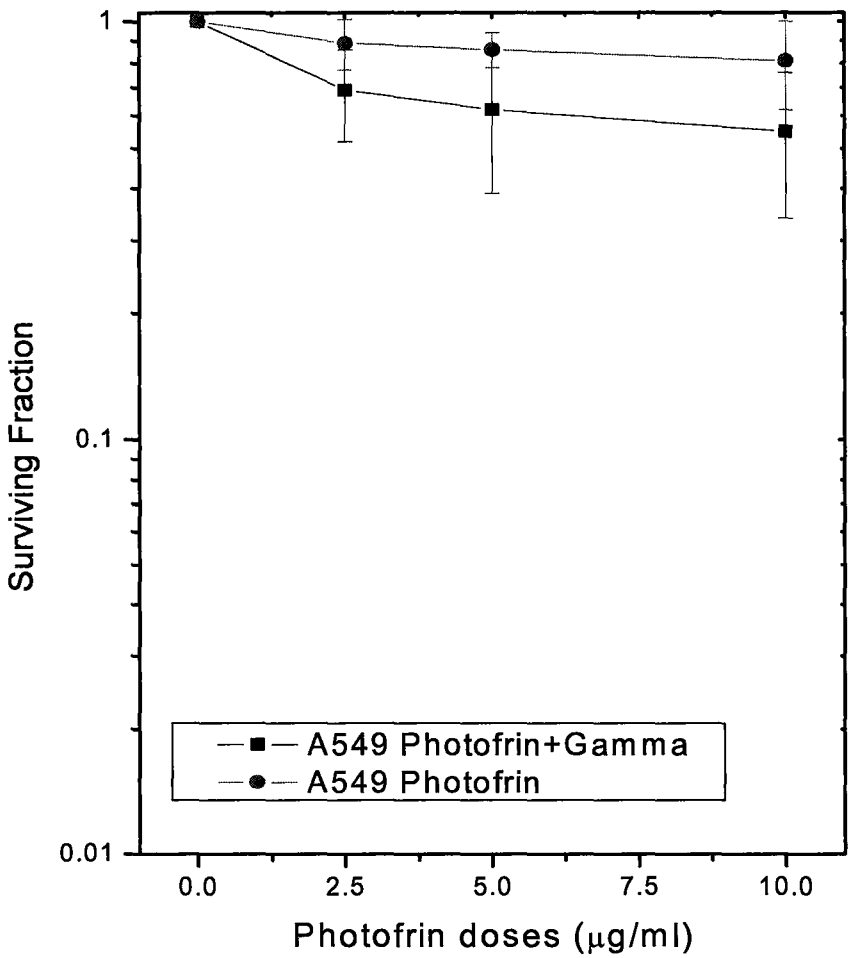


Figure 2.8 Effect of Photofrin drug followed by gamma rays on the survival of A549 cell line. Confluent monolayers of cells were overlayed with Photofrin for 18-24 hr and either irradiated with 7Gy of gamma rays (■) or left unirradiated (●) and subsequently seeded and scored for colonies. Results are from three experiments conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

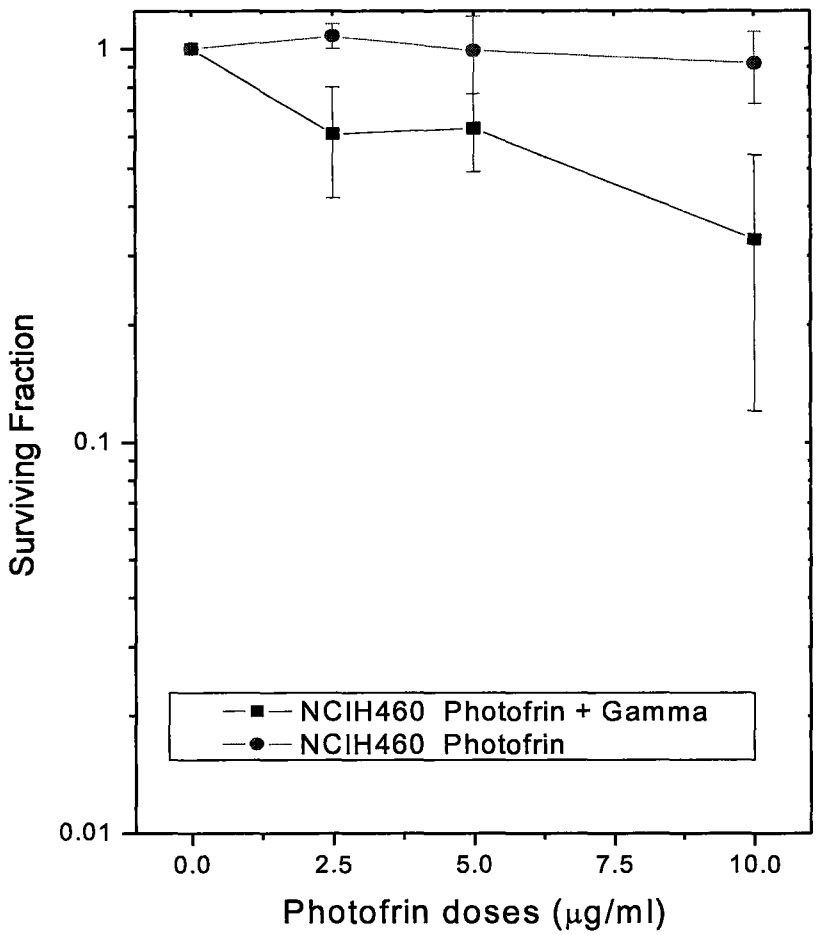


Figure 2.9 Effect of Photofrin drug followed by gamma rays on the survival of NCIH460 cell line. Confluent monolayers of cells were overlayed with Photofrin for 18-24 hr and either irradiated with 7Gy of gamma rays (■) or left unirradiated (●) and subsequently seeded and scored for colonies. Results are from three experiments conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

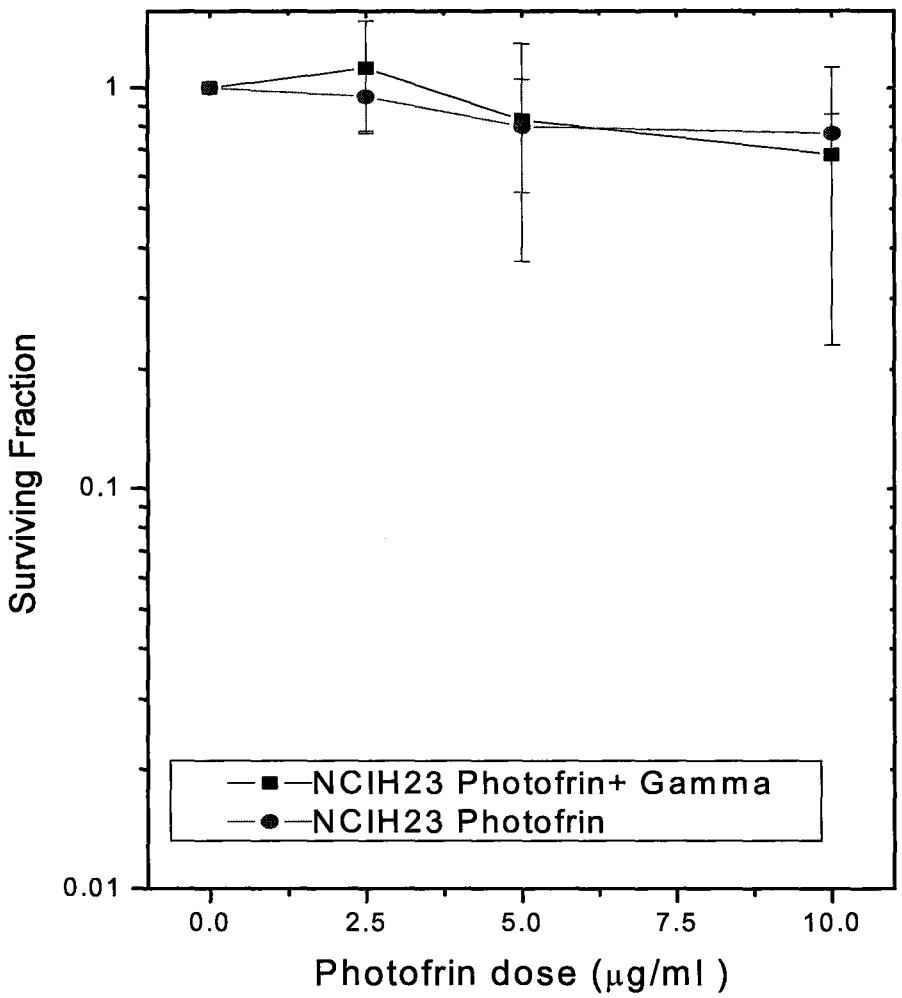


Figure 2.10 Effect of Photofrin drug followed by gamma rays on the survival of NCIH23 cell line. Confluent monolayers of cells were overlayed with Photofrin for 18-24 hr and either irradiated with 7Gy of gamma rays (■) or left unirradiated (●) and subsequently seeded and scored for colonies. Results are from three experiments conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

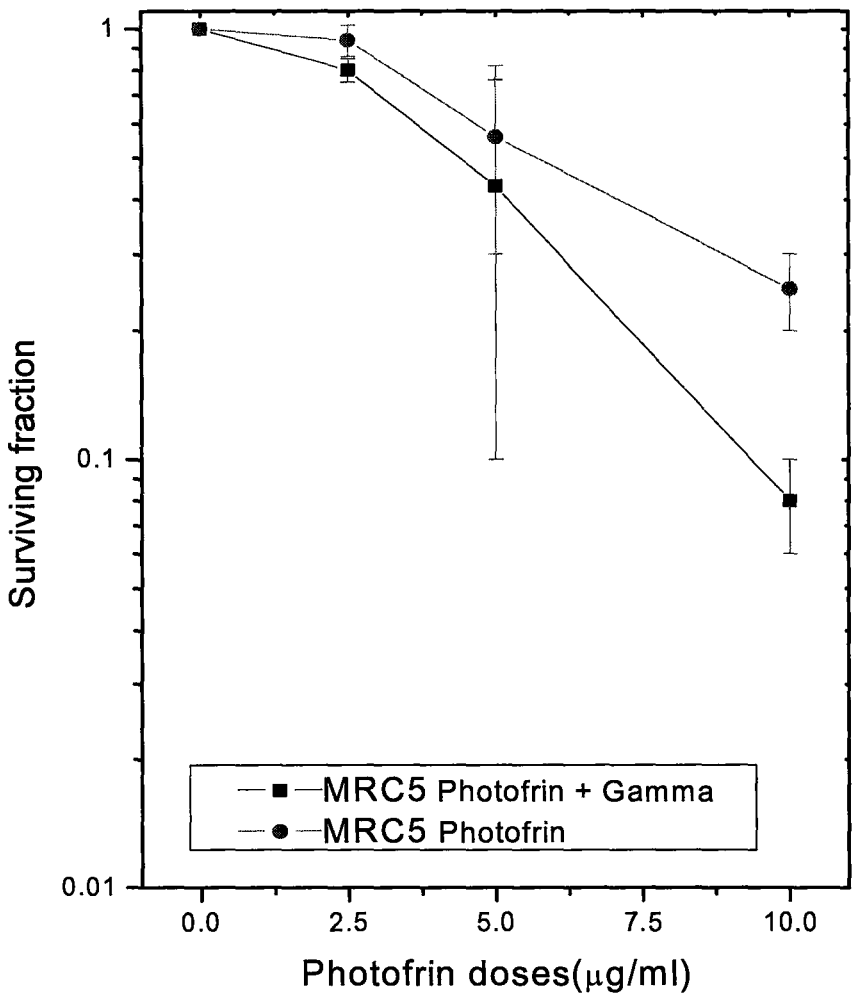


Figure 2.11 Effect of Photofrin drug followed by gamma rays on the survival of MRC5 cell line. Confluent monolayers of cells were overlayed with Photofrin for 18-24 hr and either irradiated with 7Gy of gamma rays (■) or left unirradiated (●) and subsequently seeded and scored for colonies. Results are from three experiments conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

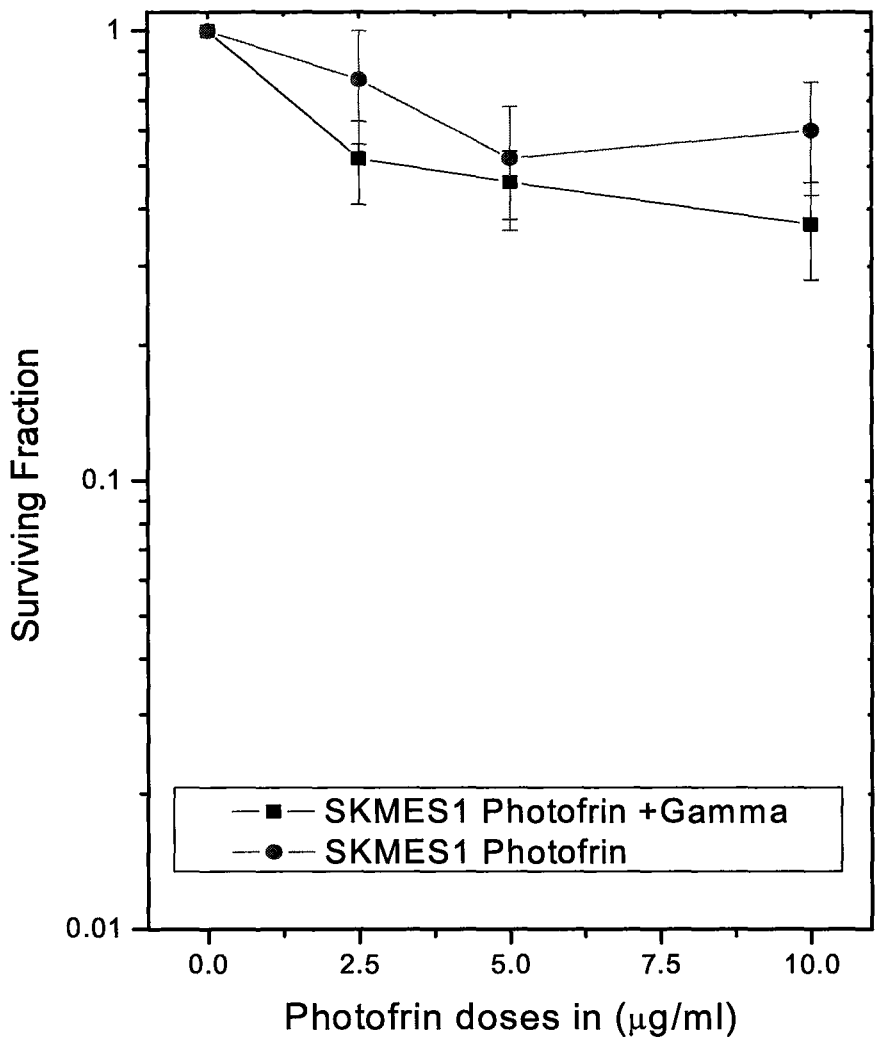


Figure 2.12 Effect of Photofrin drug followed by gamma rays on the survival of SKMES1 cell line. Confluent monolayers of cells were overlayed with Photofrin for 18-24 hr and either irradiated with 7Gy of gamma rays (■) or left unirradiated (●) and subsequently seeded and scored for colonies. Results are from three experiments conducted in triplicate. Each bar represents the arithmetic mean ± standard error.

Table 2.5 Effect of order of combined treatment on NSCLC cell lines

Mean surviving fractions ± SE of NSCLC cells and normal lung fibroblasts MRC5 are shown for cells exposed to Photofrin and then either (a) exposed to light and 15-30 minutes later exposed to gamma radiation or (b) exposed to gamma radiation and 15-30 minutes later exposed to light. Ratio of (b)/(a) for all the experiments on a given cell line is shown as the mean ratio ± SE in addition to the number of experiments (N) used to determine the values by using a high cell density protocol.

Cell Lines	No of Exp	Surviving fraction (a) P+L then Gamma	Surviving Fraction (b) P+G then Light	Ratio (b)/(a)	P value*
SKMES1	3	0.0265 ± 0.020	0.0267 ± 0.019	1.024±0.022	0.236
A549	4	0.056 ± 0.040	0.073 ± 0.036	2.289±0.882	0.141
NCIH460	4	0.0036 ± 0.0019	0.0043 ± 0.0020	2.454±1.333	0.178
NCIH23	3	0.057 ± 0.022	0.069 ± 0.027	1.268±0.095	0.053
MRC5	3	0.026 ± 0.003	0.032 ± 0.009	1.272 ± 0.382	0.274

* Not significantly greater than one by one sample one tailed t-test

Table 2. 6 Effect of combined treatment of PDT and gamma-rays on NSCLC cell lines and normal lung fibroblasts.

The survival of NSCLC cells following combined treatments (a) & (b) was compared to predicted survival values based on survival following PDT and gamma-rays alone (c). The survival of NSCLC cells following combined treatment was not significantly different from that expected (based on an additive effect of PDT plus gamma radiation) by a two-sample independent t-test for all the four NSCLC cell lines but not for MRC5 cell line. Ratios obtained from (a)/(c) and (b)/(c) were pooled as there was no significant difference between the two treatments for each of the cell lines.

Cell Lines	No of Exp	Surviving fraction (a) P+L then Gamma	Surviving fraction (b) P+G then Light	(c) Expected Survival	Ratio (a)/(c) and (b)/(c)	One sample *t* test P value	Two sample ind *t* test P value	Effect
SKMES1	3	0.0265 ± 0.020	0.0267 ± 0.019	0.0135 ± 0.0115	2.593 ± 0.423	0.016 (d)	0.52	Less than additive
A549	4	0.056 ± 0.040	0.073 ± 0.036	0.077 ± 0.037	0.733 ± 0.122	0.04 (e)	0.77	Synergistic
NCIH460	4	0.0036 ± 0.0019	0.0043 ± 0.0020	0.0033 ± 0.0011	1.664 ± 0.530	0.125	0.77	Less than additive
NCIH23	3	0.057 ± 0.022	0.069 ± 0.027	0.034 ± 0.014	2.01 ± 0.174	0.001 (d)	0.28	Less than additive
MRC5	3	0.026±0.003	0.032±0.009	0.006±0.003	12.84± 6.42	0.06	0.01(f)	Less than additive

(d) Is significantly greater than one–by one sample 1 tailed *t*-test

(e) Is significantly smaller than one–by one sample 1 tailed *t*-test

(f) Is significantly different by the two sample independent *t*-test ($P < 0.05$)

CHAPTER 3

Summary and Future Initiatives

Summary

In the present study using a panel of 4 different non-small cell lung cancer (NSCLC) cell lines A549, NCIH460, NCIH23 and SKMES1 and a normal lung fibroblast cell line (MRC5), we have examined their sensitivity to UVC, ionizing radiation alone, radiation with Photofrin but no light, Photofrin alone, Photofrin-mediated PDT alone and combination of radiation and PDT using colony forming assay. Because drug uptake is a potential factor influencing cellular sensitivity to PDT, we examined the uptake of Photofrin to see how much Photofrin was absorbed per cell by each cell line in NSCLC cell lines and the normal lung fibroblast strain MRC5. We reported that all the cell lines showed a similar Photofrin uptake per cell and observed that as the Photofrin concentration increases, its uptake by cancer cell also increases.

D_{37} values calculated from the survival curves obtained using a conventional clonagenic assay indicated a 2-fold difference in UVC sensitivity and 6-fold difference in sensitivity to gamma-ray of the four NSCLC cell lines and normal lung fibroblast. The rank order of UVC and gamma–ray resistance obtained in the NSCLC panel was same: A549 >> NCIH460 > SKMES1 > MRC5 > NCIH23. Although MRC5 cells showed increased sensitivity compared to the NSCLC cell lines, following treatment with 10 μg/ml of Photofrin alone, 2.5 and 5 μg/ml of Photofrin alone had no significant effect on colony survival for MRC5 or any of the NSCLC cell lines showing that Photofrin does not exhibit dark toxicity at low doses. Further, the sensitivity of cells to PDT was examined using the high cell density clonagenic assay protocol with a standard Photofrin concentration of 2.5 μg/ml with varying light exposure in order to more closely simulate

the clinical conditions of PDT. D_{37} values calculated from the survival curves indicated an 8-fold difference in sensitivity to PDT. The rank order of PDT resistance obtained in the NSCLC panel was A549 >> NCIH460 > SKMES1 > MRC5 > NCIH23. All the cell lines showed same trend in order of resistance in all the sets of colony survival experiments showing that a cell line which is resistant to UVC or gamma rays is also resistant to PDT and visa versa. As summarized before, Photofrin alone at concentrations up to 10 μg/ml had no significant effect on the survival of the NSCLC cell lines, whereas 10 μg/ml of Photofrin alone reduced survival significantly in MRC5 cells indicating that at high drug concentration normal cells are more sensitive than tumor cells. A radiosensitizing effect of Photofrin was detected in MRC5 and NCIH460 cells, but not in A549, SKMES-1 and NCI-H23 cells. For the combination treatment, we examined the effects of varying the time between treatments as well as the effect of the order in which treatment is given. Light followed by gamma rays resulted in a greater tumor cell kill for all the cell lines compared to gamma rays followed by light, although this was only significant when data for all NSCLC cell lines were pooled. The combined treatment with high dose rate HDR radiation and PDT was not significantly different from an additive effect of the individual treatment modalities for *in vitro* survival of the 4 NSCLC cells. In contrast the combined treatment was less than additive for the MRC5 cells suggesting that the combined treatment would have the potential advantage of doing less damage to the normal lung cells.

2.0 Future Initiatives

2.1 Cell signaling Pathways

Several aspects of this research work could be further explored. PDT can lead to several cellular responses including cell cycle arrest, necrosis, and apoptosis, as well as trigger many signaling pathways. The mitogen-activated protein kinase (MAPK) pathway is an evolutionary conserved signaling cascade that plays a critical role in cell growth, differentiation, and cell survival. (Oleinick *et al.,* 1998). The best-characterized pathway leading to activation of MAPK is the growth factor-induced ERK pathway (extra cellular signal-activated protein kinase). It has been suggested that the ERK pathway plays a crucial role in cell survival after various stress stimuli (Buckley *et al.,* 1999). ERKs are mainly activated by a variety of mitogenic or stress stimuli and lead to the production of proteins required for cell proliferation and/or differentiation (Graves *et al.,* 2000). The interaction between various signaling pathways may play an important role in the efficiency of PDT.

It has been reported previously that immortalized LFS cells expressing mutant p53 are significantly more resistant to Photofrin-mediated PDT compared to NHF that express wild-type p53 (Tong *et al.,* 2000). The role of ERKs in the sensitivity of LFS and NHF cells to Photofrin mediated PDT was also examined and showed that Photofrin-mediated PDT at equivalent cellular Photofrin levels resulted in increased phosphorylation of ERK1/2 detectable in both Li-Fraumeni syndrome (LFS) and normal human fibroblast (NHF) cells at 30 min after PDT. For the NHF cells, the increased phosphorylation of ERK1/2 was transient and decreased to levels lower than that in

untreated cells by 3 h after PDT. In contrast, LFS cells showed a prolonged activation of ERK1/2 for at least 11 h after PDT. Therefore, the duration of PDT-induced ERK activity correlated with the cellular resistance to PDT (Tong *et al.,* 2002).

We have carried out preliminary experiments based on the report of Tong *et al.* in order to examine the response of the ERK1/2 in NSCLC cell lines and the normal lung fibroblasts that displayed varying degrees of sensitivity to Photofrin-mediated PDT. The rank order of PDT resistance obtained in the cell lines was A549 >> NCIH460 > SKMES1 > MRC5 > NCIH23. The sensitivity of cells to PDT was examined using the high cell density protocol at Photofrin concentration of 2.5 μg/ml with varying light exposure in order to more closely simulate the clinical conditions of PDT. D_{37} values (Photofrin concentration resulting in 37% colony survival after light exposure) calculated from the survival curves indicated an 8-fold difference in sensitivity to PDT. Using Western blot analysis, we have conducted a single experiment on each of the NSCLC cell lines and the normal lung fibroblasts MRC5 to examine the response of ERK1/2 following PDT. Appendix-2, figure 3.1-3.4 shows the response of ERK1/2 in NSCLC cell lines and normal lung fibroblast following Photofrin-mediated PDT.

Preliminary results show that for MRC5 cells Photofrin-mediated PDT at 2.5μg/ml of Photofrin and 100sec light exposure resulted in decreased phosphorylation of ERK1/2 when compared to controls at 30 min after PDT. In addition, phosphorylation of ERK1/2 further decreased after 12 hrs. However phosphorylation of ERK1/2 in the untreated, no drug, and no light (NDNL) also decreased between 1/2h and 12h. For SKMES1 cells, it is seen that Photofrin-mediated PDT at 2.5μg/ml of Photofrin resulted

in decreased phosphorylation of ERK1/2 when compared to controls at 30 min after PDT for 100 and 400 sec light exposure. Phosphorylation of ERK1/2 in the PDT treated samples was similar (100 secs) or increased (400secs) after 12 hrs, whereas phosphorylation of ERK1/2 in the untreated, no drug, and no light (NDNL) was less than in the control 30 min sample. In contrast, there was no phosphorylation of ERK1/2 in control cells or following Photofrin-mediated PDT at 2.5μg/ml of Photofrin at 30 min or 12 hrs after PDT in A549 and NCIH460 cells. Finally for NCIH23 cells, although the control sample at 30 minutes is missing, the results suggest that Photofrin-mediated PDT at 2.5μg/ml of Photofrin and 100 and 400 sec light exposure increased phosphorylation of ERK1/2 detectable at 30 min after PDT. This increase was transient and much lower levels of phosphorylation of ERK1/2 were detected 12 h after PDT.

In summary we detected no ERK1/2 phosphorylation in control A549 and NCIH460 cells, whereas ERK1/2 phosphorylation was detected in control cells from MRC5, SKMES-1 and NCIH23 cells. For MRC5 and SKMES-1 cells, 2.5μg/ml of Photofrin and 100 sec light exposure resulted in a decrease in ERK1/2 phosphorylation at 1/2h, which remained at a lower level for up to 12h when compared to the control 1/2h sample. In contrast, there was no detectable ERK1/2 phosphorylation in A549 and NCIH460 cells following Photofrin-mediated PDT. It appears possible that the difference in the kinetics of PDT-induced ERK1/2 phosphorylation contributes to the difference in cell sensitivity to PDT among the different cell lines tested. The PDT resistant A549 and NCIH460 cells both showed no detectable ERK1/2 phosphorylation even in untreated cells. In addition, at 2.5μg/ml Photofrin concentration and 100 sec light we see the

greatest survival in A549 and NCIH460 cells, whereas MRC5 and NCIH23 cells show the lowest survival (Figure 2.6 and 2.7) there may be some correlation of PDT sensitivity of cells to the ERK1/2 phosphorylation in untreated (control) cells as well as the response of ERK1/2 phosphorylation following PDT. These results are preliminary and will require further study.

2.2 Cell cycle Analysis.

An investigation into the differential effects of PDT on the cell cycle will be of interest. Tong et al. also showed that the mutation in p53 in LFS cells may result in a pronounced G2/M arrest that contributes to the resistance of LFS cells to Photofrin-mediated PDT while NHF cells did not show marked apoptosis or G2/M arrest. These results indicate also that PDT is able to cause p53-independent apoptosis (Tong et al., 2000). These results are in contrast to our results, which show that the A549 cell line with wild type p53 is most resistant to Photofrin-mediated PDT and the NCIH23 cell line with mutant p53 is most sensitive to PDT treatment. Thus, the efficacy of PDT in relation to the cell cycle should be explored in NSCLC cell lines in terms of p53 protein status as it is an important modulator of the cell cycle. Secondly, it is known that cellular photosensitizer levels differ among the different cell cycle phases, which is associated with the cell-cycle-dependent efficacy of PDT with photosensitizer (Sano et al., 2005). For example, intracellular levels of water-soluble photosensitizer ATX-S10(Na) in different cell cycle phases were determined and it was found that human cervix adenocarcinoma cells (HeLa S3) in the S and G_2/M phases were hypersensitive to PDT with ATX-S10(Na) in comparison with those in the G_1 phase, and that cellular levels of

ATX-S10(Na) were increased in cells in the S and G_2/M phases compared to those in the G_1 phase (Sano et al., 2005). Thus, it is possible that the difference in sensitivity to PDT of NSCLC cell lines and normal lung fibroblast results from differential effects of PDT on the cell cycle and thus it will be of interest to examine the cell cycle changes in NSCLC following PDT.

2.3 Effect of low doses of light on survival of cell lines

In an initial experiment using the NSCLC A549 cells we examined PDT effects on colony survival at low light exposures and found increased colony numbers in the controls that had light exposure alone compared to control cells with no light or Photofrin treatment. In this experiment a Photofrin dose of 2.5 μg/ml and light exposures of 25, 50, 100 secs at a power output of 20mJ/cm^2/sec were given. Results of this colony survival experiment are shown in Appendix 2, Figure 3.5. It can be seen that that there are approximately 2 fold more colonies on the plates treated with light alone (50 and 100 secs) compared to the controls plates that have no light or Photofrin. This result was unexpected and is surprising; especially since the plating efficiency of control A549 cells is 46.22±11.29%. This suggests that the low light exposure increases the plating efficiency of A549 cells to close to 100%. A similar increase in plating efficiency has been reported for SCC-25 human squamous carcinoma cells (Caney *et al.* 1999) and CHO cells (Marples *et al.* 1997) when exposed to combinations of low-dose radiation and cisplatin treatment and for Chinese hamster V79 cells when exposed to combinations of low dose neutrons and X-rays (Marples and Skov, 1996). It is possible that the low levels

of light exposure to A549 cells results in the induction of protective mechanisms that result in a decrease in the number of cells that loose viability throughout the course of the colony assay due to spontaneously occurring cell damage compared with the corresponding controls. The possibility that low doses of light alone can induce protective mechanisms in cells is worth investigating and warrants further experiments.

Appendix-1

NCI H23								
	Well					Std. Error	Surviving Fraction	
UVC Fluence (J/m^2)	1	2	3	Average	Std. Dev.	(SE)	(SF)	SEof SF
0	63	73	94	76.67	15.82	9.13	1.00	0.12
5	60	39	29	42.67	15.82	9.13	0.56	0.12
10	6	8	13	9.00	3.61	2.08	0.12	0.03
15	0	1	5	2.00	2.65	1.53	0.03	0.02

Table-3.1 Representive summary of results from a single colony survival experiment using the NSCLC cell line NCIH23 that was exposed to different fluences of UVC. Cells were seeded into 6 well plates at a low cell density of 1000 cells/well, and irradiated with 5, 10, or 15 J/m^2 UVC or left unirradiated in triplicates. Colonies were stained with crystal violet and counted 6-8 days after treatment. Plating efficiency (the percentage of untreated cells seeded that grow into macroscopic colonies) of the cell line was calculated to be approximately 8% for this particular experiment. Surviving fraction of the treated plates was calculated as a fraction of the control colonies (without UVC treatment).

A 549									
	Well						Std. Error	Surviving Fraction	
COBALT 60 DOSE(Gy)	1	2	3	4	Average	Std. Dev.	(SE)	(SF)	SE of SF
0	175	185	201	200	190.25	12.53	6.26	1.00	0.033
2	163	169	164	157	163.25	4.92	2.46	0.86	0.013
4	119	110	119	114	115.50	4.36	2.18	0.61	0.011
6	68	77	81	83	77.25	6.65	3.33	0.42	0.018
8	30	33	36	30	32.25	2.87	1.44	0.17	0.008

Table-3.2 Representive summary of results from a single colony survival experiment using the NSCLC cell line A549 that was exposed to different doses of gamma rays. Cells were seeded into 6 well plates at a low cell density of 300 cells/well, and irradiated with 2, 4, 6 or 8 Gy of gamma rays or left unirradiated in quadruplicates. Colonies were stained with crystal violet and counted 6-8 days after treatment. Plating efficiency (the percentage of untreated cells seeded that grow into macroscopic colonies) of the cell line was calculated to be approximately 63% for this particular experiment. Surviving fraction of the treated plates was calculated as a fraction of the control colonies (without gamma-ray treatment).

SKMES1										
		Well					Std. Error	Surviving Fraction		
Photofrin (µg/ml)		1	2	3	Average	Std. Dev.	(SE)	(SF)	SE of SF	Cells Seeded
NDNL	0	173	188	181	180.67	7.51	4.33	1.00	0.02	500
DNL	2.5	185	178	179	180.67	3.79	2.19	1.00	0.01	500
DNL	5	134	123	112	123.00	11.00	6.35	0.68	0.04	500
DNL	10	133	137	142	137.33	4.51	2.60	0.76	0.01	500

Table-3.3 Representive summary of results from a single colony survival experiment using the NSCLC cell line SKMES1 that was exposed to different concentrations of Photofrin alone using a high cell density protocol. Confluent monolayers of cells were overlayed with different concentrations of Photofrin (2.5, 5 and 10 µg/ml) or left untreated (without Photofrin) and after 18-24 hr fresh medium without Photofrin was changed in all the plates. 3hr after medium change cell monolayers were trypsinised, diluted and plated on 6 well plates. Colonies were stained with crystal violet and counted after 7 days. Surviving fraction of the treated plates was calculated as a fraction of the control colonies (no drug, no light) NDNL

SKMES1									
	Well					Std. Error	Surviving Fraction		
PHOTOFRIN+Light	1	2	3	Average	Std. Dev.	(SE)	(SF)	SE of SF	Cells seeded
DNL 2.5	368	378	353	366.33	12.58	7.26	1.00	0.02	1000
DL 2.5 + 200 sec	160	151	158	156.33	4.73	2.73	0.43	0.01	1000
DL 2.5 + 300 sec	77	80	71	76.00	4.58	2.65	0.21	0.01	1000
DL 2.5 + 400 sec	58	31	29	39.33	16.20	9.35	0.11	0.03	1000
NDNL	295	218	260	257.67					1000

Table-3.4 Representive summary of results from a single colony survival experiment using the NSCLC cell line SKMES1. A Photofrin concentration of 2.5 μg/ml with varying light exposure at a power output of 20mW/cm^2/sec was given to cells using a high cell density protocol. The cells were pre incubated with Photofrin for 18-24 hr and exposed to visible light. 3hr after irradiation the cell monolayers were trypsinised, diluted and plated on 6 well plates. Colonies were stained and counted after 7 days. Surviving fraction of the treated plates was calculated as a fraction of the control colonies (drug, no light) DNL.

SKMES1									
	Well					Std. Error	Surviving Fraction		
PHOTOFRIN (µg/ml)+C0-60(Gy)	1	2	3	Average	Std. Dev.	(SE)	(SF)	SE of SF	Cells seeded
NDL 7Gy	108	102	113	107.67	5.51	3.18	1.00	0.03	2500
DL 2.5+7Gy	83	90	88	87.00	3.61	2.08	0.63	0.02	2500
DL 5 +7Gy	56	58	49	54.33	4.73	2.73	0.54	0.03	2500
DL 10 +7Gy	38	35	37	36.67	1.53	0.88	0.46	0.01	2500

Table-3.5 Representive summary of results of a single colony survival experiment using the NSCLC cell line SKMES1. Photofrin concentrations of 2.5, 5 and 10 µg/ml and a single gamma ray dose of 7 Gy at a dose rate of 1.5-1.9 Gy were given to cells using a high cell density protocol. The cells were pre incubated with Photofrin for 18-24 hr and exposed to gamma rays. 3hr after irradiation the cell monolayers were trypsinised, diluted and plated on 6 well plates. Colonies were stained and counted after 7 days. Surviving fraction of the treated plates was calculated as a fraction of the control colonies (no drug, gamma ray) NDL.

Experimental Sets	No. of wells 1	2	3	Average	Std. Dev.	Std. Error (SE)	Surviving Fraction (SF)	SE of SF	No. of cells seeded
1) NDNL	145	160	153	152.67	7.51	4.33	1.00	0.028	250
2) NDL(600 Secs)	143	155	131	143.00	12.00	6.93	0.94	0.045	250
3) DNL	121	127	126	124.67	3.21	1.86	0.82	0.012	250
4) PDT	41	40	46	42.33	3.21	1.86	0.14	0.006	500
5) Gamma 7Gy only	68	69	65	67.33	2.08	1.20	0.22	0.004	500
6) Photofrin+Light+ 7 Gy	2	7	4	4.33	2.52	1.45	0.007	0.002	1000
7) Photofrin+7 Gy+Light	14	15	22	17.00	4.36	2.52	0.03	0.004	1000

Table-3.6 Representive summary of results of a colony survival experiment for combined treatment of PDT and gamma rays for the NSCLC A549 cell line. 1) Survival was calculated in comparison to no drug, no light (NDNL) samples using an average of three determinations for each experimental set (±SE), 2) no drug light (NDL) shows the effect of light alone given for 600 secs at a power output of 20mW/cm^2/sec, 3) drug no light (DNL) shows the effect of Photofrin alone at a concentration of 2.5μg/ml, 4) shows the effect of PDT on cells at a Photofrin concentration of 2.5μg/ml followed by light for 600 secs, 5) shows the effect of 7 Gy of gamma rays on cells, 6 & 7)- cells were exposed to 2.5ug/ml Photofrin and then either exposed to light for 600 secs and 15-30 minutes later exposed to gamma radiation or exposed to gamma radiation and 15-30 minutes later exposed to light. High cell density protocol was used for the experiment. Colonies containing at least 32 cells were counted as surviving colonies.

Table -3.7 Combination data spread sheet for surviving fractions data for all colony survival experiments for the NSCLC cell lines and normal lung fibroblasts. The survival of NSCLC cells following combined treatments (A) & (B) was compared to predicted survival values based on survival following PDT and gamma-rays alone (c). Ratio of (B)/ (A) `or (A)/ (B) for all the experiments was also observed to see the effect of order of combined treatment on NSCLC cell lines. Survival was calculated in comparison to no drug, no light (NDNL) samples for each experimental set for all the cell lines.

Cell Lines	Experiments	NDNL	DNL	NDL	PDT	Gamma Ray	P+Light+ Gamma (A)	P+Gamma+ Light (B)	Light dose in secs Gamma dose in Gy	PDT X Gamma predicted survival (C)	B/C	A/C	B/A	A/B
SKMES1	1	1	1.25	1.31	0.02	0.13	0.0065	0.0068	400 sec/7Gy	0.0020	3.40	3.25	1.05	0.96
	2	1	1.15	0.86	0.00	...	...	...	400 sec/7Gy	...				
	3	1	1.02	1.01	0.27	0.09	0.0465	0.0466	400 sec/7Gy	0.0250	1.86	1.86	1.00	1.00
A549	1	1	1.13	1.09	...	0.32	...	...	600 sec/7Gy	...				
	2	1	0.92	0.90	0.46	0.33	0.1360	0.1450	400 sec/7Gy	0.1510	0.96	0.90	1.07	0.94
	3	1	0.97	0.88	0.21	0.25	0.0250	0.0450	400 sec/7Gy	0.0510	0.88	0.49	1.80	0.56
	4	1	0.82	0.94	0.14	0.22	0.0070	0.0280	400 sec/7Gy	0.0300	0.93	0.23	4.00	0.25
NCIH460	1	1	0.62	0.94	0.06	0.08	0.0070	0.0080	400 sec/7Gy	0.0040	2.00	1.75	1.14	0.88
	2	1	0.92	0.84	0.01	0.04	0.0003	0.0020	400 sec/7Gy	0.0004	5.19	0.81	6.45	0.16
	3	1	0.99	0.98	0.01	0.03	0.0002	0.0003	400 sec/7Gy	0.0002	1.05	0.89	1.18	0.85
	4	1	0.98	1.01	0.17	0.05	0.0068	0.0071	300 sec/7Gy	0.0086	0.83	0.79	1.04	0.96
NCIH23	1	1	0.90	0.90	0.04	0.01	...	0.0040	100 sec/7Gy	0.0002	21.05	...	...	...
	2	1	0.84	not done	0.53	0.19	...	...	100 sec/2Gy	0.1025	...	...	...	...
	3	1	1.07	1.71	0.63	0.30	...	...	100 sec/2Gy	0.1873	...	...	...	...
	4	1	0.90	1.01	0.04	0.16	0.0120	0.0170	100 sec/2Gy	0.0060	2.81	1.99	1.42	0.71
	5	1	0.76	0.64	0.28	0.16	0.0770	0.0840	100 sec/2Gy	0.0440	1.91	1.75	1.09	0.92
	6	1	0.83	0.73	0.31	0.17	0.0810	0.1050	100 sec/2Gy	0.0516	2.04	1.57	1.30	0.77
MRC-5	1	1	0.76	0.87	0.12	0.10	0.0290	0.0450	100 sec/7Gy	0.0116	3.87	2.50	1.55	0.64
	2	1	0.79	0.85	0.08	0.09	0.0200	0.0350	100 sec/7Gy	0.0070	5.00	2.85	1.75	0.57
	3	1	0.83	0.85	0.04	0.02	0.0290	0.0150	100 sec/7Gy	0.0007	21.42	41.42	0.52	1.93

APPENDIX-2

Western Blotting Analysis

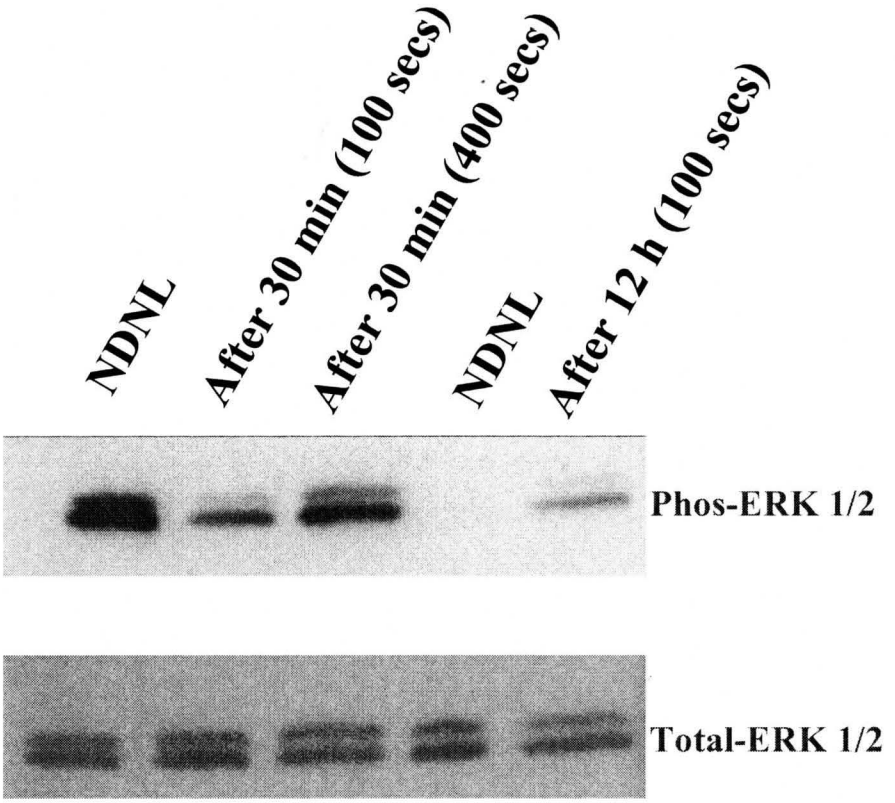
Exponentially growing cells were plated at $3x10^6$ cells/dish on a 100mm petri plates and incubated for 6 hr to allow cells to adhere to the bottom of plates. For PDT treatment, cells were exposed to 2.5 μg/ml Photofrin for 18-24 h followed by replacement with fresh culture medium followed by light exposure for 100 sec and 400 sec (power output of $20mW/cm^2$). Cells were harvested at 30 min and 12 hrs after PDT in different sets for which cells were first washed twice with ice-cold PBS buffer and lysed in a buffer containing 50 mM of Tris (pH 8.0), 150 nM of NaCl, 0.5% NP40, 2 mM of EDTA, 100 mM of NaF, 10 mM of sodium orthovanadate, and a protease inhibitor tablet (Roche Diagnostics Canada) for 30 min on ice. The debris of cells then was pelleted by centrifugation (>10,000 x *g*) for 15 min and discarded. The protein concentration of the cell lysate (supernatent) was determined using the Bradford micro assay procedure (BioRad, Munich, Germany). Equal amounts of protein (60μg) were resolved on 12% SDS-PAGE and electrophoretically transferred to an enhanced chemiluminescence membrane (Amersham Pharmacia Biotech). The blots were blocked in 5% skim milk in a Tris-buffered saline with 1% Tween 20 for 1 h at room temperature and then incubated at 4°C overnight with corresponding antibodies in the same buffer as the block buffer. Specific antibody-labeled proteins were detected by using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence plus Western blotting detection system (Amersham Pharmacia Biotech). Thereafter, blots were stripped and reprobed with

antibodies to actin or total ERK1/2. The data were quantified using Phosphor Imager analysis and normalized with total ERK1/2. (as modified from Tong *et al.*, 2002).

Figure 3.1. Activation of ERK1/2 in MRC5 (normal lung fibroblast) by Photofrin-mediated PDT.

An autoradiograph of a Western Blot (top) from cells treated with Photofrin (2.5 μg/ml) for 18-24 hrs before exposure to red light at a light exposure of 20 mW/cm^2 for 100 secs and 400 secs) or untreated cells (NDNL, no drug and no light). Cell lysates were harvested at the indicated times after PDT (1/2h and 12h after PDT). The activity of ERK1/2 was calculated as the ratio of the phosphorylated ERK1/2 to total ERK1/2 and is shown relative to the ERK1/2 activity in the untreated, no drug, and no light (NDNL) control cells at 1/2h. The result is from a single experiment. It is seen that Photofrin-mediated PDT at 2.5μg/ml of Photofrin resulted in decreased phosphorylation of ERK1/2 at 30 min after PDT, which further decreased after 12 hrs. There is also a decrease in phosphorylation of ERK1/2 in the untreated, no drug, and no light (NDNL) between 1/2h and 12h.

MRC5


NDNL
After 30 min (100 secs)
After 30 min (400 secs)
NDNL
After 12 h (100 secs)
Phos-ERK 1/2
Total-ERK 1/2

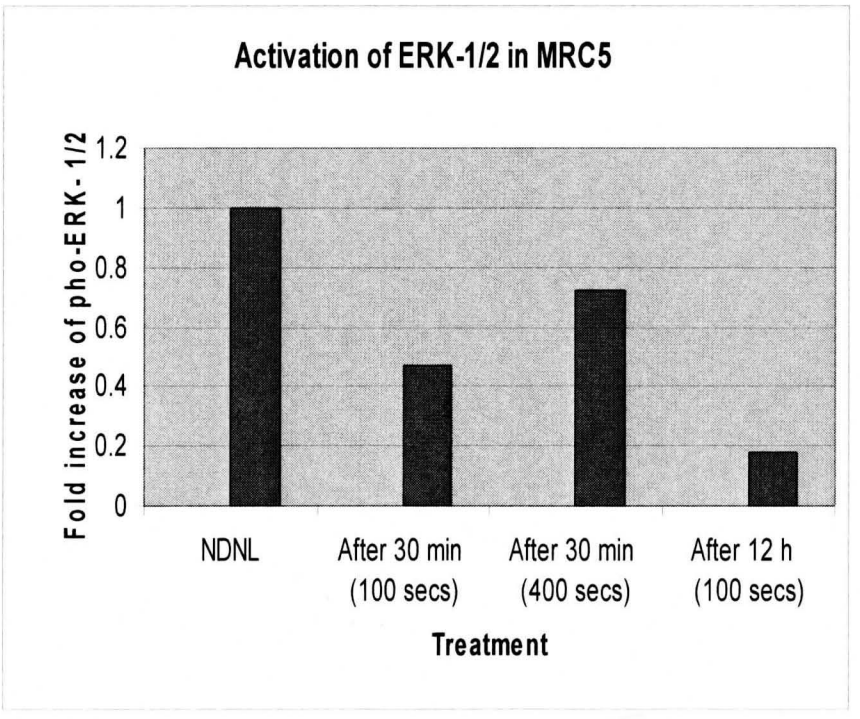
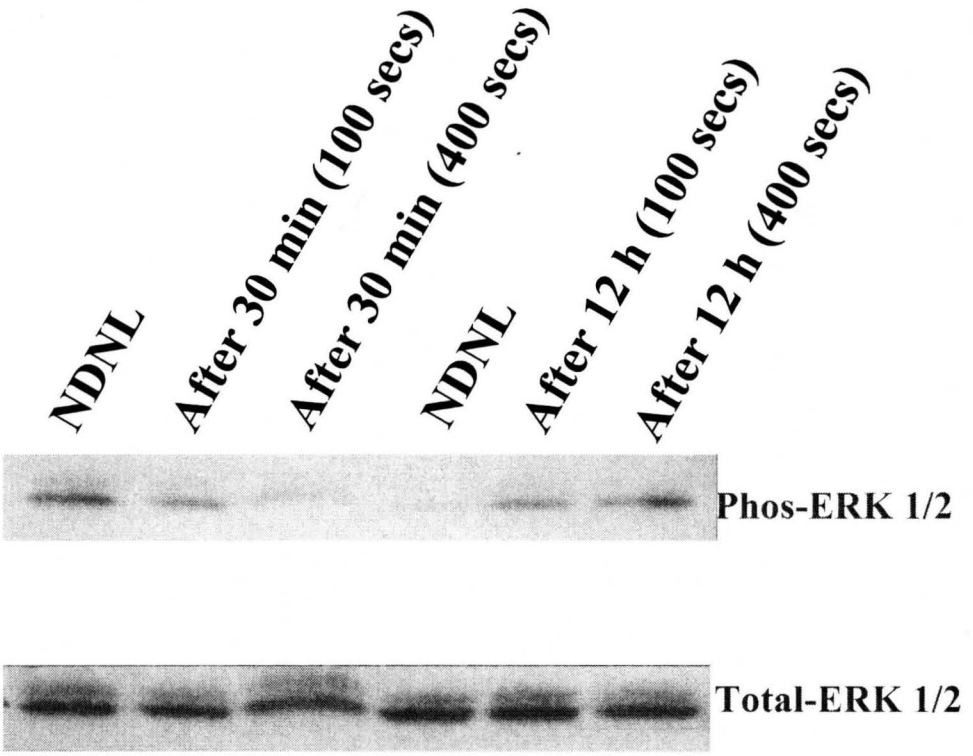

Activation of ERK-1/2 in MRC5
Fold increase of pho-ERK- 1/2
1.2
1
0.8
0.6
0.4
0.2
0
NDNL
After 30 min (100 secs)
After 30 min (400 secs)
After 12 h (100 secs)
Treatment

Figure 3.2. Activation of ERK1/2 in SKMES-1 cell line by Photofrin-mediated PDT

An autoradiograph of a Western Blot (top) from cells treated with Photofrin (2.5 μg/ml) for 18-24 hrs before exposure to red light at a light exposure of 20 mW/cm^2 for 100 secs and 400 secs) or untreated cells (NDNL, no drug and no light). Cell lysates were harvested at the indicated times after PDT (1/2h and 12h after PDT). The activity of ERK1/2 was calculated as the ratio of the phosphorylated ERK1/2 to total ERK1/2 and is shown relative to the ERK1/2 activity in the untreated, no drug, and no light (NDNL) control cells at 1/2h. The result is from a single experiment. It is seen that Photofrin-mediated PDT at 2.5μg/ml of Photofrin resulted in decreased phosphorylation of ERK1/2 at 30 min after PDT for 100 and 400 sec light doses. Phosphorylation of ERK1/2 in the PDT treated samples was similar (100 secs) or increased (400secs) after 12 hrs, whereas phosphorylation of ERK1/2 in the untreated, no drug, and no light (NDNL) was less than in the control 30 min sample.

SKMES-1


NDNL
After 30 min (100 secs)
After 30 min (400 secs)
NDNL
After 12 h (100 secs)
After 12 h (400 secs)
Phos-ERK 1/2
Total-ERK 1/2

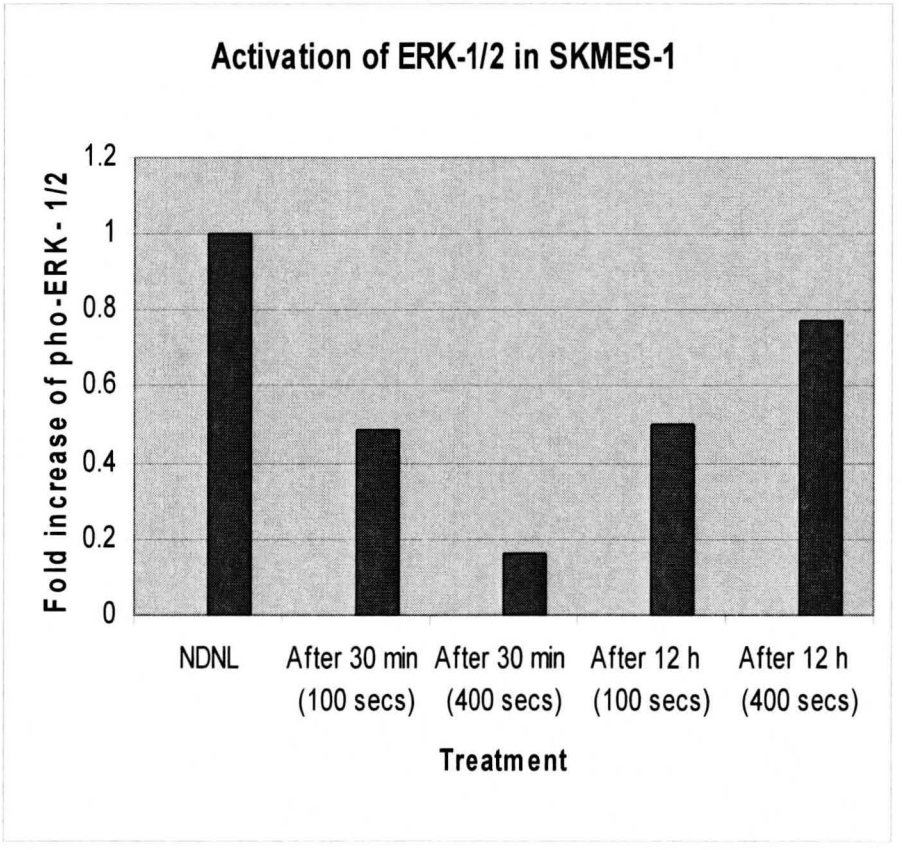
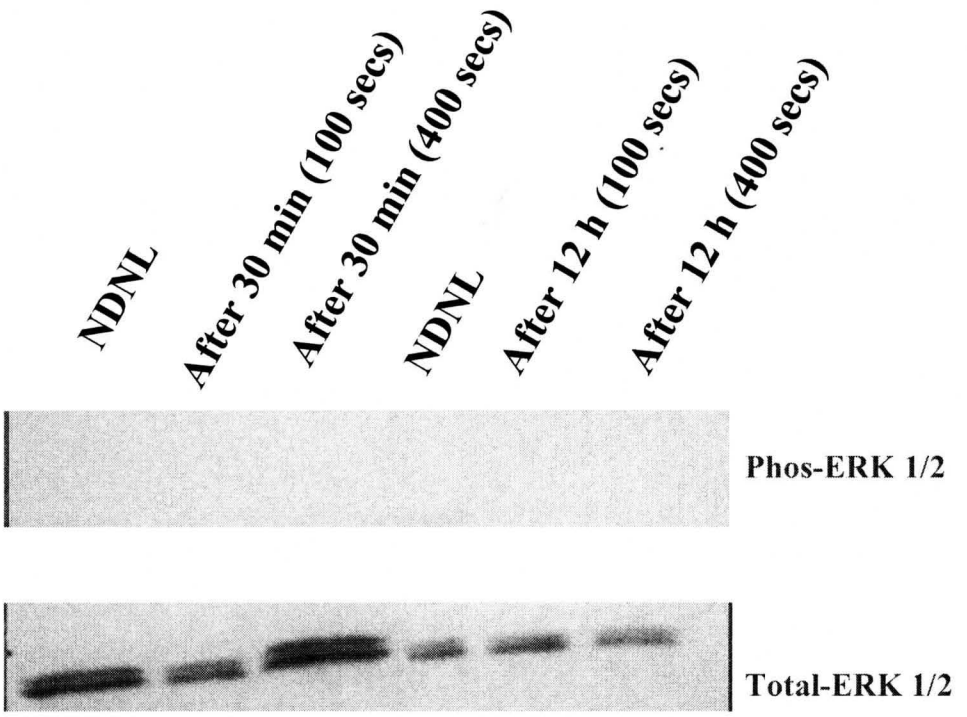

Activation of ERK-1/2 in SKMES-1
Fold increase of pho-ERK - 1/2
1.2
1
0.8
0.6
0.4
0.2
0
NDNL
After 30 min (100 secs)
After 30 min (400 secs)
After 12 h (100 secs)
After 12 h (400 secs)
Treatment

Figure 3.3. Response of ERK1/2 in A549 and NCIH460 by Photofrin-mediated PDT.

An autoradiograph of a Western Blot (top) from cells treated with Photofrin (2.5 μg/ml) for 18-24 hrs before exposure to red light at a light exposure of 20 mW/cm^2 for 100 secs and 400 secs) or untreated cells (NDNL, no drug and no light). Cell lysates were harvested at the indicated times after PDT (1/2h and 12h after PDT). The activity of ERK1/2 was calculated as the ratio of the phosphorylated ERK1/2 to total ERK1/2 and is shown relative to the ERK1/2 activity in the untreated, no drug, and no light (NDNL) control cells at 1/2h. The result is from a single experiment. It is seen that there was no phosphorylation of ERK1/2 in control cells or following Photofrin-mediated PDT at 2.5μg/ml of Photofrin at 30 min and 12 hrs after PDT in A549 and NCIH460 cells.

A549



NCIH460

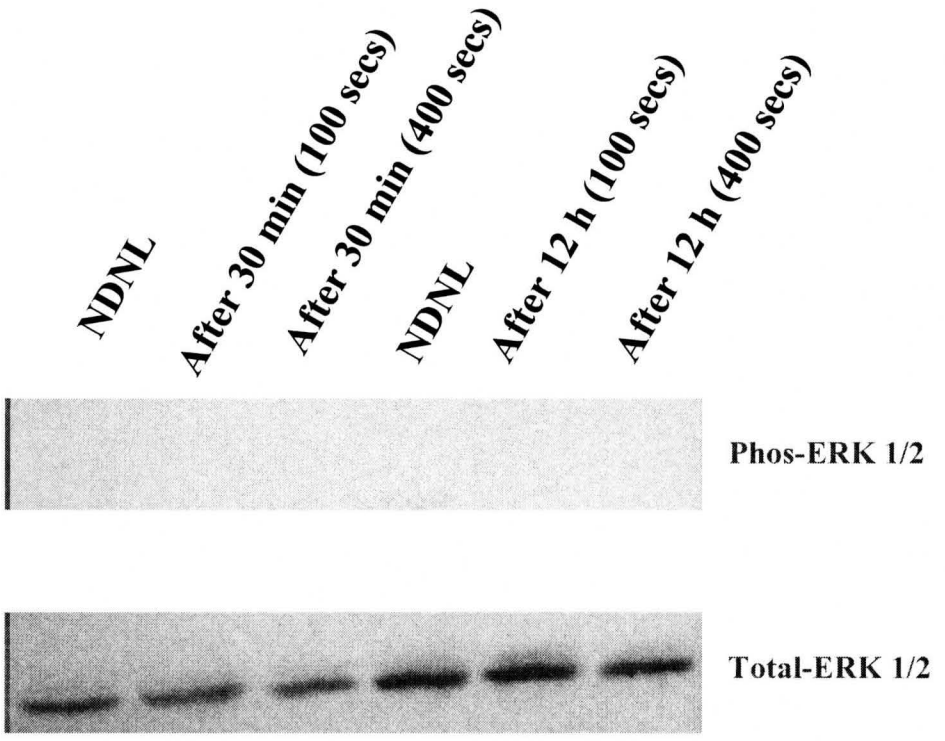
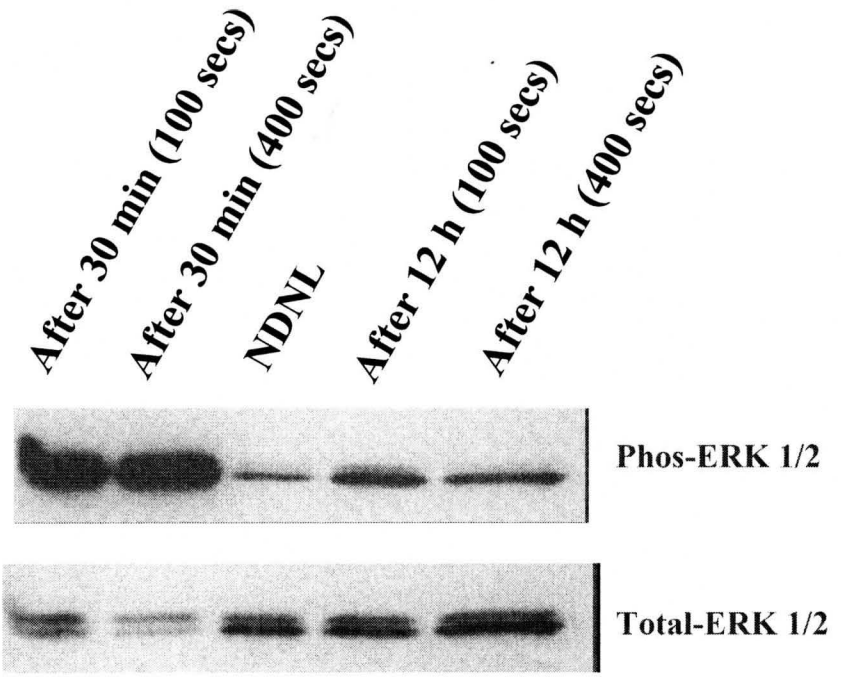
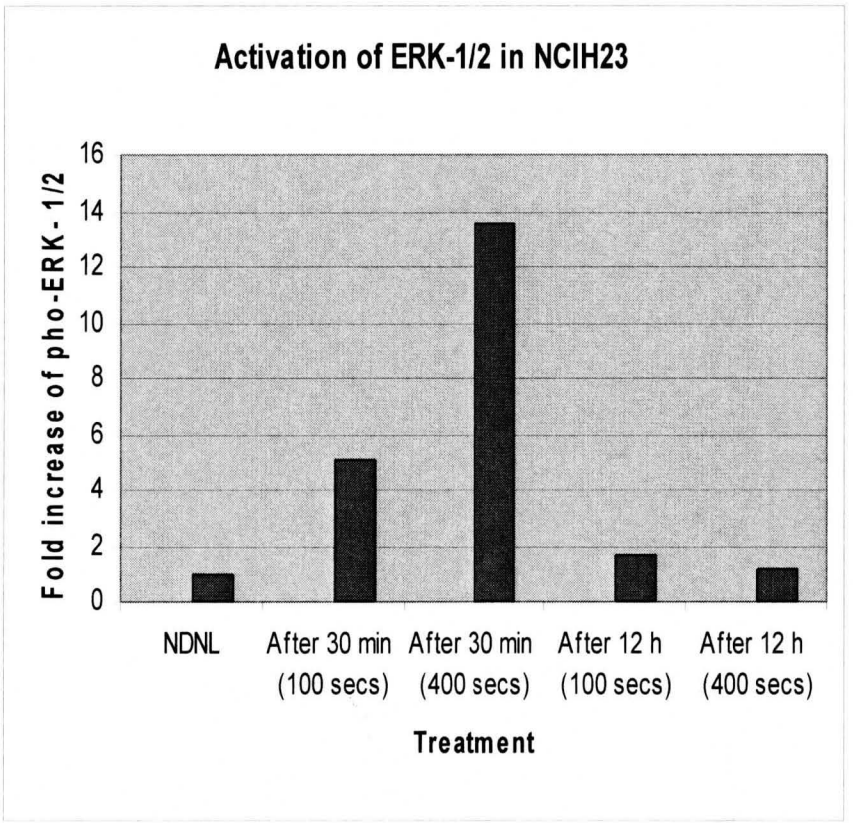


Figure 3.4. Activation of ERK1/2 in NCIH23 by Photofrin-mediated PDT.

An autoradiograph of a Western Blot (top) from cells treated with Photofrin (2.5 μg/ml) for 18-24 hrs before exposure to red light at a light exposure of 20 mW/cm^2 for 100 secs and 400 secs) or untreated cells (NDNL, no drug and no light). Cell lysates were harvested at the indicated times after PDT (1/2h and 12h after PDT). The activity of ERK1/2 was calculated as the ratio of the phosphorylated ERK1/2 to total ERK1/2 and is shown relative to the ERK1/2 activity in the untreated, no drug, and no light (NDNL) control cells at 12h (the result for control cells at 30 min was discarded due to experimental error during the loading of wells in the gel). The result is from single experiment. The results suggest that Photofrin-mediated PDT at 2.5μg/ml of Photofrin resulted in increased phosphorylation of ERK1/2 detectable at 30 min after PDT in the NCIH23 cells and the increased in phosphorylation of ERK1/2 was transient such that phosphorylation of ERK1/2 was detected at lower levels by 12 h after PDT.

NCIH23


After 30 min (100 secs)
After 30 min (400 secs)
NDNL
After 12 h (100 secs)
After 12 h (400 secs)
Phos-ERK 1/2
Total-ERK 1/2


Activation of ERK-1/2 in NCIH23
Fold increase of pho-ERK-1/2
0
2
4
6
8
10
12
14
16
NDNL
After 30 min (100 secs)
After 30 min (400 secs)
After 12 h (100 secs)
After 12 h (400 secs)
Treatment

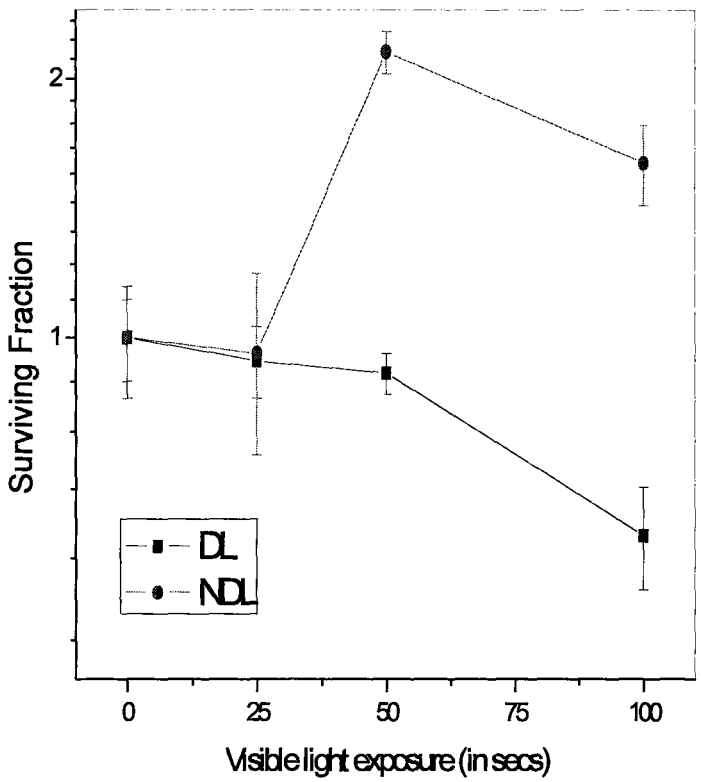


Figure-3.5 Effect of low light exposures on A549 cells in comparison to effect of (PDT). Effect of PDT on the survival of the A549 cell line when a Photofrin dose of 2.5 μg/ml and light exposures of 25, 50 and 100 secs at a power output of 20mJ/cm^2/sec were given to cells using a high cell density protocol. Results of the colony survival assay displays the effect of low light exposure (without Photofrin) (NDL●) on A549 cells in comparison to effect of (PDT) (Photofrin with various doses of light) on A549 (DL■).

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