THE ROLE OF STRESS PROTEINS IN CELLULAR RESISTANCE TO PHOTODYNAMIC THERAPY
THE ROLE OF STRESS PROTEINS IN CELLULAR RESISTANCE TO
PHOTODYNAMIC THERAPY IN BLADDER CANCER T24 CELLS
AND COLON CANCER HT29 CELLS

By:

JOHN G. HANLON, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University
© Copyright by John G. Hanlon, June 2001
MASTER OF SCIENCE (2001)  McMaster University
(Biology)  Hamilton, Ontario

TITLE: The Role of Stress Proteins in cellular resistance to Photodynamic Therapy in Bladder Cancer T24 Cells and Colon Cancer HT29 cells

AUTHOR: John G. Hanlon, B. Sc. (McMaster University)

SUPERVISOR: Professor G. Singh

Number of Pages: xiii, 164
ABSTRACT

As Photodynamic Therapy (PDT) becomes increasingly popular as a treatment modality for some solid tumours, the need for a better understanding of the mechanism(s) of action and resistance are paramount. To this end we have generated Photofrin® PDT-induced resistant variants to numerous cell lines including the colon cancer cell line HT29.

There is significant evidence indicating that stress proteins play an important role in determining the outcome of PDT on a cell. In this thesis the roles of the mitochondrial Heat Shock Protein 60 (Hsp60) as well as the endoplasmic Glucose Related Protein 78 (GRP78) were examined in the HT29 cells and their Photofrin induced resistant variant HT29-P14. The expression and role of these two stress proteins were also examined in T24 Bladder carcinoma cells and their GRP 78 stable-overexpressing clones.

Hsp60 protein was expressed at slightly higher basal levels in the resistant HT29-P14 cells relative to the parental HT29 cells. After incubation alone or PDT action, a temporal and dose dependent induction of Hsp60 was observed and this too was found to be significantly greater in the resistant cells. In the T24 model, no Hsp60 induction was observed following drug incubation or PDT.

GRP78 protein levels were increased by PDT action but not by Photofrin® incubation alone in all cell lines tested. In the T24 model, GRP78 transfection resulted in a stable 2-fold increase in protein levels and a 10-20-fold increase in cell survival after PDT at the highest dose tested. A temporal and dose dependent response was noted in all cells and induction of GRP78 protein was lower in the stable overexpresser such that all cell lines had similar post induction levels. In the HT29 and HT29-P14 resistant cells,
GRP78 protein levels were similar at basal level, and, both cell lines exhibited the same temporal and dose dependent increases in expression post PDT.

Finally, broad scale expression profiling using a “stress” microarray in the HT29 and HT29-P14 resistant variants revealed a very similar expression profile for the 168 of the 169 stress proteins tested with the exception of the small Heat Shock Protein 27 (Hsp27). As confirmed by northern and western blot analysis, Hsp27 is over 20 fold greater at the transcriptional level and 10-15 fold greater at the translational level in the HT29-P14 resistant variant.

These findings implicate Hsp27, Hsp60 and GRP78 as possible mediators of cellular sensitivity to Photofrin-mediated PDT. Specifically, Hsp27 appears to play a role in the increased resistance of our induced resistant HT29-P14 cells.
ACKNOWLEDGEMENTS

I would like to thank my Supervisor, Dr. G. Singh for this opportunity as well as his time, support and guidance throughout this enriching experience. I would also like to thank Dr. A. J. Rainbow and Dr. R. S. Gupta, the other members of my supervisory committee for their help and feedback. I also thank Dr. R. Austin for the T24 cell model and helpful discussions.

Moreover, I must thank our "lab-mommy" Myrna Espiritu, for teaching me many techniques and answering my endless questions. Also Sarka Lhotak for teaching me immunohistochemistry. Thanks also to everyone else on the fourth floor, for friendship support and fun times.

Finally a special thank you to Mum, Dad and Sarah Jane for their love and support from the beginning.
PREFACE

This thesis is presented in four chapters. Preceding these chapters is an introduction, which reviews the general concepts necessary for an understanding of the thesis. The first chapter is an original manuscript documenting induction of a mitochondrial heat shock protein (Hsp60) by PDT; it has been submitted for publication. The second chapter examines the effect of overexpression of a glucose related protein (GRP78) on PDT as well as the response of GRP78 to PDT in various cells and was written in preparation to be submitted for publication. In the third chapter, a cDNA microarray was used to perform broad scale stress protein expression profiling; this too has been written in preparation for publication. Finally, chapter four present's preliminary work documenting the absence of Hsp60 induction following PDT in T24 cells. It also documents the similar post PDT GRP78 induction and expression in HT29 and HT29-P14 cells following PDT. These chapters are followed by a general summary of the thesis and conclusions; this includes possible future directions for the project. The references for chapter one can be found following the discussion while the remaining references are listed at the end of the thesis. Finally, appendices contain additional pertinent data. Page numbers of the manuscript have been adjusted for continuity within the thesis.

All work presented in this thesis has been carried out by the author with two exceptions. Myrna Espiritu carried out clonogenic survival experiments for the work presented in chapter one and Kathryn Adams carried out the experiments and collected the Hsp60 data from the RIF cells that are also presented in chapter one.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><strong>Photodynamic Therapy</strong></td>
<td>2</td>
</tr>
<tr>
<td>1.1 History</td>
<td>3</td>
</tr>
<tr>
<td>1.2 Photosensitizers</td>
<td>4</td>
</tr>
<tr>
<td>1.3 Incubation Protocols</td>
<td>7</td>
</tr>
<tr>
<td>1.4 Light Sources</td>
<td>8</td>
</tr>
<tr>
<td>1.5 Oxygen</td>
<td>9</td>
</tr>
<tr>
<td>1.6 Mechanisms of Action</td>
<td>9</td>
</tr>
<tr>
<td>1.7 Cellular and Tumour Effects</td>
<td>11</td>
</tr>
<tr>
<td>1.8 Intrinsic PDT Sensitivity</td>
<td>16</td>
</tr>
<tr>
<td>1.9 Altering PDT Sensitivity</td>
<td>21</td>
</tr>
<tr>
<td><strong>Stress Proteins and the Stress Response</strong></td>
<td>25</td>
</tr>
<tr>
<td>2.1 The Heat Shock Proteins and the Stress Response</td>
<td>25</td>
</tr>
<tr>
<td>2.11 Hsp100-110</td>
<td>27</td>
</tr>
<tr>
<td>2.12 Hsp90</td>
<td>28</td>
</tr>
<tr>
<td>2.13 Hsp70</td>
<td>28</td>
</tr>
<tr>
<td>2.14 Hsp60</td>
<td>29</td>
</tr>
<tr>
<td>2.15 Hsp40</td>
<td>30</td>
</tr>
<tr>
<td>2.16 Hsp27</td>
<td>30</td>
</tr>
<tr>
<td>2.2 The Glucose Related Proteins</td>
<td>31</td>
</tr>
<tr>
<td>2.21 GRP 78</td>
<td>31</td>
</tr>
</tbody>
</table>
CHAPTER ONE
Induction of Hsp60 in Sensitive and Resistant HT29 Colon Cancer Cells by Photofrin Incubation and Photofrin Mediated Photodynamic Therapy

Abstract

Introduction

Materials and Methods

Results and Discussion

References

CHAPTER TWO
Overexpression of GRP78 Protein Causes Cellular Resistance to Photodynamic Therapy: An Examination of Stable Transfected T24 Cells and GRP78 levels in the HT29 Model

Abstract

Introduction

Materials and Methods

Results

Discussion

CHAPTER THREE
Photofrin-Induced Resistance to Photodynamic Therapy Leads to
LIST OF ABBREVIATIONS

ALA, 5-aminolevulinic acid
BSA, Bovine Serum Albumin
CHO, Chinese hamster ovary
Cpn, Chaperonin
DHE, Dihematoporphyrin ether
EOR, Endoplasmic Reticulum Overload Response
ER, Endoplasmic Reticulum
GAPDH, Glyceraldehyde 3-Phosphaste Dehydrogenase
GRP, Glucose Related Protein
HpD, Hematoporphyrin derivative
Hsp, Heat Shock Protein
Ig, Immunoglobulin
LDL, Low Density Lipoprotein
MDR, Multidrug resistance
PpIX, Protoporphyrin IX
P II, Photofrin
PDT, Photodynamic Therapy
RIF, Radiation induced fibroblast
sHsp, Small Heat Shock Protein
UPR, Unfolded Protein Response
$^{1}\text{O}_2$, Singlet Oxygen
$^{3}\text{O}_2$, Triplet Oxygen
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Introduction:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. Structure of Photofrin</td>
<td>5</td>
</tr>
<tr>
<td>Figure 2. Absorption spectrum of Photofrin</td>
<td>6</td>
</tr>
<tr>
<td>Figure 3. Mechanism of singlet oxygen generation by activated Photosensitizers</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter One:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. Hsp60 content of RIF-1 and RIF-8A cells 6 hours post (a) Photofrin incubation and (b) PDT action</td>
<td>55</td>
</tr>
<tr>
<td>Figure 2. Survival curves for HT29 and HT29-P14 cells post PDT</td>
<td>57</td>
</tr>
<tr>
<td>Figure 3. Hsp60 content of HT29 and HT29-P14 cells 6 hours post (a) Photofrin incubation and (b) PDT action</td>
<td>55</td>
</tr>
<tr>
<td>Figure 4. Hsp60 and Mitotracker® double stained HT29 and HT29-P14 cells</td>
<td>61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter Two:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. Survival curves for T24 cells and the A4 and pcDNA clones following PDT</td>
<td>77</td>
</tr>
<tr>
<td>Figure 2. Doubling times for T24 parental cells and their clones</td>
<td>79</td>
</tr>
<tr>
<td>Figure 3. Drug uptake for T24 parental cells and their clones</td>
<td>81</td>
</tr>
<tr>
<td>Figure 4. GRP78 levels in T24 parental cells and their clone at 16 hours Post PDT</td>
<td>83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter Three:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. cDNA “stress” microanalysis of HT29 and HT29-P14 cells</td>
<td>99</td>
</tr>
<tr>
<td>Figure 2. Northern blot analysis of Hsp27 in HT29 and HT29-P14 cells</td>
<td>101</td>
</tr>
<tr>
<td>Figure 3. Western blot analysis of Hsp27 in HT29 and HT29-P14 cells</td>
<td>103</td>
</tr>
</tbody>
</table>
Chapter Four

Figure 1. Western Blot analysis of Hsp60 in T24 cells and A4 clone post PDT 113

Figure 2. GRP78 expression in HT29 and HT29-P14 at basal and post PDT 120

Appendix

(CHAPTER 1)
Appendix 1. Survival curves for HT29 and HT29-P14 cells post Photofrin II incubation 145
Appendix 2. Doubling times for HT29 and HT29-P14 cells 147
Appendix 3. Drug uptake for HT29 and HT29-P14 149
Appendix 4. Western Blot time course experiment for HT29-P14 cells 151
Appendix 5. Western Blot dose response experiment for HT29-P14 cells 153

(CHAPTER 2)
Appendix 6. Initial survival curves for T24 cells and all clones post PDT 155
Appendix 7. Western Blot analysis of GRP78 basal expression in T24 cells, pcDNA and overexpressing A4 clone 157
Appendix 8. Western Blot time course experiment for T24 A4 clone 159
Appendix 9. Western Blot dose response experiment for T24 A4 clone 161

(CHAPTER 3)
Appendix 10 Additional Microarray data from HT29 and HT29-P14 cells 163
INTRODUCTION
The general objective of this thesis has been to examine the role of stress proteins in Photodynamic Therapy (PDT) and specifically study their role in resistance to this emerging cancer therapy. This introductory chapter provides background and insight into Photodynamic Therapy as well as stress proteins and the stress response. Although a complete understanding of the therapy and the associated cellular stress responses are still not available, the background information contained in the following pages highlights the need for such understanding and also makes clear the rationale for this thesis.

Photodynamic Therapy

Photodynamic therapy, commonly referred to as PDT is a treatment modality which uses a photosensitizing drug and light to kill cells. It has received increasing attention over the last decade as a result of the first approvals granted by the Canadian government who recognized its efficacy in a range of diseases including cancer (Dougherty et al., 1992). Clinical use of PDT requires three components to be present simultaneously; a photosensitizing agent, oxygen and light of a wavelength that matches the absorption spectrum of the photosensitizer. When this photosensitizer is activated, it interacts with molecular oxygen to form a toxic short-lived oxygen species know as singlet oxygen, which in turn mediates cell death (Weishaupt, 1976). While cell kill is easy to achieve by many treatments, the appeal of PDT in oncology stems form the fact that photosensitizers tend to be retained in tumour tissue for longer time periods than normal tissue and PDT is very effective at generating an immune response (Schunthaker, 1996).
The following pages provide insight into many of the important considerations for clinical PDT as well as basic science research concerning this young science. Specific attention is paid to the history of PDT, the photosensitizer Photofrin (PII), the impact of various incubation protocols, light sources and oxygen. This is followed by a discussion of the mechanisms of action, cellular effects and differences in inherent as well as induced resistance to PDT.

History

Photodynamic action was discovered a century ago when the scientist Marcacci claimed that the toxicity of quinine and cinchonamine to enzymes, plants and frog eggs was greater in the light than in the dark. Ten years later, Oscar Raab discovered that certain drugs killed Paramecium better with increasing sunlight. However, real interest in Photodynamic Therapy (PDT) did not occur until mid-century when it was reported that injected hematoporphyrin accumulated in tumours in rats and, upon illumination, led to necrosis of the tumour (Spikes, 1968). Modern PDT was initiated in the 1970s and was led by Dr. T.J. Dougherty, who recognized the potential of PDT for tumour treatment and who demonstrated that hematoporphyrin derivatives had efficacy for the treatment of human tumours metastatic to the skin. These hematoporphyrin derivatives (HpD) have served as the basis of modern PDT, their purification further increased the tendency to accumulate in tumour tissue and eventually lead to the development of the first commercially available photosensitizer, Photofrin® (Moan et al., 1979; Moore, 1997).

The first health agency approval for PDT (with Photofrin®) was obtained in 1993 in Canada for the prophylactic treatment of bladder cancer. Since then Photodynamic Therapy has been tested for the treatment of many other tumours. The most accessible
site for PDT is obviously the skin, and PDT has been used in primary basal and squamous cell carcinomas and many others conditions connected to the skin. In addition to Photofrin® (PII), which has numerous approved clinical uses, there are various other "second generation" photosensitizers in clinical trials for cancers of the bladder, brain, head, neck, ovary, lung and eye (Oleinick and Evans, 1998).

Photosensitizers

There are various properties of a photosensitizer used in PDT which are critical determinants of its success. The ideal photosensitizer will absorb in the red or near infrared regions as these are the only wavelengths that are not appreciably absorbed by the skin. It is also important that the photosensitizer does not absorb outside of that region because this will cause skin problems due to light and sun sensitivity. A second important criterion is that of localization; the larger the tumour-tissue to normal-tissue differential, the better the cure rates. Additionally, the sensitizer should be well photoactivated and capable of generating the necessary cytotoxic species. Pure compounds, or at least those with a defined chemical composition are also important for consistency in localization and effect while compounds that fluoresce have the added bonus of enabling the monitoring their localization and accumulation (Hsi et al., 1999; Dougherty et al., 1998)

The sole sensitizer used in the completion of this thesis was Photofrin® (PII). Photofrin is a commercially available partially purified hematoporphyrin derivative (HpD) and was supplied by Quadra Logic Technologies Inc. of British Colombia. It is a mixture of monomeric and oligomeric derivatives of hematoporphyrin units linked via
ether or ester bonds. Figure 1 is modified from Dougherty and Marcus (1992) and displays its chemical structure

\[ R = \text{HO-CH- or } -\text{CH=CH}_2 \text{ and } n = 0-7 \]

Figure 1: Structure of Photofrin II (Dougherty and Marcus, 1992)

Photofrin is a first generation photosensitizer; it was the first to receive full clinical approval and remains the most commonly used photosensitizer. However, there are several properties of PII which limit its use and have been the catalysts for the development of second-generation photosensitizers. First, Photofrin’s absorption of light is problematic. As can be seen below in Figure 2, PII absorbs in both the visible (500-700nm) and the UV (360-400nm) region of the spectrum. However it’s absorption at the higher wavelengths (>600nm) are relatively poor and it is these wavelengths in the red region of the spectrum which are most effective due to their ability to penetrate tissue (Gomer et al., 1989; Almond, 1992).
Additionally, Photofrin has a poor clearance rate causing patients to be highly photosensitive and requiring them to stay out of sunlight for up to six weeks (Bissonnette and Lui, 1997). Despite these problems, PII continues to be increasingly used in the treatment of cancer and its properties have been extensively studied. 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 PII has also been determined. Following short incubations, porphyrins have been shown to localize in plasma membranes, cytoplasm, nuclear membrane and nucleoli (Hisazumi et al., 1984; Kessel, 1986). Following longer incubations (>16 hours), intracellular fluorescence is pronounced and the binding is stronger in intracellular areas such as nuclear membrane, the mitochondria and other cellular organelles. (Hisazumi et al., 1984, Wilson and Singh, 1997). While not the exclusive site, mitochondria have been repeatedly implicated as the major subcellular site of porphyrin localization. (Singh et al., 1987; Roberts and Berns, 1989). This increased affinity has been suggested to be due to partitioning based on the
lipophilicity of the porphyrins (Afonso et al., 1999). Mitochondrial localization is desirable due to the increased probability of death by apoptosis using such sensitizers.

**Incubation Protocols**

As noted above intracellular localization changes over time, and the drug ratio in tumour versus normal tissue also changes over time. For these two reasons incubation protocols are very important. The change in intracellular localization has a large impact on effectiveness, as different sites in the cell will be damaged depending on the time of illumination. The specific localization and kinetics of intracellular distribution of Photofrin is depend on factors including hydrophobicity, the type and number of charges, the charge-to-mass ration, the number of ring and core substituents and whether entry into the cell occurs by diffusion (Afonso et al., 1999; Oleinick and Evans, 1998).

Of even greater importance than intracellular localization is tissue localization. The greater the tumour-to-normal cell ratio, the better the outcome and this parameter is paramount when deciding on incubation protocols. Although the mechanism of preferential photosensitizer localization is not fully understood, there are a number of factors which have been implicated. These factors include LDL receptor-mediated exocytosis which are often more abundant in cancerous cells, the increased hydorphobicity and decreased pH of cancer cells, and finally the leaky vasculature and poorly developed tumour lymphatics all contribute to the preferential uptake and retention in cancer cells (Moore et al., 1997; Hsi et al 1999).

As a result of the temporal changes in tissue and intracellular localization this aspect of dosimetry is very important. In clinical PDT the light source is often applied 48 hours after drug administration (Pass, 1993). In the petri dish, where only cancerous
cells are present this period of time may be reduced and photosensitization usually occurs 18-24 hours after drugging the cells.

**Light Sources**

As one of the three essential components for effective PDT, light dosimetry is highly important. The depth of penetration of the light through tissue is also an important consideration in the efficacy of PDT and therefore, differences between individuals and differences between organ/tumour type must be considered. While any light source that has sufficient power within the photoactivation region can be used, lasers are preferential for many reasons. Lasers can be set to emit at the desired longer wavelengths of visible light, which penetrate tissue more effectively than shorter ones. More importantly, a laser beam has three useful characteristics; monochromaticity, coherence and collimation. Finally, lasers and optical fibres also allow light delivery to deep-seated tumours (McCaughan, 1999; Hsi et al., 1999).

Light dose or fluence is expressed as joules over length or area (J/cm, J/cm²). While this allows for the standardization use of different light sources, it can be problematic because such dose determination is in terms of incident light fluence. Differences in light penetration in individual tumours are not accounted for in this calculation and in the future must be considered in order to get an accurate estimate of PDT dose (Wilson et al., 1989).

In the petri dish, many of these complexities are avoided, we therefore use a red-filtered light box for a defined time and were thereby able to administer a known and controlled light dose or fluence.
Oxygen

Sufficient oxygen levels are critical for successful PDT. It has been shown both in vivo and in vitro that there is little to no PDT action or tumour destruction in the absence of measurable oxygen (Henderson and Dougherty 1992). It has also been clearly shown that the rates of oxygen consumption and therefore the rate of singlet oxygen production can be affected by fluence rate and fractionation (McCaughan, 1999; Hsi 1999). 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 (Weishaupt et al., 1976). This $^1O_2$ has a short half-life of approximately 0.6μs in cells and a limited diffusion distance of about 0.1μm, as a result, oxygen must be present at the site of activation and will damage those structures close to it (Afonso et al., 1999).

In the petri dish, oxygen limitation is not a serious concern and no additional sources of oxygen beyond those naturally present in the laboratory are required (Khanum and Jain, 1989).

Mechanisms of Action

To date, the exact mechanisms of action of PDT remain unclear. However very strong evidence suggests that oxygen, light and the photosensitizing agent interact as depicted in Figure 3 (Weishaupt et al., 1976, Hsi 1999). The stable photosensitizer is first activated and excited by light of the appropriate wavelength at this higher energy level the photosensitizer interacts with molecular oxygen and thereby causes the formation of the highly unstable singlet oxygen ($^1O_2$). This above reaction is termed a
Type 2 reaction and the singlet oxygen will react with biomolecules thereby forming the cytotoxic oxy products (Levy, 1995).

Another reaction, known at the Type 1 reaction involves the direct reaction of the excited sensitizer with a biomolecule. This Type 1 photo-oxidation involves electron transfers and yields free radicals which in turn react with molecular oxygen to create the cytotoxic oxyproducts (Levy, 1995). Finally a third and non-detrimental fate for the activated photosensitizer is to fluoresce when returning to the ground state. This can also be useful as it provides an opportunity to determine its localization via fluorescence (Wilson et al., 1998)

![Absorption spectra for Photofrin II (DHE), monoaspartyl chlorin e6 (NPe6) and aluminum phthalocyanine sulfonate (APCS). Drugs were evaluated at a concentration of 200 μg/ml in MEM media containing 0.8% fetal calf serum.](image)

**Figure 2.** Absorption spectra for Photofrin II (DHE), monoaspartyl chlorin e6 (NPe6) and aluminum phthalocyanine sulfonate (APCS). Drugs were evaluated at a concentration of 200 μg/ml in MEM media containing 0.8% fetal calf serum.

**Figure 3:** Photosensitizer Activation and Formation of Cytotoxic products (Adapted from Hsi, 1999)
Cellular and Tumour Effects

Treatment of tumours with PDT can cause tumour ablation within a few days and there is evidence for three main mechanisms of destruction involved in this rapid response, which are discussed below. First, PDT may damage the malignant cells of the tumour directly. Second, PDT may produce profound changes in the tumour vasculature. Third, PDT causes the release of numerous mediators including cytokines that can produce an inflammatory response (as reviewed by Oleinick and Evans, 1998).

The direct cellular effects are a result of the singlet oxygen generated by photosensitizers that are usually incorporated into cellular membranes. Such damage can be noted by swelling, bleb formation, shedding of vesicles containing cytosolic enzymes and inhibition of membrane enzymes such as Na⁺, K⁺-ATPase (Henderson et al., 1994; Moan et al., 1979; Singh et al., 1987; Gibson et al., 1988; Moore et al., 1997). Additionally, functional impairment of isolated mitochondria (Salet and Moreno, 1981) and inhibition of mitochondrial enzymes have been demonstrated (Singh et al., 1987).

Other known direct cellular effects of PDT include a rise in Ca²⁺ (Joshi et al., 1994), lipid peroxidation which may lead to protein crosslinking (Thomas et al., 1989), up and down regulation of surface antigens (Davies, 1986) and damage to multidrug transporters (Kessel et al., 1995).

While there is evidence for numerous signal transduction pathways being activated by PDT, it appears that there are many cell line, photosensitizer and incubation time specific considerations (Moore et al., 1997). That notwithstanding, it is apparent that PDT causes the release of many inorganic and lipid secondary messengers as well as the release of calcium ions from their intracellular stores. Additionally, several protein
kinase signaling cascades are activated which can lead to apoptosis (stress kinases) or can help with cell survival (NF-κB). (Olenick and Evans 1998).

Since mitochondria are important cellular targets of PDT, and have been repeatedly implicated at the primary site of porphyrin-mediated PDT induced damage (Moan et al., 1982; Salet and Moreno, 1990) an examination of the direct cellular effects in the mitochondria is essential. Both fluorescence microscopy and cell fractionation studies have identified mitochondria as the major site of porphyrin accumulation (Berns et al., 1982). This has been shown in both in vivo and in in vitro studies (Hisazumi et al., 1984). Electron microscopy has revealed that mitochondria damage occurs rapidly following PDT and that loss of mitochondria membrane integrity occurs before loss of plasma membrane integrity (Singh et al., 1987). There are many components of the mitochondria that are known and/or suspected to be sensitive to PDT. Inner mitochondrial membrane enzymes such as complex I and IV of the Electron Transport Chain (ETC) are known to be highly sensitive. Moreover, this mitochondrial damage impairs oxidative phosphorylation enzymes including cytochrome c oxidase, ATPase and succinate dehydrogenase (Salet and Moreno, 1990; Hilf et al., 1984; Hilf et al., 1986). There is some evidence suggesting that the primary target for the uncoupling of oxidative phosphorylation following PDT is through the ADP/ATP translocator (Atlante et al., 1989) and a decrease in ATP has been noted post PDT which parallels the decrease in cell viability (Perlin et al., 1985). Finally experiments using oligomycin and or iodoacetate have lead to the proposal that oxidative phosphorylation is an important target of porphyrin-mediated PDT and that the disruption of oxidative phosphorylation is
Cellular apoptosis is an obvious endpoint of such direct PDT effects and PDT has been shown to cause apoptosis in addition to necrosis (Kessel et al 1998). It is strongly believed that PDT damage resulting in the release of cytochrome c and other mitochondrial factors into the cytoplasm can initiate this apoptotic response (Liu et al, 1996). Such an effect would primarily be seen using sensitizers such as PII, which preferentially localize to the mitochondria.

There is also evidence for a small role of DNA damage in the expression of in vitro PDT lethality for most photosensitizers. Following PDT, inhibition of DNA and RNA polymerases (Curte et al 1986) as well inhibition of the synthesis of DNA, RNA and protein have been demonstrated (Davies et al 1988). While double- and single-strand breaks as well as various chromosome aberrations have been noted (Gomer et al 1983, Penning et al 1994) these effects do not seem to be lethal and recovery has been shown to occur.

Two conclusions may be made about the direct effects of PDT. First, some of these effects are contingent on the incubation protocol, the photosensitizer and the cell line studied. Second and more importantly, it appears unlikely that there is a single universal mechanism for the response of cells to PDT. Instead, numerous reactions for death and survival appear to occur simultaneously and it is only the net result of these competing pathways that determine cellular fate.

A second major action of PDT has been shown to occur on vasculature. PDT is known to cause large changes in the conformation and permeability of vessels and water
balance within a few hours. Vasoconstrictions have been noted in both tumour and normal tissue as a result of PDT. These changes have been attributed to both thrombosis (Foster et al., 1991) as well as genuine vasoconstrictions (Fingar et al., 1993). The overall effects of these vasculature events are an increase in tumour fluid pressure, the release of vasoactive molecules including: prostaglandins, leukotrienes and tromboxanes (Stern et al, 1992) and persistent post-PDT tumor hypoxia/anoxia (Moore et al., 1997). Again, it must be noted that the vasculature effects of PDT are contingent on the photosensitizer used. Our photosensitizer, Photofrin causes vessel constriction, macromolecular vessel leakage, leukocyte adhesion and thrombosis formation, all of which appear to be linked to platelet activation and release of thromboxane (Fingar et al 1997).

The third major mechanism of PDT action is via the release of numerous mediators that can cause an immune response. These events include antitumour activity of inflammatory cells and a tumour sensitized immune reaction; the effects of which can be both long- and short-term (Moore et al 1997). Photo-oxidate lesions of membrane lipids are followed by a rapid activation of membranous phospholipases; this then leads to release of lipid fragments and metabolites of arachidonic acid, which are powerful inflammatory mediators (Korbelik, 1996). Additionally, due to their role in cell adhesion and antigen presentation, some PDT-induced stress proteins can also help start this inflammatory/immune response (Ochsner et al., 1997).

The inflammatory and immune responses start by a rapid accumulation and attachment of circulating neutrophils and platelets, leading to a progressive impairment of vascular function which is accompanied by the release of inflammatory mediators. These
mediators include vasoactive substances, components of compliment and clotting
cascades, acute phase proteins, peroxidases, radicals, leukocyte chemoattractants,
cytokines and immunoregulators (Ochsner, 1997; Fingar, 1996). While these specific
and nonspecific immune effector cells have been shown to have a impact on PDT
mediated destruction of cancerous tissue, there are large inconsistencies in the data and
more work is required to fully understand the phenomena (reviewed by Dougherty et al.,
1998). It should also be noted that cancer immunity elicited by PDT is possible and has
attributes of an “inflammation primed immune development process” (Korbelik, 1996).
Two other important immune-related findings are that PDT can induce immunity, even
against less immunogenic tumours and secondly, the generation of immune memory cells
sensitized to PDT treated tumour (Korbelik et al. 1996). These inflammatory/immune
characteristics of PDT suggest that PDT could potentially be successfully combined with
various immunotherapy protocols for long term tumour control.

It is clear that, in addition to the direct cellular effects, there are various other
mechanisms of action. Again, the contributions of each of these mechanisms are
dependent on both the photosensitizer and the tumour. Since there are numerous
photosensitizers available and many of these sensitizers work by different modes of
action, much more work is required in characterizing these photosensitizers.
Accordingly, different tumors will have unique cellular environments, which depend on
tissue and even the individual treated. This variable aspect of PDT also requires much
more study in order to understand the source of these differences and how to control or
quantify them in order to ensure optimal dosimetry and effectiveness for this cancer
therapy.
Intrinsic PDT Sensitivity

Differences in PDT sensitivity are well accepted and continue to limit the usefulness of PDT as a clinical procedure (Schuitmaker, 1996). This lack of understanding stems from two main factors. First, the entire field of PDT in oncology is a relatively young science and second, the laboratories studying this phenomenon are still using very different techniques, cells and sensitizers. This causes confusion and difficulty when comparing the findings of different groups or even different protocols of the same group, therefore a brief examination of the limitations and problems is warranted. Undoubtedly the most controlled and measurable environment is the petri dish, even though the desired endpoints are the \textit{in vivo} measures. None the less, even in the petri dish there are many operational definitions which must be established, for example, if cell A and B take up different amounts of the photosensitizing drug, is it possible to compare the sensitivity between cell lines? Do you compare the concentrations of drug used or the cellular drug levels at time of irradiation? Accordingly are such factors as drug uptake a measure of sensitivity? Additionally, certain cells are generally more prone to apoptosis or necrosis and this must also be considered when choosing experimental protocols and evaluating cellular responses. While there has recently been a great deal of interest, time, and effort comparing the sensitivities and mechanisms of PDT induced damage in cell lines, progress remains slow. Perhaps the biggest confounding variable is that fact that laboratories use different amount of drug, different drug exposure times, different fluence rates and wavelengths which all make direct comparisons difficult and controversial (as reviewed by Moore et al, 1997).
The condition of the cells being treated may also influence cell survival. Some studies have shown that rapidly proliferating vascular endothelial cells are more sensitive to PDT in vitro than those proliferating slowly. It is further proposed that this finding may be one of the reasons behind the greater damage/sensitivity of tumours versus normal tissue (Gomer et al., 1996). Such results and theories make measures of cellular doubling time an important consideration.

Regardless of the limitations and problems listed, knowledge of different sensitivities is of interest mechanistically and also of great clinical relevance. Differences in sensitivities of PDT in vitro, of cell types have been the subject of a number of papers. In a comprehensive review by Moore and others (1997), they highlight the variance in findings of different groups and differences between cell lines. The group review papers in which malignant cells have been reported to have i) higher levels of porphyrins and show greater sensitivity to PDT than normal cells. ii) similar uptake/sensitivity, or iii) lower sensitivity. Such findings highlight the vast differences in sensitivity that can be found towards PDT. In studies that have compared vascular endothelia with other cells, endothelium was very commonly the most sensitive cell type, whether compared to normal tissue or tumour cells. Although there are many theories, the reasons behind these variances in sensitivity are not clear and further investigations are still required (Moore et al., 1997). One variable is photosensitizer uptake into the cells and for Photofrin, the cellular sensitivities are often in accordance with the amount of drug per cell. It has also been claimed that Photofrin® containing compounds such as hematoporphyrin derivatives (HpD), which circulates around the body in association with lipoproteins and albumin might be expected to be taken up to a greater extent by cells
which have a large number of LDL receptors. Endothelia (especially proliferating endothelia as in tumours) fulfill the latter criteria of LDL receptors. If this pathway is indeed proven to be important for uptake, this will provide a means of improving effectiveness of PDT (Gomer et al., 1996). That notwithstanding, it must be acknowledged that total photosensitizer concentration per cell is in no way the sole parameter in determining relative sensitivity.

*In vivo* determination of relative cellular sensitivities are even more complicated than *in vitro*, as there are many other factors which can confound the PDT action. It has been shown that the endothelium of normal and tumour tissue is quickly and effectively damaged by a variety of PDT treatments. This promptness of effect has been assumed and used as an indicator of high sensitivity, it is also believed that sometimes the death of tumour cells is the result of this rapid vascular damage and not direct cytotoxicity (Henderson et al., 1985; Nelson et al., 1987). However, this theory has been challenged by various groups who have shown that vascular damage alone is not enough to produce optimal cures (McMahon et al., 1994), accordingly, our group has shown that Photofrin-induced resistance to PDT *in vitro* is maintained *in vivo* (Adams et al., 1999). One finding that is agreed upon by the scientific community is that drug concentration and the time of illumination always influence 'sensitivity' (Moore et al., 1997). The importance and consequences of these two factors were highlighted by Reed et al. (1989), who found that by changing the time of DHE administration, the number of patients who suffered severe bladder contracture went from 0% to 80%. It could be argued that these findings are reflective of changes in sensitivity and if the two experiments were non run concurrently, one would not be able to recognize that there was a timing issue. The
second instance would be recorded as cells or patients who have extremely high sensitivity, when that was not the case. Such changes in tissue response must be taken seriously and the timing variable must be understood as an important factor in determining sensitivity and treatment outcome.

Perry et al (1990) undertook an in-depth study of the sensitivity of different human lung cancer histologies to PDT. They compared the relative sensitivities of six established lung cancer cell lines and one normal fibroblast cell line using the clonogenic assay. They were able to establish dose-response curves for varying total energies. The finding showed that, while none of the cell lines were sensitive to the sensitizer alone, or light alone there were significant inherent differences in PDT sensitivities as evaluated by survival curve parameters n, Do, and light dose required to yield 1% survival. While they found no correlation between sensitizer uptake and inherent sensitivity, they noted that a general association existed between PDT sensitivity and the plating efficiency of the cell line (Perry et al, 1990). Such studies illustrate the pronounced differences inherent to PDT in in vitro systems.

A recent study focused on the cell cycle phase and how it influences tumour cell sensitivity to aminolaevulinic acid (ALA)-induced PDT in vitro. ALA, is the prodrug of the photosensitizer protoporphyrin IX (PpIX) and this type of PDT depends on the rate of cellular synthesis of the photosensitizer. In synchronized cells, after 1 hour of ALA incubation, cells in G1 produced less PpIX than those in S-phase or G2, and were significantly less sensitive to ALA-induced PDT. Findings such as these are evidence of how delicate and prone to change the response can be. This differential response in
tumour cells may have implications for clinical PDT, and may be the cause of treatment resistance or failure in complete tumour cure (Wyld, 1998).

Within the last few years, some studies have examined the effect of PDT in cells after abrogation of p53 function. It was found through cell cycle analysis that abrogation of p53 had minimal effects on an observed PDT-induced G1 block. The overall conclusion of this study was that p53 expression did not directly modulate tumour cell sensitivity to PDT in either apoptosis-responsive or non-responsive cells (Fisher et al, 1999). Conversely, we have examined the clonogenic survival of normal human fibroblasts that express wild-type p53 and immortalized Li-Fraumeni syndrome (LFS) cells that express only mutant p53, following Photofrin-mediated PDT and shown a role of the p53 tumor suppressor in the response to PDT (Tong et al., 2000).

Boehncke and others performed a comparison of PDT sensitivity in cutaneous resident and infiltrating cell types in vitro. They analyzed the sensitivity of cell lines established from resident cutaneous cells and transformed lymphocytes towards PDT, using a number of photosensitizers. Their findings showed consistency both in vitro and in vivo, with the general conclusion that the photosensitizer applied topically on the lesion of a patient with mycosis fungoides was found to accumulate preferentially in the lymphocytic infiltrate. From this, they deduced that such treatment might be useful for the treatment of cutaneous lymphomas (Boehncke et al, 1996). More importantly, this highlights differences between resident and infiltrating cell types.

As the data demonstrates, our current understanding of intrinsic cellular sensitivity to PDT is at best, fragmented. Confounding this are the diverse cells, methodologies and sensitizers used by various laboratories. It will be only through
cooperation and the power of our new molecular biological techniques that a clear and integrated picture of the determinants of cellular sensitivity will emerge and only then will clinicians be able to fully exploit the potential of this cancer therapy.

**Altering PDT Sensitivity**

A fruitful approach to the study of mechanisms of action by many antineoplastic drugs and other physical agents has been the creation of resistant cell lines (Morrow and Cowan, 1988). Comparative studies using PDT-sensitive and resistant cells derived from survivors of PDT treatment have been important in studying sensitivity and elucidating critical targets for PDT killing *in vitro*. Moreover, comparisons of the photosensitivity of tumours *in vivo*, grown from cells with different PDT sensitivities *in vitro* can also be helpful as it allows differentiation between direct and indirect tumour killing *in vivo* (Adams, 1997). The reason is that indirect effects would be expected to operate independently of intrinsic tumour cell sensitivity (Singh et al, 1991). This approach has been used primarily by two research groups; our group here at McMaster University, specifically at the Hamilton Regional Cancer Centre and the Gomer laboratory in Los Angeles. Both groups initially used the cell line RIF-1 which is a mouse derived radiation induced fibroblast cell line and from this, developed PDT resistant cell lines, namely RIF-8A by the Singh group (Singh et al., 1991), and various resistant clones by the Gomer group (Luna and Gomer, 1991). The levels of induced resistance were 1.8 and 1.5 fold respectively as determined by colony forming assay. RIF models were selected because of their ability to grow both in vivo and in vitro. In addition it has been well-characterized in PDT studies with Photofrin (Sharkey et al, 1992).
The findings of both groups have shown that some of the possible mechanisms responsible for the decreased sensitivity in these newly generated cell lines include altered drug uptake, or intercellular distribution, increase levels of scavenger molecules and enhanced repair activity. The efforts in elucidating the factors in these differences, have been intense, and through comparative studies of the RIF-1 and RIF-8A a number of interesting changes have been documented. RIF-8A has a significantly increased content of ATP per cell. Moreover it also has higher succinate dehydrogenase levels per cell (Sharkey et al, 1992). These findings along with others have generated a number of interesting hypotheses. The difference in ATP production between the two cells is likely through an induction of oxidative phosphorylation pathways. This theory is supported by the morphologic observation of an increase in mitochondrial cristae in RIF-8A, as compared to RIF-1. In addition, the finding that the ultrastructural appearance of the mitochondria can be correlated with their metabolic state, also agrees with this theory. Specifically condensed mitochondria may be enhanced in oxidative phosphorylation, however, the relationship if any, between these morphological changes and PDT resistance remains to be conclusively established (Sharkey et al, 1992). Another interesting finding in this murine model has been that the Heat Shock protein (Hsp 60) which is also found in the mitochondria is upregulated in a dose dependent manner by incubation with Photofrin® and then is phosphorylated after activation by light. The finding that the upregulation is greater in the resistant line suggests that this protein refolding pathway may be an important factor in determining cell sensitivity (Adams, 1997).
Gomer's group used mRNA differential display in an attempt to identify unique transcripts. They identified decreased expression and function of α-2 macroglobulin receptor/low density lipoprotein receptor-related protein in their Photodynamic Therapy resistant mouse tumor cells. The binding and endocytosis of activated α-2 macroglobulin and lipoproteins by α-2 MR/LRP are consistent with modulating uptake and localization of photosensitizers (Luna et al., 1995).

More recently, our group has used multiple human tumour cell and multiple photosensitizers in a comprehensive study of induced resistance to PDT in cancer cells in vitro (Singh et al., 2001). The specific aims of this current project were (a) to generate resistant variants and (b) elucidate the mechanism(s) by which PDT resistance is induced by the three photosensitizers in each of three human tumour cell lines. The three tumour cell lines selected were human neuroblastoma (SK-N-MC), human colon adenocarcinoma (HT29) and human bladder carcinoma (HT1376). These cell lines were chosen because induced resistance in human tumour cell lines is of clinical relevance and each tissue type is suitable for PDT. Multiple cell lines were used because there is a large body of information indicating that there are significant differences in inherent sensitivity for different organs, tissues, and individuals. Moreover, this same review highlighted recently published data suggesting that how individual cells die in vitro also varies with cell type (Moore et al., 1997). By comparing these three cell lines in both their inherent sensitivity as well as ability to become resistant, it is hoped that general principles may be extracted concerning mechanisms and degrees of possible induced resistance.

Use of multiple photosensitizers, combined with multiple cell lines, should allow for identification of cell line specific or sensitizer specific changes involved in resistance.
Selection of AlPcS₄, Nile Blue A and Photofrin® as the three photosensitizers was based on the localization properties for each of the different photosensitizers. Since the intracellular localization sites are unique, consequently the target(s) for direct tumour phototoxicity were also expected to be unique for each photosensitizer. As such, generation of resistant variants using these distinct photosensitizers has generated useful tools to determine of the mechanisms involved in conferring induced-resistance to each sensitizer. Moreover, it has facilitated cross-resistance studies and may be useful in identifying more general principles and cellular changes that may be involved in conferring resistance to multiple sensitizers. The generation of these resistant variants now serves as a useful model to understand the molecular mechanism(s) of sensitivity to various photosensitizers based on inherent and induced resistance in various cell lines.

This above cited work represents the first comprehensive study in generation of various human resistant variants to three unique photosensitizers. These attempts to induce resistance were partially successful with varying degrees of resistance being observed. In the HT29 cell and their variants, which are most relevant to this thesis, it was found that the induced resistance was not the result of changes in uptake of the drug. These three induced variants of the HT29 cell line were the most extensively studied as they represent the only cell line from which resistant variants could be generated to all three photosensitizers. Their ratios of increased survival at the LD₉₀ level range between 1.5 and 2.62 fold more resistant. The HT29-P14 resistant variant was found to be the least resistant with decreased sensitivity of approximately 1.5-1.8 fold. As mentioned previously, these variants from the HT29 cell line were examined for cross-resistance using the three photosensitizers. Only HT29-P14 (Photofrin derived) cell line was found
to be resistant to all three photosensitizers, conversely the variants made resistant to Nile Blue A and AlPcS₄ showed no cross-resistance. Additionally all HT29 derived cells were characterized by morphological and biochemical properties and subtle differences in morphological features of the cells were observed.

Stress Proteins and the Stress Response

The general objective of this thesis was to study the role of stress proteins in PDT and specifically their role, if any, in modulating PDT sensitivity. This following section provides background into two important and related families of stress proteins, the Heat shock proteins (Hsp) and the Glucose Related Proteins (GRP).

In all cells there are both ongoing and acute problems of protein aggregation. These problems exist because of the extremely high intracellular protein concentrations both in the cytoplasm and in most cellular compartments. Estimates of this concentration range from 100-150 mg/ml. Of even greater concern is the continuous presence of hydrophobic interactions, and with increased temperatures this problem becomes even more pronounced. At higher temperatures still, massive protein aggregation occurs, both of newly synthesized and pre-existing folded proteins. In order to deal with these closely related problems of high protein concentration and potential aggregation a set of universally conserved proteins exists. These proteins are called the molecular chaperones and their general role is to minimize protein aggregation, while ensuring proper protein folding and transport (Ellis and van der View, 1991; Georopoulos and Welch, 1993; Sarto et al., 2000).
While now often referred to as molecular chaperones, this phenomena and response was initially referred to as the heat shock response because it was first characterized in the presence of heat, by extremely rapid increase of a select group of proteins, the so-called heat shock proteins (Hsp's). As our understanding increased we learned that in addition to heat shock, these proteins are increased when cells are exposed to a number of other metabolic insults. These include but are not limited to: amino acid analogs, heavy metals, ionophores, glucose analogues, microbial infections, ischemia, nitric oxide, hormones, metabolic poisons, antibiotics, infections and cancer (as reviewed by Sarto et al., 2000). As a consequence of this increased understanding about these so called Hsp's, the response is now often referred to more generally as the stress response, and those proteins whose expression increases have now become known as the stress proteins (Welch, 1992). It must however be noted that not all molecular chaperones belong to the heat shock family of proteins, conversely, not all heat shock proteins function as molecular chaperones (as reviewed by Morimoto et al., 1994).

Many of the stress proteins or Hsp's and molecular chaperones are parts of large gene families with often related but distinct functional homologues in each cellular compartment such as the cytosol, nucleus and in organelles including the mitochondria and endoplasmic reticulum. These proteins are present in all living cells and are highly conserved in evolution. Some studies of human and bacterial stress proteins show a sequence similarity of above 50% (Hartl, 1996), and additionally the evolution of these chaperones, especially the chaperonin’s have been extensively studied (Gupta, 1995). While such studies have revealed both subtle and major differences between these heat shock proteins, the enormous effort in elucidating their roles and mechanisms of action
do facilitate a basic and generalizable description of the group as a whole. Most of these stress proteins function as molecular chaperones and by binding to partially denatured proteins as well as dissociating proteins aggregates and regulating correct folding, they are able to protect cells from various environmental stresses. There is significant evidence that the induction of these heat shock proteins resulting from a sublethal stress then protect these cells from a subsequent stress that otherwise may have been lethal (Lindquist, 1986; Schlesinger, 1994). Additionally they can be thought of as a “quality control and triage system” (Sarto et al, 2000) where they cooperated in transporting newly synthesized polypeptides to target organelles for final packaging, repair or degradation. Finally, many are inducible under many physiological conditions such as cell cycle, division, differentiation, tissue development, hormonal stimulation, growth factor activity and apoptosis (Jaattela, 2000).

The heat shock proteins can be grouped according to their molecular masses and are generally classified as belonging to one of six subfamilies. These include the large Hsps of 100-110kDa, the Hsp 90 family, the Hsp 70 family the Hsp60 family, the Hsp40 family and the small heat shock protein (sHsp) family of 18-30kDa (Jaattela, 1999; Lindquist and Craig, 1988). It must be noted that various other classification systems exist and hence the families may be described differently (Sarto et al., 2000).

**Hsp100-110**

These large Hsp’s are highly conserved and found in a vide variety of organisms. They function in stress tolerance and are found in the cytoplasm, nucleus and nucleolus. After
heat shock, levels increase and it is found primarily in the nucleolus region where ribosomal RNA transcription occurs (Oh et al., 1997).

**Hsp 90**

The Hsp90 family forms oligomers under stress conditions. It is believed to form stable complexes with actin when ATP levels fall, it is also involved in proteasome action and binding with actin may mediate the structural organization of the proteasome. Additionally it is known to act as a chaperone and often found in association with certain protein kinases (Morimoto et al. 1994).

**Hsp70**

The Hsp70 family is the best studied class of Hsp’s, it is also highly conserved with many family members. These members include both constitutive and highly stress inducible proteins including a mitochondrial Hsp75 protein (mtHsp75) and a Glucose Related Protein (GRP) known as GRP78 or BiP which is localized in the endoplasmic Reticulum. (Tavaria et al., 1996). This glucose related protein (GRP78) is examined in this study and will be discussed in depth later in the context of glucose related proteins.

**Hsp60**

The Hsp60 family of molecular chaperones are highly conserved and are termed chaperonins (Cpn60). They occur in both prokaryotes and eukaryotes; other members include it’s homologue in E. coli and the protein GroEL, the ribulose 1,5-bisphosphate carboxylase-oxygenase (rubisco) subunit binding protein in chloroplasts of higher plants.
The TCP-1 system, which operates in the cytosol of eukaryotic cells and replaces GroEl in archaebacteria is also related (Gupta, 1995; Frydman and Hartl, 1994).

The primary role of Hsp60 is in the folding and assembly of many mitochondrial proteins (Cheng et al 1989). Various localization studies have shown Hsp60 to be primarily located in the mitochondria where it is a major component. More recent studies have also demonstrated that it can be found at discrete sites outside of the mitochondria, including the plasma membrane, endoplasmic reticulum and peroxisomes (Soltys and Gupta, 1996). Hsp60 is encoded by an essential nuclear gene and it is then transported to the mitochondrial matrix by a targeting pre-sequence located in the N-terminus of the protein (Singh et al., 1990). It’s transcription has been shown to increase two- to threefold upon temperature shift to 39°C at which point it can represent up to 0.3% of total cellular protein (Reading et al 1989).

Hsp60 is an ATP dependent protein that has been shown to mediate the folding of many mitochondrial proteins, that have been encoded in both the nucleus and mitochondria, moreover studies have also shown that it prevents the denaturation of a large number of preexisting proteins as well (as reviewed by Langer and Neupert 1994). It accomplishes this by recognizing hydrophobic surfaces in the context of collapsed (globular or molten) conformations (Burkau and Horwich, 1998).

As a chaperonin (promotes folding of proteins to their native state), Hsp60 is composed of two rings of back-to-back, identical or closely related rotationally symmetrical subunits in bacteria and single ringed in mammals (Bukau and Horwich 1998). This creates a large central channel within ring where newly translocated or damaged proteins are protected from aggregation and manipulated into their native forms.
In the presence of a lid-like co-chaperonin (Hsp10 or Cpn 10), the Hsp60 chaperonin provides kinetic assistance to the folding process which under normal in vivo conditions can end up in kinetic traps (Langer and Neupert 1994, Bukau and Horwich 1998)

**Hsp40**

The Hsp40 protein is known to be stress inducible and have amino acid sequence homology with bacterial DnaJ. This protein localizes to the nucleus and particularly the nucleolus of heat-shocked cells, it returns to the cytoplasm during recovery. It too, is known to act like a chaperone and interact with Hsp70 (Hattori et al 1993).

**Hsp27**

The small heat shock protein (sHsp27) plays an important role in responses to stress. Elevated levels appear to confer resistance to a number of challenges, including but not limited to heat shock, reactive oxygen species (ROS), various drugs including cancer therapies (i.e. cisplatin) and heavy metals (reviewed by Arrigo and Landry 1994). It is found primarily in the cytosol and upon stress, is phosphorylated, induced and relocalized to the nucleus. It shows structural and functional similarity to that of α-crystallins and is also known to exhibit chaperone function, primarily by preventing polypeptide aggregation (Arrigo and Landry, 1994; Ehrnsperger, 1997). It has been shown to trigger an adaptive stress response at the micorfilament and actin level by stabilizing them and reducing damage during oxidative stress. Additionally, Hsp27 is known to protect cells by increasing cellular glutathione (GSH) levels (Mehlen et al., 1997). Even more recent evidence has implicated it in directly inhibiting the apoptosis pathway by modulating proteins that control cytochrome c release and also further
downstream during the cytochrome c dependent activation of procaspase-9 (Jaattela, 1999; Garrido, 1999; Arrigo, 2000).

The Glucose Regulated Proteins

These Endoplasmic Reticulum (ER) stress proteins were first named Glucose-Regulated Proteins (GRP) because of their induction by glucose starvation. The group includes the heavy-chain binding protein GRP78 (also know as BiP), GRP94, protein-disulfate isomerase (PDI/Erp59), Erp72 and GADD153 (also known as CHOP). Their general role is to facilitate protein folding in the ER, thereby reducing the number of misfolded proteins and alleviating ER stress (Gething et al., 1994; Gething 1999).

GRP78

GRP78 or BiP, is an HSP70 molecular chaperone located in the lumen of the endoplasmic reticulum (ER), binds newly-synthesized proteins as they are translocated into the ER and maintains them in a state competent for subsequent folding and oligomerization (Gething et al., 1994). It has been shown to interact with both immunoglobulin (Ig) heavy and light chains before assembly into their final protein product, however it does not interact with native polypeptides (Welch et al 1992). GRP78 is an essential component of the translocation machinery and plays a role in retrograde transport of aberrant proteins across the ER membrane. These proteins are destined for degradation by the proteasome. GRP78 is an abundant protein under all growth conditions and its synthesis is highly induced under conditions that lead to the accumulation of unfolded polypeptides in the ER. Agents or events that induce GRP78 as a result of such protein accumulation include glucose starvation, calcium ionophores,
amino acid analogues or drugs that inhibit glycosylation (Morris et al., 1997; Gething, 1999; Gething et al., 1994). As a result the activated signal transduction pathway has been called the Unfolded Protein Response (UPR). More recent work has also shown that ER Ca\(^{2+}\) depletion can also induce GRP transcription and this represents a separate signaling pathway. Finally, it is cytokine growth factors which have been shown to regulate basal transcription of GRP genes under non-ER-stressed conditions (Pahl, 1999; McCormick et al., 1997).

**GRP94**

The GRP94 exhibits significant sequence homology with Hsp90. It is found primarily in the Endoplasmic Reticulum but more recent evidence also implicates the plasma membrane. GRP94 exhibits calcium-binding properties, is highly abundant in cells with secretory activity and its synthesis is increased in response to agents that perturb protein secretion. There is increasing evidence that it may also regulate the activities of other ER resident proteins (Welch, 1992; Gething, 1999)

**Stress Proteins and Cancer**

The final stage of tumourigenesis has been reached when cells become able to metastasize and have acquired resistance against cancer therapies. Since heat shock proteins primary roles are protecting cells from death and mediating stress, these proteins have an obvious impact in cancer therapy and are important determinants in the fate of cells after various treatments.

The Hsp27 and Hsp70 chaperones are of special relevance in cancer therapy as both have been shown to inhibit apoptosis. Additionally, Hsp27 is overexpressed in
numerous human cancers and anti-cancer drugs, such as cisplatin trigger the accumulation of Hsp27. Moreover, it has been repeatedly implicated as a strong negative prognostic marker in a variety of these cancers (Arrigo, 2000; Sarto et al., 2000).

Other studies have also implicated overexpression of Hsp’s as negative factors in survival. Hsp60 has been implicated as a negative marker in osteosarcoma and Hsp90 has been reported to be correlated with poor prognosis in breast cancer (Uozaki et al., 2000).

**PDT and the Stress Response**

Since PDT causes significant oxidative stress to cells, a stress response including induction of various heat shock proteins is not surprising. Gomer and his group reported the first evidence of this in the early nineties. They observed induction of GRP78 and GRP94 following PDT. In the same study, they found that incubation with the calcium ionophore A23187, which is known to induce GRP78 caused cellular resistance to Photofrin mediated PDT (Gomer et al 1991). This work has been repeated by other groups using different sensitizers, including a mitochondrial targeting dye Victoria Blue (Morgan et al., 1998). However, no direct causal relationship between GRP78 expression and PDT sensitivity currently exists in the literature.

The role of Hsp70 in PDT has also been extensively studied. Gomer and his group have shown that *in vitro*, chlorin or purpurin based sensitizers induce Hsp70 expression while Photofrin does not. Conversely, *in vivo*, all three sensitizers induce Hsp70 (Gomer et al., 1996). However, this same group has shown that cellular
sensitivity to PDT is not altered in heat resistant cell lines that constitutively overexpress Hsp70, when compared to control cells (Gomer et al 1990).

Finally, increased transcription and translation of heme oxygenase (Hsp 34) has been documented following Photofrin incubation alone as well as after Photofrin mediated photosensitization and Rose Bengal mediated photosensitization (Gomer et al., 1991). Again no information regarding the role of Hsp34 in cellular sensitivity to PDT has been published to date.

It should also be noted that there is evidence that incubation protocols, just like the photosensitizer chosen do effect the induction of stress proteins. In general it appears that shorter incubations (1-3hr) can fail to elicit a stress response that longer incubations (16-24hr) with the same degree of cell kill do elicit (Gomer et al 1996). It appears that only oxidative damage at specific sites induces this response and it is not simply a response to oxidative stress in general. It is clear that more work is necessary to fully understand the relationship between PDT and stress proteins as well as the differences between the various photosensitizers and incubation protocols that are used.

The Proposed Study

Previous work conducted here at the Hamilton Regional Cancer Centre has led to the development of multiple models for studying PDT resistance and mechanisms of action. Currently, we possess human cancer cell models where, through repeated in vitro treatments with Photofrin mediated PDT followed by regrowth of single surviving colonies, we have generated PDT resistant variants. Previously, the RIF model proved
very useful in elucidating mechanisms of action and changes associated with PDT resistance.

This current work utilizes the Human colon cancer cell line HT29 and its PDT induced resistant variant HT29-P14 (indicating 14 cycles of PDT and regrowth of single surviving colonies using the drug Photofrin). Similar to the murine model (Singh et al., 1991; Sharkey et al., 1993), there exists preliminary evidence for mitochondrial alterations in our human PDT resistant variants.

The general objective of this thesis is to examine the role of stress proteins in PDT. In this work, I examine the expression, localization and induction of both the mitochondrial heat shock protein Hsp60 and the endoplasmic reticulum glucose related protein, GRP78 before, during and after PDT in the both sensitive and PDT resistant variants. To gain a better understanding of the stress protein status of the sensitive and resistance variants I also apply broad scale “stress protein” expression profiling using a cDNA microarray thereby facilitating an understanding of the basal level expression of 234 stress proteins simultaneously.

The second component of this thesis employs a bladder cancer cell line; namely T24 and its GRP78 stable overexpressing clones. This model is used to directly study the role of GRP78 in PDT sensitivity. Hsp60 expression is also determined before, during and after PDT in these cells.
HYPOTHESIS AND OBJECTIVES

Hypothesis One

The HT29-P14 cells have a differential stress response with an increase in the expression and activity of the mitochondrial heat shock protein (Hsp60) as well as the endoplasmic reticulum glucose related protein (GRP78) following Photofrin PDT.

Objectives to Confirm Hypothesis One

(1a) To determine the basal expression of the two proteins in the HT29 and HT29-P14 cells and any changes that occur following PDT or Photofrin incubation. Multiple doses of drug and light will be used and the expression as well as localization will be determined at various times during and after PDT.

(1b) To determine if there are other changes in Hsp60 such as the decrease in mobility seen in the murine model believed to be due to phosphorylation.

Hypothesis Two

Overexpression of the endoplasmic reticulum glucose related protein (GRP78) in the bladder cancer T24 cell line causes cellular resistance to PDT.

Objectives to confirm hypothesis two

(2) To determine the clonogenic survival of stable overexpressing clones, empty vector clones and parental cells.

(3) To determine the basal expression of GRP78 protein in all cell lines and any changes that occur following PDT. The expression of Hsp60 will also be determined.
Hypothesis Three

The HT29-P14 cells have a differential stress expression profile with the overexpression of numerous stress proteins relative to their parental HT29 cells

Objectives to Confirm Hypothesis Three

(4a) Determine the expression of 234 stress proteins using a cDNA microarray in both the HT29 and HT29-P14 cells at basal levels.

(4b) Determine if these findings are reproducible by Northern blot analysis

(5) Determine if differences at the messenger (RNA) level result in expression differences in protein levels
CHAPTER 1

Induction of Hsp60 in Sensitive and Resistant HT29 Colon Cancer Cells and RIF-1 by Photofrin Incubation and Photofrin Mediated Photodynamic Therapy.

John G. Hanlon¹,², Katherine Adams¹, Andrew J. Rainbow², Radhey S. Gupta³ and Gurmit Singh¹,²,³,⁴.

¹Hamilton Regional Cancer Centre, ²Department of Biology, ³Department of Biochemistry, ⁴Department of Pathology and Molecular Medicine, McMaster University.

Running Title: INDUCTION OF HSP60 BY PHOTOFRIN-MEDIATED PHOTODYNAMIC THERAPY

Keywords: PDT, Photofrin II®, Heat shock Proteins, Hsp60,
ABSTRACT

Photodynamic therapy (PDT) invokes a number of cellular responses. Other studies have shown that PDT induces transcription and translation of heat shock proteins (Hsp’s). The expression of mitochondrial heat shock protein, Hsp60, was measured following in vitro Photofrin-mediated PDT in the colon cancer cell line HT29 and its PDT-induced resistant variant HT29-P14 as well as RIF-1 and its PDT-induced resistant variant, RIF-8A. Basal levels of Hsp60 were found to be similar in the two murine cell lines. In the human model, the resistant HT29-P14 cell line showed a small increase in basal levels relative to its parental population. Incubation with Photofrin® (PIT) alone or PDT action caused a significant increase in Hsp60 levels in all cell lines as determined by western blot analysis and flow cytometry. A dose dependent and temporal relationship for PDT response was observed and maximum levels were detected 6-8 hours post PDT. At which time, Hsp60 induction was found to be significantly greater in the two resistant variants. These results indicate that the presence of PIT and the subsequent oxidative stress of PDT induce Hsp60. It also implicates Hsp60 as a common factor that may play a role in the increased survival of these resistant cell lines.
INTRODUCTION

Photodynamic therapy uses the localized delivery of light to activate photosensitizing dyes which are preferentially retained by tumors (1-3). Through \textit{in vitro} studies, singlet oxygen has been identified as the cytotoxic agent that is most likely responsible for the cellular damage and the primary subcellular targets are the plasma membrane, mitochondria and DNA (3-7).

Induction of resistance has been extensively used to study the mechanisms of resistance to many anti neoplastic drugs as reviewed by Morrow and Cowan (8). Exposure to high dosage PDT is a strong selective pressure allowing only the more resistant cells in a population to survive. Repeated cycles of treatment and regrowth are expected to amplify the biochemical or intracellular changes associated with resistance.

We have previously generated a population of cells namely, RIF-8A, which show a degree of resistance to PDT as compared to their parental line, RIF-1 (9). The protocol for inducing resistance consisted of repeated \textit{in vitro} photodynamic treatments with the drug Photofrin® (PII) to the 1-10% survival level followed by regrowth of single surviving colonies. This model has proven very useful in understanding the mechanisms of PDT action and resistance and this RIF model has been extensively characterized by us (10-12). Of relevance to this present study, were the findings of mitochondrial alterations on a physical, functional and chemical level. Also of importance is the fact that these cells had a mechanism of resistance different from that of classical MDR or plieotropic resistance (12).

More recently, we have developed resistant variants from human tumour cell lines. HT29 is a human colon adenocarcinoma and we developed a PII resistant variant named HT29-P14. The degree of resistance measured by colony forming assay and seen in these cells is approximately 1.5 fold greater than that of the parental population, analysis of the mechanisms of
resistance involved are currently being determined in our laboratory. These cells also do not display the classical MDR phenotype.

The oxidative stress associated with PDT increases the expression of a number of stress proteins such as Hsp34 (also known as heme oxygenase), Hsp70, Hsp90, Hsp110 and the glucose-regulated proteins Grp74, Grp78 and Grp100 (13-15). Heat shock proteins are rapidly induced during the stress response following exposure to numerous adverse environmental factors (16,17). Their expression is ubiquitous and increases are known to confer transient resistance to subsequent challenges, which would otherwise be lethal. These proteins are also essential for normal cellular functions such as development and protein degradation (17-20). Hsp60 is one of the major molecular chaperones, which binds to nascent or denatured proteins and mediates their proper folding and/or secretion from the cells (20,22). In eukaryotic organisms ranging from S. Cerevisiae to human cells, Hsp60 is mainly found in the mitochondrial compartment although smaller amounts are also present at other specific locations including secretory granules and plasma membrane (23).

Our sensitizer, PII, a commercially available product containing enriched amounts of DHE (Dihematoporphyrin Ether), is known to localize to the mitochondria by 18-24 hours which is the length of our incubation (10). As a result much of the damage caused by PII occurs in the mitochondria (altered in our resistant lines) and will be in proximity to Hsp60 (mitochondrial matrix) and the proteins that Hsp60 folds. In this study, we investigated the characteristics of Hsp60 expression in the PDT-resistant RIF-8A cells compared to the parental RIF-1 cells following PII-mediated PDT. Similar studies were conducted in human colon carcinoma cells (HT29) and its resistant variant (HT29-P14).
MATERIALS AND METHODS

Photosensitizer. Photofrin® was provided by Quadra Logics Phototherapeutics, Inc. (Vancouver, British Columbia, Canada) and was reconstituted in 5% dextrose in water at 2.5 mg/ml and stored at -20°C. The stock solution was diluted to the appropriate concentration in media immediately before use.

Cells and Culture Conditions. All four cell lines (RIF1 and RIF8A as well as HT29 and HT29-P14) were grown as a monolayer in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 1% Antibiotic (Gibco-BRL, Burlington, Ontario, Canada). All cell lines were routinely trypsinized with 0.05% trypsin/0.53 mM EDTA three times a week and kept in a humidified atmosphere with 5% CO₂.

In vitro Treatment. Prior to PDT treatment, 1x10⁶ cells/ml were seeded in 100-mm plastic culture dishes and allowed to adhere overnight. Cells were incubated for 18 hrs with increasing concentrations of Photofrin (2.5-30µg/ml) at 37°C. Following incubation, the media containing photosensitizer was removed and replaced with fresh media immediately prior to light exposure and irradiated as described previously (9). Plates were returned to the incubator for 0-24 hrs. All procedures after plating of the cells were carried out in ambient light. At the end of the incubation period plates were washed in cold PBS, trypsinized and the cells used immediately for flow cytometry. Cells used for electrophoresis were lysed directly on the plate and frozen until ready to be run.
Flow Cytometry. The protocol was similar to published protocols (24). Cells were trypsinized washed with cold phosphate buffered saline (PBS) and fixed in freshly prepared PBS supplemented with 4% paraformaldehyde and 2% bovine serum albumin (BSA) for 10 min. After permeablizing the cells with 0.25% saponin (Sigma Chemical Co., Mississauga, Ontario, Canada) for 5 min the samples were labeled using rabbit polyclonal Hsp60 antibody raised against human recombinant Hsp60 (1:30) (23). This primary antibody was suspended in the permabilizing solution as described above, a Goat anti-rabbit fluorescein isothiocyanate (FITC) secondary antibody in PBS (1:400) (Gibco-BRL, Burlington, Ontario, Canada) for 1 hr. Cells were then washed 3 times in cold PBS and analyzed using the EPICS IV flow cytometer (Coulter Electronics, Haleigh, CA).

Electrophoresis and Immunoblotting. Cell were lysed on the plate, on ice for 30 min in 500 μl of lysis buffer (150mM NaCl, 100mM Tris-HCl, 1% Noniodet P-40 (v/v) (pH 8.0)) supplemented with fresh 1mM dithiothreitol, 20 μg/ml leupeptin and 40 μg/ml aprotinin. Insoluble material was pelleted at 15 000 rpm for 10 min at 4° C and the supernatant was removed and stored at -80° C. The protein concentration of the samples was determined according to the Bio-Rad protein assay (25) (Bio-Rad, Burlington, Ontario, Canada) and 10μg of protein were resolved on 0.75-mm thick 7.5% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to Hybond-C nitrocellulose membrane (Amersham, B’aie d’Urfe, Quebec, Canada) and non-specific binding was blocked overnight with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 7.5% skim milk. The membranes were probed with mouse monoclonal antibodies anti-Hsp60 or anti-β-actin (Stressgen, Vancouver, BC Canada). The membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Burlington,
Ontario, Canada) for 1 hour at room temperature and the protein bands were visualized by ECL (Amersham Pharmacia Biotech, B’aie d’Urfe, Quebec, Canada).

**Immunohistochemistry.** Approximately 10,000 cells were plated on a sterilized coverslip and allowed to adhere overnight. PDT treatment was carried out as above and cells were examined 6-8 hours post PDT. First, in ambient light, the cells were incubated in pre-warmed media containing 50 nM Mitotracker red (Molecular Probes, Oregon, USA) for 15 mins. They were then washed twice, fixed and washed again. Permabilization and incubation were also carried out as described above but the more stable secondary antibody goat anti-rabbit ALEXA (Molecular Probes, Oregon, USA) at 1:1000 was used to overcome the quick quenching associated with FITC antibodies. Cells were viewed under confocal as well as fluorescent microscopes and pictures were taken of the various conditions in addition to detailed analysis of mitochondrial and Hsp60 localization within cells.

**RESULTS**

**Murine Fibrosarcoma cells (RIF-1) and resistant variant (RIF-8A)**

Prior to any treatment, the basal level of Hsp60 protein expression was measured in both the parental (RIF-1) and the PDT-resistant (RIF-8A) cell populations using the flow cytometer. There was no significant difference in the expression between the RIF-1 and RIF-8A cells (p<0.001). Figure 1a shows the increase in Hsp60 expression as detected in both RIF-1 and RIF-8A following an 18hr incubation with increasing doses of PII (2.5-10 µg/ml) without exposure to light. This increase was dose and time dependent and at the maximum dose of 10µg/ml the RIF-1 showed a 1.6 fold increase in expression over basal levels whereas the RIF-8A cells showed a
3.25 fold increase. The induction of Hsp60 expression in the resistant variant was approximately 2 times greater compared to the parental line at 6-8 hours post incubation. Levels started to return to basal beyond this time point.

The cytotoxicity assays that were performed concurrently with the expression experiments demonstrated that PII incubation alone has a small toxic effect on the cells at the highest doses. RIF-8A were more resistant to these effects of Photofrin just as they are to PDT when compared to the RIF-1 cells. Figure 1b depicts Hsp60 expression measured 6-hrs post incubation and photoactivation. An increase in the expression of Hsp60 in both the parental and the resistant variant was observed. The induction was dose-dependent in both the RIF-1 and RIF-8A. However, this was not significantly different compared to that observed following Photofrin induction alone (p<0.001). When comparing the increase in the RIF-8A and the RIF-1 cells, the resistant variant had approximately 1.5 fold greater expression of Hsp60 versus the RIF-1 following PDT. This increase in Hsp60 expression observed in the RIF cells is in agreement with other studies that have reported an induction of the stress proteins following PDT (14-16). Finally, in the RIF8A cells, at the highest doses, induction was found to be significantly higher for PII alone than PDT (P<0.05).

Immunohistochemistry revealed differential staining patterns for Hsp60 in the two cell lines (data not shown). Since Hsp60 was mainly localized in mitochondria, the observed differences in staining patterns likely correspond to the different shapes and distribution of mitochondria in each of the cell lines as determined previously in our RIF model (12).

Human carcinoma cells (HT29) and resistant variant (HT29-P14)
Since the HT29-P14 cell line has not been previously published, Figure 2 shows the relative survivals of both HT29 and the significantly more resistant HT29-P14 cells after Photofrin mediated PDT as determined by colony forming assays. HT29-P14 is approximately 1.5-1.8 fold more resistant to PIT PDT.

Prior to any treatment, Hsp60 protein expression was measured in both the smaller parental (HT29) and the larger PDT resistant (HT29-P14) cell populations using the flow cytometer. A significant difference in basal levels was found (p<0.05). The resistant variant was found to have approximately 1.4 times the amount of Hsp60 protein per cell. For these human tumor cells, similar increases in Hsp60 expression associated with incubation alone or PDT were found to be temporal and dose dependent with maximal levels present 6-8 hours post incubation period. These levels return to an amount not significantly different than basal levels within 48 hours. Incubation with drug alone was found to have minimal effect on the survival of either population. No dark toxicity was detected below 20 ug/mL. Even at concentrations of 30 ug/mL, which would kill all cells if photoactivated, a maximum of 6% cell kill were noted by colony forming assay (data not shown). Figure 3a depicts the response to incubation alone at different concentrations. At the maximum dose tested (30 ug/mL) the HT29 showed a 1.8 fold increase in expression over basal levels whereas the HT29-P14 showed a 3.4 fold increase. As in the RIF model the induction of Hsp60 expression was approximately 2 times greater compared to the parental line. Moreover, in terms of absolute cellular Hsp60 content, the resistant cells contained 2.5 times the amount of Hsp60 protein.

Expression was also measured after photoactivation. The maximal dose tested was 15 ug/mL which corresponds to slightly less than the D_{37} of HT29-P14. The increase in expression was again found to be temporal and dose dependent in both the parental and resistant variant.
Figure 3b depicts the effects of PDT on Hsp60 expression. At the highest concentration of drug tested the resistant cells showed a 2 fold increase in protein expression and the sensitive cells only a 1.5 fold increase. Similar to the RIF-8A cells, at the higher doses, induction in both of these human tumour cells following PDT was not as large as following incubation with the drug alone.

Immunohistochemistry performed on these tumour cells included doubly staining these cells for mitochondria and Hsp60. Figures 4 a-d depict the staining pattern found in a HT29 and HT29-P14 cell prior to treatment. In both cell lines the match between the two stains was almost identical as expected. The differential staining patterns of the Mitotracker dye consistently showed the gross morphological and arrangement differences in the mitochondria found in the HT29-P14 cells. HT29 cells have large vacuolated mitochondria as can be seen in Figure 4a. However, the mitochondrial dye in the HT29-P14 cells shows a more clustered and perinuclear distribution of mitochondria as seen in Figure 4c. No large vacuolated mitochondria are detectable. The significance of these changes are currently under investigation in our laboratory. Immunohistochemistry revealed no changes in localization either post incubation or post PDT.

DISCUSSION

We have shown that after PDT, Hsp60 protein content per cell is increased. In our sensitive cells this induction is small (1.5-1.8 fold) but much larger induction's were observed in our resistant variants (up to 3.4 fold). This increase is in general agreement with other previously published findings of Hsp induction by PDT. Specifically, it has been shown that cellular PDT can induce Hsp70 expression, however depending on the sensitizer used, varying degrees of induction were observed (13). Gomer et. al. also examined the glucose related proteins (GRP's) which show sequence homology with Hsp’s and are known to bind to
abnormally folded or processed proteins which accumulate in the endoplasmic reticulum. They found that PDT induced GRP78 and that resistance to PDT was observed in cells overexpressing GRP's (15). In the context of our growing knowledge PDT action, the induction of Hsp60 after the oxidative stress is an expected response.

What is most intriguing, however is the induction of Hsp60 by incubation of drug alone. These significant increases have been observed at drug concentrations that do not have any lethal effects on the cell. This incubation alone condition that was initially expected to have levels similar to the no drug condition, yet this dose dependent dark response resulted in levels of Hsp60 not significantly different from the same dosage when activated by light. From this finding, a different conceptual approach is required. Concurrent colony forming assays have confirmed that PII incubation alone has a negligible effect on cell survival and colony forming ability at the low to medium doses.

There are a number of known cellular effects as the result of the non-photodynamic actions of porphyrins. In another study, Gomer et al, observed the Hsp 32 (Heme Oxygenase, found in the mitochondria) was induced by PII incubation alone (26). It has also been found that the early response gene c-fos (a transcription regulator) is activated by porphyrin based photosensitizers in the absence of light (27). Two other findings are also of relevance 1) the activity of cytochrome c oxidase is decreased (also found in the mitochondria) and 2) a significant increase in glycolysis (glucose utilization and lactate production) along with increased ATP levels. They also found an increase in activity of the glycolytic enzyme lactate dehydrogenase, which is bound to the mitochondria (28). These findings suggest that chemical structure or possibly serum and membrane binding properties of the PII play a role in dark responses. Moreover, from these findings it appears that, especially in the mitochondria,
porphyrins exert a double effect in enzymes and proteins. The dark inactivation and changes are the result of a direct inhibition due to the conformational changes induced by the attachment of porphyrins on or near active sites. The light activation then causes the singlet oxygen induced oxidative stress associated with PDT (35).

There is evidence that porphyrins can interact with proteins, exposing their hydrophobic residues (29). Hsp60 is responsible for folding and refolding proteins. In the native (folded) state, proteins have their hydrophobic residues shielded from the aqueous environment, when these proteins are denatured or unfolded and such residues become exposed, precipitation follows (30). We propose that in our experiments, the presence of PII has led to unfolding of proteins (possibly including Hsp60). It is known that Hsp60 chaperonin recognizes hydrophobic surfaces in the context of their collapsed globular conformation and will then enclose them within its cavity and promote refolding (30). The HT29-P14 and RIF8A cells must have a mechanism that allows for greater induction or retention of Hsp60 protein. As a result these cells have more Hsp60 which will translate into less unfolded proteins and will allow for more refolding upon light activation and the subsequent oxidative stress. We also propose that the decrease in Hsp60 levels for PDT conditions relative to the incubation alone condition is the result of damage and destruction of these proteins by the cytotoxic oxygen species that are generated in their proximity during light activation.

There is considerable evidence to suggest that the stress proteins serve a protective role and their induction has been implicated as a mechanism responsible for transient resistance to subsequent cellular stress. As mentioned previously, there is a positive correlation between the induction of the glucose-regulated proteins Grp78 and Grp94 and resistance to Photofrin-
mediated PDT (16). The stress proteins Hsp70, Grp74 and Grp78 have also been shown to influence cell sensitivity to adriamycin. When Chinese hamster lung fibroblasts (V79) are pretreated with a stress which results in an increase in these stress proteins, there is a corresponding decrease in the cell’s sensitivity to adriamycin treatment (31). These data suggest that if the stress proteins are induced by the initial insult, they offer a tolerance mechanism which allows protection from the stress to follow (31,32), our observations are consistent with this.

The significant increase in Hsp60 expression in the resistant cells compared to the sensitive ones, without exposure to light suggests that this initial induction ‘primes’ the cells for light exposure. This will result in a protective effect especially at the higher doses (7.5-20μg/ml) and therefore may be involved in the resistance of the RIF-8A and HT29-P14 cells to PDT. The effect of these differential levels of Hsp60 induction in resistance to PDT is currently under investigation as there is a positive correlation between the two. It is clear however, that the stress that the physical presence of PII places on mitochondrial function initiates a number of responses that can play a role in determining survival following light activation. It must also be noted that the findings of our study and those cited as demonstrating dark effects of PII, may have implications for PDT. Conversely this induction of Hsp60 in the dark has the potential to be used as a molecular marker for identifying and possibly quantifying the cells and tissue regions that have taken up the PII drug although the limited degree of induction found in sensitive cells could limit such applications.

Present observations, therefore, show (a) no or small differences in Hsp60 expression between PDT sensitive and PDT resistant cells; (b) significant differences in induction and expression between the PDT-sensitive and PDT-resistant cells following incubation with PII. Moreover, (c) the greater increase in the expression of Hsp60 in RIF-8A and HT29-P14 cells
relative to RIF and HT29 cells respectively implicates Hsp60 as a common factor that may contribute to the resistance observed in the induced resistant cells and finally; (d) preliminary evidence for gross morphological mitochondrial alterations in the induced resistant human tumour cell line which has been previously noted and characterized in the murine model.

ACKNOWLEDGEMENTS

This work is supported by the National Institute of Health program grant (project 3) CA 43892 and the National Cancer Institute of Canada with funds from the Canadian Cancer Society. Support for J.G.H. was also provided by NSERC fellowship 232027. Many thanks to Myrna Espiritu for generating the resistant variants and her ongoing support. We would also like to acknowledge QLT Phototherapeutics Inc for supplying Photofrin II.
REFERENCES


Figure 1a-b. Hsp60 protein content for RIF1 (○) and Rif-8A (■) cells exposed to increasing
doses of Photofrin II a.) Without light activation and b.) After activation by light. Mean
fluorescence units are determined using FITC labeled antibodies and flow cytometry. Each data
point is the average of 4 experiments and their SE.
Graph a shows the mean fluorescence units (MUF) of RIF-1 and RIF-8A at different drug concentrations (ug/ml). The concentrations range from 0 to 10 ug/ml. The error bars indicate the standard deviation.

Graph b also presents the mean fluorescence units of RIF-1 and RIF-8A, with concentrations ranging from 0 to 10 ug/ml. The error bars represent the standard error.
Figure 2. Survival curves for HT29 (□) and HT29-P14 (■) cells exposed to increasing doses of PDT as determined by colony forming assays. Cells were incubated for 18 h with Photofrin II prior to light treatment. Each data point is the average of three experiments done in triplicate and it's SE.
Figure 3 a-b. Hsp60 protein content for HT29 (□) and HT29-P14 (■) cells exposed to increasing doses of Photofrin II a.) Without light activation and b.) After activation by light. Mean fluorescence units are determined using FITC labeled antibodies and flow cytometry. Each data point is the average of 3 experiments and SE.
Figure 4. Cells doubly stained using Mitotracker® Red (left column) and anti-Hsp60 antibodies with anti-rabbit green ALEXA secondary antibody (right column). Hsp60 staining shows mitochondrial localization and for HT29 cells (a & b) their large vacuolated mitochondria are obvious. In the resistant HT29-P14 cells (c & d), a consistently altered mitochondrial shape and arrangement was noted.
CHAPTER 2

Overexpression of GRP78 Protein Causes Cellular Resistance to Photodynamic Therapy: An Examination of Stable Transfected T24 Cells

John G. Hanlon¹,², Andrew J. Rainbow², Richard C Austin¹,³, and Gurmit Singh¹,

¹Hamilton Regional Cancer Centre, ²Department of Biology, ³Department of Pathology and Molecular Medicine, McMaster University.

Running Title: STABLE OVEREXPRESSION OF GRP78 CAUSES RESISTANCE TO PHOTOFRIN-MEDIATED PHOTODYNAMIC THERAPY

Keywords: PDT, Photofrin II®, Glucose Related Protein (GRP78), Overexpression, Resistance,
ABSTRACT

The relationship between the 78 kDa glucose related protein (GRP78) expression and cellular sensitivity to the cancer treatment, Photodynamic Therapy (PDT) was assessed in vitro in human bladder carcinoma cells (T24) that are stable overexpressors of GRP78. Previous studies have shown that the calcium ionophore A-23187, which causes GRP78 overexpression, was associated with increased survival after PDT. Our protocol consisted of an 18-hour incubation with the porphyrin photosensitizer Photofrin®, then illumination for varying time periods at a constant drug dose. Colony forming assays showed increased survival after PDT in the transfected cells when compared to the parental or vector-transfected cells and a ten to twenty fold increase in colony survival was noted in the GRP78 overexpressors at the highest light dose tested. Drug uptake and growth rate was similar in all cells, thereby eliminating these as the cause of increased survival in the GRP78 overexpressers. Western blot analysis and flow cytometry showed that PDT also caused a dose-dependent induction of GRP78 in all lines tested and a temporal relationship was found where maximal protein levels were observed 12-16 hours post PDT. This study provides direct evidence that increased GRP78 protein levels in human tumor cells causes an increase in PDT resistance.
INTRODUCTION

Photodynamic therapy (PDT) is the treatment of solid tumours employing the localized delivery of light to activate systemically administered photosensitizing dyes such as Photofrin® that are preferentially retained by tumors (Dougherty et al., 1976; Levy, 1994). Reactive oxygen species such as singlet oxygen are suspected to be the cytotoxic agents responsible for the cellular damage at such primary subcellular targets as the plasma membrane, and various subcellular organelles including mitochondria and endoplasmic reticulum (Weishaupt et al., 1976; Gomer et al., 1989; Wilson et al., 1997). This, in turn induces oxidative damage to numerous lipids, proteins and nucleic acids (Moore et al., 1997).

The oxidative stress associated with PDT has been shown to induce the synthesis of stress proteins in both the Hsp and GRP families. These include Hsp34, Hsp70, GRP78 and GRP94 (Gomer et al., 1990, 1991). The heat shock proteins can be grouped into families and classified according to their molecular masses into one of six subfamilies: large Hsps of 100-110kDa, Hsp 90 family, Hsp 70 family, Hsp60 family, Hsp40 family and the small heat shock protein (sHsp) family of 18-30kDa. The glucose related proteins GRP78 and GRP94 are members of the Hsp70 and Hsp90 families respectively. (Jaattela 1999, Lindquist and Craig Annu Rev Genet1988). There is significant evidence showing that increased expression of these Hsp’s confer thermotolerance and play a central role in cellular resistance to stresses including heat (Lindquest, 1986). While this heat shock response allowed for the initial identification of these proteins, most of them function in protein folding and translocation of proteins and by transiently binding to nascent proteins they facilitate correct folding and packing. Additionally, some of them are known to bind more permanently to damaged proteins that are to be degraded (Morimoto et al., 1994).
The endoplasmic reticulum (ER) is the site of folding for newly synthesized proteins that are to be sent to the cell surface and is the principle storage site for calcium. The calcium homeostasis achieved, is required for proper polypeptide folding, secretion of selective proteins and intracellular signaling events within the cell (Sambrook, 1990). The glucose related proteins, GRP78 and GRP94 are both found in the ER.

GRP78 binds transiently to nascent, secretory and transmembrane proteins and more permanently to damaged or abnormally folded proteins in the ER. It is known to be induced by anoxia, glucose starvation, alterations in intracellular calcium, inhibitors of glycosylation and various other cellular insults (as reviewed by Gething et al., 1994). Various conditions that cause the induction of GRP's such a glucose starvation or incubation with A23187 (a calcium ionophore) or 2-deoxyglucose are correlated with cellular resistance to various challenges. These include doxorubicin, and tumour necrosis factor (Shen et al., 1987; Shen et al., 1989). Accordingly, indirect evidence for the protective function of GRP78 to PDT was found by Gomer et al. (1991), who showed that incubation with A23187, which is known to induce GRP78 caused cellular resistance to PDT in a radiation induced fibrosarcoma cell line. Such findings support a hypothesis that GRP78 can modulate sensitivity to cellular stresses and may have a protective function during and after stress where protein processing in the endoplasmic reticulum is disturbed.

This current study was performed to directly examine the role of GRP78 expression in sensitivity to PDT employing stable overexpression of GRP78 by transfection.
MATERIALS AND METHODS

Photosensitizer. Photofrin was provided by Quadra Logics Phototherapeutics, Inc. (Vancouver, British Columbia, Canada) and was reconstituted in 5% dextrose in water at 2.5 mg/ml and stored at -20°C. The stock solution was diluted to the appropriate concentration in media immediately before use.

Cells and Culture Conditions. All cell lines (T24 and its clones) were grown as a monolayer in M199 media supplemented with 10% fetal bovine serum, 1% Antibiotic (Gibco-BRL, Burlington, Ontario, Canada). The generation of the clones is described elsewhere (Werstuck et al., 2001). All cell lines were routinely trypsinized with 0.05% trypsin/0.53 mM EDTA three times a week and kept in a humidified atmosphere with 5% CO₂.

Drug Uptake. The uptake of photosensitizer was measured by fluorescence flow cytometry (Coulter EPIC XL). Approximately 1x10⁶ cells/10 ml were seeded in 100 mm x 20 mm tissue culture plates and left in an incubator for 18 hours before drugging. Cells were then incubated with varying concentrations of Photofrin (18 hours). After which time, the media containing drug was decanted and cells were washed once in 10 ml of cold PBS, dislodged with 1 ml of 5X trypsin/EDTA and harvested with 10 ml of cold PBS. Cells were spun and the pellets were resuspended in 1 ml cold PBS for flow cytometry.

Growth Rate. Growth rate experiments were carried out over 6 days. On the first day 1000 cells were plated (each row contained 8 replicates) for each cell line to be tested; six of these 96 well
plates were made. Standard curves were generated by plating varying concentrations of each cell line (1000, 2000, 4000, 8000, 16000, 32000 and 64000) in triplicate. The cells were given 6 hours to adhere, at which point the standards and day zero cells were harvested and rinsed twice with PBS. 100ul of water was then added and they were placed in a −80°C freezer. This was repeated at 24 intervals for the next five days. The DNA fluorochrome (Hoechst 33258) was diluted to a working concentration of 50 ug/mL in TNE buffer (Tris 10mM, NaCl 2M, EDTA 1mM, pH7.4). 100 uL of this solution was added to each well and levels of fluorescence were measured in a CytoFlour™ 2350 (Millipore, Bedford MA, USA). Standard curves were then generated and used to extrapolate cell number from DNA fluorescence and deduce doubling times.

**Colony Forming Assay.** Preconfluent cells were removed from culture dish by 5 minutes incubation in 5X trypsin/EDTA. After the cells were spun at 1000 rpm for 5 minutes, they were resuspended and counted, and appropriate dilutions were made to obtain 4x10^2 cells/ml. One ml of this cell suspension was added to wells of 6-well tissue culture plates containing 1 ml of medium. Cells were allowed to adhere for 4-6 hours, at which time 5μg/ml of Photofrin in one ml of medium was added, control cells received 1 ml of medium only. Cells were drugged and after 18 hours, the medium was removed and replaced with fresh medium. The cells were then irradiated on a 100cm x 50 light diffusing surface illuminated by a bank of fluorescent tubes (Philip type TL/83), filtered with red acetate filters (Rossolux No.19, ROSCO California, U.S.A.), to provide wide band illumination above 585 nm. The energy fluence rate was 9.2 W/m² in the wavelength 525-700 nm, representing 12% of the total filtered output. Irradiation for 1-7 min. of Photofrin treated cells resulted in an incident energy fluence of 1.6 x 10^3 J/m² to 11.2 x 10^3 J/m² respectively. All procedures were carried out in minimal ambient light condition after
plating the cells. After 5 days of undisturbed growth in the dark, the colonies were stained with 0.08% methylene blue in 70% methanol. Colonies containing >20 cells were counted under an inverted microscope. Percentage survival is expressed relative to the average of three controls, namely No Drug No Light, Drug No Light and No Drug Light.

In Vitro Treatment. Prior to PDT treatment, 1x10⁶ cells/ml were seeded in 100-mm plastic culture dishes and allowed to adhere overnight. Cells were incubated for 18 hrs with a constant concentration of Photofrin (6 µg/ml) at 37°C. Following incubation, the media containing photosensitizer was removed and replaced with fresh media immediately prior to differing lengths of light exposure (1-7 min.) and irradiated as described previously (Singh et al., 1991; Singh et al., 2001). Plates were returned to the incubator for 0-24 hrs. All procedures after plating of the cells were carried out in ambient light. At the end of the incubation period plates were washed in cold PBS, trypsinized and the cells used immediately for flow cytometry. Cells used for electrophoresis were lysed directly on the plate and frozen until ready to be run.

Flow Cytometry. Protocol is similar to published protocols (Chant et al., 1995). Cells were trypsinized washed with cold phosphate buffered saline (PBS) and fixed in freshly prepared PBS supplemented with 4% paraformaldehyde and 2% bovine serum albumin (BSA) for 10 min. After permeabilizing the cells with 0.25% saponin (Sigma Chemical Co., Mississauga, Ontario, Canada) for 5 min the samples were labeled using a rabbit polyclonal GRP78 antibody (1:200) (StressGen, Vancouver, BC Canada). This primary antibody was suspended in the permabilizing solution as described above, a Goat anti-rabbit fluorescein isothiocyanate (FITC) secondary antibody in PBS (1:400) (Gibco-BRL, Burlington, Ontario, Canada) was then incubated at room
temperature for 1 hr. Cells were then washed 3 times in cold PBS and analyzed using the EPICS IV flow cytometer (Coulter Electronics, Haleigh, CA, USA).

**Electrophoresis and Immunoblotting.** Cell were lysed on the plate, on ice for 30 min in 500 μl of lysis buffer (150 mM NaCl, 100 mM Tris-HCl, 1% Noniodet P-40 (v/v) (pH 8.0)) supplemented with fresh 1 mM dithiothreitol, 20 μg/ml leupeptin and 40 μg/ml aprotinin. Insoluble material was pelleted at 15,000 rpm for 10 min at 4°C and the supernatant was removed and stored at -80°C. The protein concentration of the samples was determined according to the Bio-Rad protein assay (Bradford, 1976) (Bio-Rad, Burlington, Ontario, Canada) and 50 μg of protein were resolved on 1.0-mm thick 7.5% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to Hybond-C nitrocellulose membrane (Amersham, B’aie d’Urfe, Quebec, Canada) and non-specific binding was blocked overnight with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 7.5% skim milk. The membranes were probed with mouse monoclonal antibodies anti-KDEL and anti-β-actin (Stressgen, Vancouver, BC Canada). The membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Burlington, Ontario, Canada) for 1 hour at room temperature and the protein bands were visualized by ECL (Amersham Pharmacia Biotech, B’aie d’Urfe, Quebec, Canada).

**Immunohistochemistry.** Approximately 10,000 cells were plated on a sterilized coverslip and allowed to adhere overnight. PDT treatment was carried out as above and cells were examined 6-8 hours post PDT. First, in ambient light, the cells were incubated in pre-warmed media containing the endoplasmic reticulum stain 50 nM ER Blue (Molecular Probes, Oregon, USA) for 15 mins. They were then washed twice, fixed and washed again. Permeabilization and
incubation were also carried out as described above but the more stable secondary antibody rabbit anti mouse ALEXA (Molecular Probes, Oregon, USA) at 1:1000 was used to overcome the quick quenching associated with FITC antibodies. Cells were viewed under fluorescent microscopes and pictures were taken of the various conditions in addition to analysis of the endoplasmic reticulum and GRP78 localization within cells.

RESULTS

Stable overexpression of GRP78 causes cellular resistance to PDT.

GRP78 overexpression was accomplished using stable transfected T24 cells (Human bladder carcinoma cells) using a pcDNA 3.1 expression vector with the GRP78 gene under a CMV promoter. This promoter caused an increase in GRP78 levels to approximately 2 fold higher than basal levels as seen in the T24 parental cells or the empty vector clone (pcDNA). Constant selection using G418 (Gibco BRL, Burlington, ON, Canada) and western blot analysis were used to ensure that although selection was stopped approximately four days prior to PDT experiments, these elevated levels of protein were maintained. Cellular resistance to PDT was then determined using standard colony forming assay. Cells were incubated in Photofrin and then subject to increasing light doses. Results obtained using three categories of control cells did not differ significantly from each other. These three categories were: no drug-no light, no drug-light, and finally drug-no light. As can be seen in Figure 1, the T24 parental cells and pcDNA empty vector cells were very sensitive to PDT with close to one percent survival at a dose of 6μg/ml Photofrin and 5 min. Light. In contrast, the GRP78 overexpressing clone (A4) had approximately 20 percent survival at this same dose. These colony-forming assays were performed in triplicate at least three times and while
significant experiment to experiment variation exists, these large differences in PDT sensitivity were highly reproducible.

**Cellular characteristics are not altered following GRP78 overexpression**

In order to confirm that GRP78 overexpression was directly causing these differences in PDT sensitivity, it was necessary to measure various cellular properties. These included doubling time, drug uptake, plating efficiency and an analysis of gross morphological characteristics.

Figure 2 shows the doubling times for the three cell lines. While A4 did have a slightly higher doubling time, this difference was not significant and the doubling times of approximately (21-22 hrs.) were comparable. Similarly, drug uptake studies using flow cytometry found that drug uptake was not altered by either empty vector transfection (pcDNA) or GRP78 overexpression (A4). While the A4 clone showed slightly higher levels of drug per cell at the time of illumination, this was not significant, as can be seen in Figure 3.

Finally, plating efficiency was measured for each of the cell lines and this parameter was also similar in all lines tested. For each cell line, plating efficiency ranged between 50-60%. Gross morphological analysis, using light and fluorescent microscopes, detected no differences in growth patterns or colony size or shape. Staining with the endoplasmic reticulum dye, ER Blue® also revealed similar staining patterns corresponding to no gross changes in the endoplasmic reticulum after transfection of this ER based protein.
**GRP78 induction post PDT is temporal and dose dependent**

Maximum levels of GRP78 protein were noted 12-16 hours post photosensitization for all cell lines tested. These maximum levels of GRP78 protein for each cell line after this PDT induced induction are similar and thereby abolish the 1.8-2.0 fold higher levels of GRP78 that were found in the overexpressing clone at basal levels. Control cells that were either not drugged, not illuminated or neither drugged or illuminated did not show any changes in GRP78 expression. GRP94 levels were also determined due to the cross reactivity of the K-Del antibody and no increase in expression was found in either GRP94 or actin (which was used as a loading control) post PDT. These findings were demonstrated using both Western blot analysis and flow cytometry.

Figure 4 shows the levels of GRP78 at 16 hours post PDT at varying doses. As can be seen at the basal level, the transfected clone still has significantly higher levels but at the highest doses tested, this difference is not significant due to the larger induction in the non-overexpressing cells.

**PDT does not affect GRP78 localization**

GRP78 localization was studied by double staining cells for GRP78 and the endoplasmic reticulum specific dye ER Blue®. Since GRP78 is known to localize to the ER, this co-localization allowed for comparisons of cellular distribution of the protein of interest. Coverslip experiments failed to show any detectable changes in GRP78 localization as a result of PDT at any dose or time. Under all conditions, there was identical co-localization and no changes in the pattern of either fluorescent dye could be noted.
DISCUSSION

Decreased cellular sensitivity to PDT was documented in a cell line stably overexpressing GRP78. This finding is direct evidence for the role of GRP78 in modulating PDT sensitivity. Previously, it had been shown that the calcium ionophore A23187 which, among other things caused an increase in GRP78 also made cells more resistant to PDT (Gomer et al., 1991). These current findings therefore represent the first direct evidence for the role of GRP78 in modulating PDT sensitivity. The strong protective effect that these elevated protein levels confer is highly significant. While another member of this Hsp70 family, namely Hsp70 has been shown to be induced by PDT, various experiments have found that increases in cellular Hsp70 are not correlated with PDT sensitivity (Gomer et al., 1990)

The rapid accumulation of stress proteins is a well conserved characteristic of cells exposed to various environmental insults (Morimoto et al., 1994). We documented increased levels of GRP78 following PDT mediated oxidative stress and our findings in human tumour cells are similar to those found in a murine model by Gomer and others (1991). They documented elevated levels of mRNA encoding glucose related proteins (GRP's) as well as an increase in GRP protein synthesis in mouse radiation induced fibrosarcoma cells exposed to an extended (16 hr.) porphyrin incubation. The findings of this group however, differ from ours with respect to GRP94. They found a similar pattern of GRP78 and 94 induction while we found no detectable difference in GRP94 protein levels. It remains unclear why or even if these differences exist since their procedure involved detecting mRNA synthesis post PDT and our less sensitive method was to measure total protein levels post PDT by western blot analysis. It should be noted that cellular events and even mechanisms of cellular death can vary significantly from one population of cells or cell type to another (Moore et al., 1997). Regardless, the GRP78
data that we have generated in the human model is similar to their murine data in terms of the
temporal and dose response relationship in expression and it appears that it is GRP78 and not
GRP94 which confers the increased resistance in these transfected cells.

It should be noted that after PDT, the GRP78 induction was smaller in our stable
overexpressing transfected cell line (A4) relative to the untransfected parental (T24) line or the
empty vector (pcDNA). This finding is not surprising as GRP78 is known to be induced by
multiple pathways. One prominent pathway is the UPR, the Unfolded Protein Response where
unfolded proteins accumulate in the ER cause the cell to respond by increasing GRP78 levels,
thereby facilitating more protein folding. It has been recently shown that this response is not
directly triggered by the unfolded proteins but appears to be caused by the decrease in
concentration in free GRP78 when it is sequestered into complexes with unfolded proteins
(Kohno et al., 1993). In our model, the transfected cell line would not experience this dramatic
decrease in free GRP78 and hence would initiate a smaller and later induction. After PDT, it is
the amount of unfolded protein that indirectly determines the extent of GRP78 induction and
hence at the higher doses (with similar high levels of protein unfolding), the post induction
GRP78 levels for all cells would be expected to be similar.

The immunohistochemical analysis did not reveal any differences between the parental
and transfected cell lines or between the same cell lines both pre- and post-PDT. This is
consistent with another report which shows no significant changes in GRP78 localization after
stress (Gething, 1999). Our method used was sufficiently sensitive to allow visual identification
of the transfected cells (A4) based on the increased fluorescence owing to the increased GRP78
levels.
Our observation that GRP78 confers significant resistance to PDT in vitro and is induced by PDT suggests an important role for this protein in mediating cellular resistance simply by its initial levels and also a role in the immediate stress response to the PDT oxidative stress induced injury. These results suggest that GRP78 expression is an important consideration with respect to PDT and could represent a target for therapeutic intervention. Its rapid induction would also permit application as a diagnostic tool where its induction could serve as a specific molecular marker for identifying the initial cell, tissue regions and/or tumour depths that are actually being affected by PDT. While these two applications are initially attractive, much more work including in vivo studies must be carried out to fully assess the importance and role of GRP78 in PDT.

ACKNOWLEDGEMENTS

This work is supported by the National Institute of Health program grant (project 3) CA 43892 and the National Cancer Institute of Canada with funds from the Canadian Cancer Society. Support for J.G.H. was also provided by NSERC fellowship 232027. Thanks also to Sudesh K. Sood for generating the GRP78 overexpressing clones. We would also like to acknowledge QLT Phototherapeutics Inc for supplying Photofrin II®.
Figure 1. Survival curves for T24 (□), pcDNA (◇) and A4 (○) cells that were treated with 5μg/mL of Photofrin II; after 18 hours the drug was removed and cells were irradiated for varying time periods. Survival was determined by colony forming assay and percent survival for each cell line is relative to the average of the No-Drug-No-Light, Drug-No-Light and Light-No-Drug conditions. Shown are results of a single experiment, data repeated in triplicate ±SD. Experiments were repeated at least three times with similar results.
Colony Forming Assay for GRP78 transfected cells (A4), empty vector cells (pcDNA) and the T24 parental cells
Figure 2: Doubling times of T24 cells and their pcDNA and A4 clones were determined using a Heoscht assay. Known numbers of each cell line were used to generate standard curves and growth rate was determined by harvesting cells every 24 hours for 4 days. Data repeated in triplicate ±SD. Experiment was repeated twice.
Doubling Times for T24 cells

- T24 (parental)
- pcDNA (empty vector)
- A4 (GRP78 overexpressor)
Figure 3: T24 cells and their pcDNA and A4 clones were incubated in two concentrations of Photofrin II for 18 hours. Flow cytometry was then used to measure the mean florescence per cell for 10,000 cells. Cells without drug were used to determine auto-fluorescence. Data repeated in triplicate ±SD. Experiment was repeated twice.
Cellular Drug Levels After 18 Hour Incubation with Photofrin

Drug Concentration (ug/mL)

Mean Fluorescence Units

T24 pcDNA A4
Figure 4: GRP78 protein levels in the T24 cells as well as the pcDNA and A4 clones were measured using immunohistochemistry and quantified by flow cytometry. For all three cell lines basal level expression as well as the maximal GRP78 levels which occurred 16 hours post irradiation with a Photofrin® dose of 5 μg/ml and 4 minutes of irradiation are shown. Data was obtained in triplicate ±SD, repeated three times and normalized to the T24 parental cells fluorescence for the No-Drug-No-Light condition. The post photosensitization GRP78 levels shown for the overexpressing clone are at a sub-optimal concentration of Photofrin® but at the same drug concentration as the T24 and pcDNA cells.
GRP78 Protein Levels Before and After Photosensitization

Normalized Mean Fluorescence Units

<table>
<thead>
<tr>
<th></th>
<th>Basal Protein Levels</th>
<th>Maximal Levels After Photosensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td>T24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pcDNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4 Clone</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3

Photofrin-Induced Resistance to Photodynamic Therapy Leads to Specific Changes in Expression of Stress Genes in Human Colon Cancer Cells

John G. Hanlon\textsuperscript{1,2}, Andrew J. Rainbow\textsuperscript{2}, and Gurmit Singh\textsuperscript{1,2†}.

\textsuperscript{1}Hamilton Regional Cancer Centre,
\textsuperscript{2}Department of Biology, McMaster University.

\textit{Running Title:} EXPRESSION PROFILING OF STRESS GENES IN PHOTOFRIN-INDUCED PHOTODYNAMIC THERAPY RESISTANT CELLS REVEALS SPECIFIC CHANGES IN GENE EXPRESSION

\textit{Keywords:} PDT, Photofrin II®, Resistance, Hsp27, stress proteins, expression profiling
ABSTRACT

Photodynamic Therapy (PDT) is known to induce a number of heat shock and stress response genes. In this study we examine a model of Photofrin-induced PDT resistance using the human colon cancer cell line HT29 and its induced resistant cell variant, namely HT29-P14. To determine if this induction of resistance is caused by specific changes in gene expression of stress proteins, cDNA microarrays were screened using radioactively labeled CDNA probes generated from mRNA derived from either the sensitive HT29 cells or the 1.8 fold resistant HT29-P14 cells. Independent experiments identified only one stress protein to be significantly (>5 fold) altered between the two lines. This protein, heat shock protein 27kDa (Hsp27) was found to be expressed at approximately a 20 fold higher level in the resistant HT29-P14 cells. Good correlation was observed between the expression levels determined using microarray analysis and Northern blotting. Furthermore, Western blot analysis confirmed this large difference in mRNA expression was maintained at the level of protein expression. These results successfully demonstrate the use of cDNA microarrays in identifying specific changes in gene expression of PDT induced resistant cell lines.

INTRODUCTION

Photodynamic therapy (PDT) is an increasingly popular treatment for solid tumours requiring three components: a photosensitizing agent such as Photofrin®, the localized delivery of light and molecular oxygen. Together these cause the activation of the systemically administered photosensitizing dyes which are preferentially retained by tumors (Dougherty et al., 1976; Levy, 1994). The primary subcellular targets of our sensitizer, Photofrin® (PII), a commercially available porphyrin product include the plasma membrane, and various subcellular
organelles including mitochondria and endoplasmic reticulum (Gomer et al., 1989; Wilson et al., 1997). It is believed that the reactive oxygen species, singlet oxygen is the primary cytotoxic agent and PDT is known to induce oxidative damage to numerous lipids, proteins and nucleic acids (Moore et al., 1997). While our understanding of PDT is increasing steadily, much remains unknown about the determinants of intrinsic sensitivity and resistance to PDT as well as the mechanisms behind induced resistance to this cancer therapy.

Induction of resistance has been extensively used to study the mechanisms of resistance to many anti neoplastic drugs as reviewed by Morrow and Cowan (1988). Exposures to repeated high doses PDT is a strong selective pressure allowing only the more resistant cells in a population to survive. Therefore, repeated cycles of treatment and regrowth are expected to amplify the biochemical or intracellular changes associated with resistance. We have used this method to study mechanisms of action in both murine RIF cells (Singh et al 1991) and three human tumour cells using three different photosensitizers (Singh et al., 2001). One such cell line, HT29 is a human colon adenocarcinoma to which we developed a PII resistant variant named HT29-P14. The degree of resistance seen in these cells is approximately 1.5-1.8 fold greater than that of the parental population based on colony forming assay and analysis of it’s mechanism(s) of resistance involved are currently being determined in our laboratory. These cells have similar growth and drug uptake rates and do not display the classical MDR phenotype.

PDT, and the oxidative stress associated with it have been shown to induce the synthesis of stress proteins including such Heat shock protein family members as Hsp34, Hsp70, GRP78 and GRP94 (Gomer et al, 1990, 1991). Heat shock proteins are considered some of the most important stress proteins and there is significant evidence showing that increased expression of Hsp’s confer thermotolerance and play a central role in cellular resistance to stresses such as heat
In addition to these Hsp's are a host of other stress proteins including, the survival proteins, various other DNA and protein repair proteins, proteins that mediate cell death and apoptosis and others which help cells tolerate toxic metals, drugs, and other insults (Sarto et al., 2000). Collectively these proteins can all be classified as stress proteins and their expression and action are often responsible for determining the fate of a cell and its resistance or sensitivity to a particular stress.

To explore this relationship between stress protein expression and resistance to PDT, cDNA "stress" microarrays were screened using radiolabeled cDNA probes generated from mRNA transcripts from both the PDT sensitive HT29 and PDT resistant HT29-P14 cells.

Herein, we provide a first step in understanding the relationship between stress protein expression and PDT sensitivity through the use of such commercially available "stress" microarrays, which facilitate broad scale expression profiling of these proteins. Our data implicates the small Heat shock protein (Hsp27) as the only "stress" protein to display significant changes in expression as a result of repeated PDT treatments and its highly significant upregulation is correlated with the increased resistance to PDT noted in the HT29-P14 cells.

MATERIALS AND METHODS

Cells and Culture Conditions. Both cell lines (HT29 and HT29-P14) were grown as a monolayer in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 1% Antibiotic (Gibco-BRL, Burlington, Ontario, Canada). All cell lines were routinely trypsinized with 0.05% trypsin/0.53 mM EDTA three times a week and kept in a humidified atmosphere with 5% CO₂.
Preparation of total RNA. Total RNA was collected from sub-confluent HT29 and HT29-P14 cells. An Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA, USA) was used to isolate this RNA which was suspended in RNAse free H2O as described by the manufacturer. Quantification and purity of the RNA by the A260/A280 absorption and RNA samples with ratios greater than 1.6 were stored at -70°C for further analysis.

Analysis of differential gene expression using a human cDNA microarray. Poly (A)^+ RNA was isolated from total RNA using the Atlas Pure Total RNA Labeling System. To generate radiolabeled cDNA probes, Clontech protocol was followed using their reagents. Poly (A)^+ RNA was reverse-transcribed with Moloney murine leukemia virus (MMLV) in the presence of [α-32P]dATP (NEN). The radiolabeled cDNA probes were purified from unincorporated nucleotides by gel filtration in Chroma Spin-200 columns (Clontech) and hybridized overnight at 68°C to a human “stress” cDNA consisting of 234 known human genes under tight transcriptional control, as described by the manufacturer (Clontech). After a series of high stringency washes (three 20-minute washes in 2x saline-sodium citrate (SSC), 1% SDS followed by two 20-minute washes in 0.1X SSC, 0.5%SDS) at 68°C, the membranes were exposed to x-ray film (Kodak OMAT, Rochester, NY, USA) and subjected to autoradiography. The membranes were also exposed to the Storm 680 phosphoimager and changes in gene expression were quantified using the Image Quant 5.0 analysis software (Molecular Dynamics, Sunnyvale, CA, USA).
Northern Gel Analysis. As described above total RNA was isolated, qualified and quantified. Samples were first precipitated (2.5X EtOH 0.1X NaOAC) then suspended in 20μL of loading buffer (720μL Formamide, 160μl 10X MOPS, 260μL of 37% Formaldehyde, 100μl of 80% Glycerol, 80μl Bromophenol Blue). Samples were denatured at 70°C for 10 minutes then cooled on ice for 5 minutes. 15-25 μg of RNA was then run in a 1.2% agarose gel (1.2% agarose, 0.66 M formaldehyde, 1X MOPS buffer). RNA was separated at 60 V for 3 hours in 1X MOPS buffer. Transfer was performed overnight to a nylon membrane (Boehringer-Mannheim, Laval, Canada) via a gravity assisted capillary action transfer in 10X SSC (1.5M NaCl, 0.15M sodium citrate). Then RNA was then crosslinked onto the membrane using UV light of 1.2J/cm² (Bio-Rad, Mississauga, Canada) and stained with 0.05% methylele blue to visualize the RNA. A copy of this membrane was then made using the photocopier to record the locations and positions of the 18S and 28S ribosomal RNA bands.

Specific Hsp27 probes were made by a restriction digest of 10μg Hsp27 DNA probe in a pUC19 vector (StessGen, Victoria, BC) using the Psi enzyme (Gibco BRL, Burlington, ON, CA) running the fragment as described above and extracting the Hsp27 fragment using a QIAquick Gel Extraction Kit (Quiagen, Santa Clarita, CA). These probes were labeled with [α-32P]dCTP (NEN) using a random labeling kit (Gibco, BRL, Burlington, ON, CA). The membrane was pre-hybridized for one hour then hybridization with the probe was carried out at 48°C overnight using the same hybridization buffer (5X SSC, 50% formamide, 5% Denhardts Solution, 1% SDS, 10% Dextran Sulphate and 100μg/mL denatured sheared salmon testes). The membrane was then washed as follows: 30 minutes (2X SSC 0.5% SDS) at room temperature, two times 45 minutes (0.1X SSC 0.5% SDS) at 65°C and finally for five minutes (0.1X SSC 0.5% SDS) at room temperature. The membrane was then sealed in plastic and visualized using x-ray
radiography or a phosphor-imaging screen (Molecular Dynamics, Sunnyvale, CA USA). Bands were quantified using Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA USA).

To correct for differences in gel loading, integrated optical densities were normalized to human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using an identical protocol to that described above.

**Electrophoresis and Immunoblotting.** Cell were lysed on the plate, on ice for 30 min in 500 μl of lysis buffer (150mM NaCl, 100mM Tris-HCl, 1% Noniodet P-40 (v/v) (pH 8.0)) supplemented with fresh 1mM dithiothreitol, 20 μg/ml leupeptin and 40 μg/ml aprotinin. Insoluble material was pelleted at 15 000 rpm for 10 min at 4° C and the supernatant was removed and stored at -80° C. The protein concentration of the samples was determined according to the Bio-Rad protein assay (Bradford, 1976) (Bio-Rad, Burlington, Ontario, Canada) and 50μg of protein were resolved on 1.0-mm thick 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to Hybond-C nitrocellulose membrane (Amersham, B’ai e d’Urfe, Quebec, Canada) and non-specific binding was blocked overnight with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 7.5% skim milk. The membranes were probed with mouse monoclonal antibodies anti-K-Hsp27 and anti-β-actin (Stressgen, Vancouver, BC Canada). The membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Burlington, Ontario, Canada) for 1 hour at room temperature and the protein bands were visualized by ECL (Amersham Pharmacia Biotech, B’aie d’Urfe, Quebec, Canada).

**Immunohistochemistry.** Approximately 10,000 cells were plated on a sterilized coverslip and allowed to adhere overnight. Cells were washed with cold phosphate buffered saline (PBS) and
fixed in freshly prepared PBS supplemented with 4% paraformaldehyde and 2% bovine serum albumin (BSA) for 10 min. After permeabilizing the cells with 0.25% saponin (Sigma Chemical Co., Mississauga, Ontario, Canada) for 5 min the samples were labeled using mouse monoclonal Hsp27 antibody (1:200 overnight) (StresGen, Vancouver, Canada). This primary antibody was suspended in the permeabilizing solution described above. Finally, the stable fluorescent secondary antibody rabbit anti-mouse ALEXA (Molecular Probes, Oregon, USA) (1:200) was then used. Cells were viewed under fluorescent microscopes and pictures were taken of the both cell lines in addition to detailed analysis of Hsp27 localization and expression within cells.

RESULTS

Microarray Analysis of HT29 and HT29-p14 cells

The relationship between PDT induced resistance and gene expression was determined using cDNA microarrays. Previous studies have shown that cDNA arrays provide a rapid and effective method of monitoring differential gene expression (DeRisi et al., 1996; Schena et al 1996). To investigate the possibility that Photofrin-induced resistance to PDT reflect specific changes in expression of various “stress” genes, a human cDNA microarray that contained 234 known human “stress” genes was screened and analyzed by autoradiography and phosphor-imaging. The levels of nonspecific hybridization were low since the negative DNA controls, including M13mp18(+) strand DNA, γDNA and pUC18 failed to show any hybridization signal. To ensure accurate comparisons in the expression levels of each gene on the cDNA array, hybridization signals were normalized to the signals obtained from nine housekeeping gene controls (i.e., ubiquitin, glyceraldehyde 3-phosphate dehydrogenase, α-tubulin, human leukocyte
antigen [HLA] class 1 histocompatibility antigen C-4, β-actin, 23-Kd highly basic protein and ribosomal protein S9) on the same array.

As shown in Figure 1, the hybridization patterns between the HT29 cells and the PDT induced resistant HT29-P14 cells were similar for the 168 of 169 genes that had signals sufficiently above background and could be analyzed. Analysis of the cDNA array showed that a number of genes showed slight variations in expression, however as determined before the experiment and recommended by the manufacturer, a five fold difference in expression was set as the minimum difference which would be investigated further. Only one gene met this criteria of a high level of differential gene expression, and was found in position K5. This gene, Hsp27 was found to be expressed at approximately 22 fold higher levels in the resistant variant, HT29-P14.

**Northern blot analysis confirms Hsp27 overexpression**

To test the reliability of the cDNA microarray result of Hsp27 overexpression, we analyzed it by Northern blot analysis. As seen in Figure 2, the relative expression levels of HSp27 mRNA observed on Northern blots were consistent with the differential gene expression identified by microarray hybridization. Using GAPDH as a loading control and image Quant analysis software, the level of overexpression was determined to be greater than 20 fold higher in the resistant variant.

**Hsp27 protein is also expressed at greater levels in the resistant variant.**

To test whether the differential levels of mRNA expression corresponded with a realized difference in protein expression of Hsp27, Western blot analysis was performed. As seen in
Figure 3, basal level expression of Hsp27 protein was determined in both HT29 and HT29-P14 cells under various levels of confluence as previous studies have identified Hsp27 expression to be confluence dependent (Garrido et al., 1997). Hsp27 levels were found to be confluence independent in the resistant HT29-P14 cells whereas in the HT29 cells they were found to increase with increasing confluence. That notwithstanding, even at the highest levels confluence, Hsp27 protein was found to be expressed at approximately 10-15 fold higher levels in the resistant HT29-P14 cells. This finding is consistent with the higher levels of Hsp27 mRNA determined by cDNA microarray analysis and Northern blot analysis.

**Immunohistochemical analysis localization in both cell lines**

Hsp27 is has been shown to be found in the cytosol of unstressed cells, however under stress, Hsp27 localization becomes nuclear and perinuclear (MacRae, 2000). Immunohistochemistry using Hsp27 primary antibodies and fluorescent secondary antibodies was performed in both the HT29 and HT29-P14 cells. The large difference in Hsp27 protein expression was obvious as HT29-P14 had a much stronger signal. Accordingly, while both had a mainly cytosolic distribution, in the HT29-P14 cells much of this staining was at the extremities and filopoedia, whereas in the HT29 cells this cytosolic distribution was more even. It was also noted that in approximately 2-4% of HT29 cells, the Hsp27 protein had a nuclear localization pattern. The cause of this localization for this distribution is currently under investigation in our laboratory.

**DISCUSSION**

In the rapidly expanding and evolving area of microarray technology, their most common application remains transcript profiling which is the gene by gene determination of differences in
transcript abundance between two mRNA preparations. We performed such a comparison in this current study. A pair of mRNA samples from PDT sensitive and resistant cells were independently copied as labeled cDNAs with reverse transcriptase then hybridized to spotted microarrays so that radiolabel signals could be computed and compared.

We employed expression profiling to identify a change in gene expression as a result of repeated PDT treatments, which after 14 cycles had resulted in a PDT resistant variant. Instead of investigating the innumerable biological effects that could have occurred by analyzing single genes of putative importance one after the other, this technology allowed us to test the expression of the most important stress proteins at once. In doing so this allowed us to correlate which genes are involved in a biological event and in the future to analyze in detail their (inter)actions and responses to PDT, it is these applications which make microarrays such an indispensable tool.

Our microarray analysis identified the small Heat shock protein of 27 kDa (Hsp27) to be highly overexpressed in the HT29-P14 cell line. This finding was highly reproducible, as was the general pattern of expression for the other genes that were analyzed. Northern bolt analysis confirmed this finding at the messenger level and Western blot analysis showed this difference in abundance to be maintained at the protein level. By identifying the most differentially expressed stress protein between PDT sensitive parental cells and their PDT resistant variant we have rapidly identified a very significant change resulting from repeated PDT treatments which correlates with increased resistance. This is therefore suggestive of Hsp27 playing a protective role in mediating PDT damage. The importance and/or role(s) of Hsp27 in PDT are currently under investigation in our laboratory and there is significant evidence to support such a hypothesis.
It should be noted that other groups employing HT29 cells found an inconsistent association between Hsp27 content and doxorubicin resistance in these colon cancer cells (Garrido et al., 1996). This group later showed Hsp27 to be drastically increased when these HT29 cells reach confluence and designated Hsp27 as a mediator of confluence-dependent resistance to cell death induced by anticancer drugs (Garrido et al., 1997). We too noted changes in the expression of Hsp27 with an increased expression at the higher confluence in the parental HT29 cells. These experiments also showed that the overexpression of Hsp27 in HT29-P14 cells was not confluence dependent and even at the highest confluence, this overexpression is still approximately 20 fold greater in the PDT resistant cells than in the parental HT29 cells.

Through immunohistochemistry, we noted the primarily cytoplasmic localization of Hsp27, which is consistent with the literature. However, our finding of Hsp27 in the nucleus in some HT29 cells under unstressed conditions is surprising and requires further investigation. Such nuclear localization has previously only been associated with stresses that induce Hsp27 and cause the accumulation into insoluble cytoplasmic structures as well as a redistribution into the nucleus (Arrigo, 1998; MacRae, 2000).

Small heat shock protein such as Hsp27 are involved in cellular defense mechanisms against several different types of aggressions and play an important role in responses to stress. They are members of the Heat shock family of proteins as well as being members of the family of so-called ‘survival proteins’ which include other anti-apoptotic proteins (Hsp70, Bcl-2 and IAP and survivin) which are often upregulated in cancer cells (Jaattela, 1999). These proteins participate in essential physiological processes such as regulation of cell cycle, differentiation, programmed cell death and tumourigenicity (Sarto et al., 2000; Arrigo 2000). Of special interest, high levels have been shown confer resistance to a number of challenges, including but not
limited to heat shock, reactive oxygen species (ROS), various drugs including cancer therapies such as cisplatin and other insults such as heavy metals (reviewed by Arrigo and Landry 1994). Hsp27 is known to exhibit chaperone function, primarily by preventing polypeptide aggregation (Hansperger, 1997). It has been shown to mediate an adaptive stress response at the microfilament or intermediate filaments and actin level by stabilizing them and reducing damage to them during oxidative stress (Huot et al., 1991). Hsp27 can also directly increase cellular glutathione (GSH) levels and this is also known to be protective (Mehlen et al 1997). A lot of recent and strong evidence has directly implicated it in inhibiting the apoptotic pathway by modulating cytochrome c release from the mitochondria and further downstream, blocking cytochrome c dependent activation of procaspase-9. Additionally, Hsp27 has been shown to interfere with both programmed cell death and necrotic cell death induced by TNF-α and the Fas ligand (Jaatela, 1999; Garrido, 1999; Bruey et al., 2000; Arrigo, 2000)

Such an understanding of Hsp27 lends credibility to the hypothesis that Hsp27 overexpression in HT29-P14 cells is an important component of PDT resistance. Hsp27 therefore represents an exciting target for further investigation and this is currently underway in our laboratory. If such investigation confirms a role in PDT resistance, Hsp27 then represents a promising target for therapeutic intervention as Hsp27 antisense technology has already been shown to be effective in vitro (Ambrosini, 1998; Arrigo, 2000)

ACKNOWLEDGEMENTS

This work is supported by the National Institute of Health program grant (project 3) CA 43892 and the National Cancer Institute of Canada with funds from the Canadian Cancer Society.

Support for J.G.H. was also provided by NSERC fellowship 232027. Many thanks to Myrna
Espiritu for generating the resistant variants and her ongoing support. We would also like to acknowledge QLT Phototherapeutics Inc for supplying Photofrin II.
Figure 1. cDNA microarray analysis of changes in “stress” gene expression in PDT sensitive HT29 cells and their Photofrin-induced PDT resistant variant HT29-P14 at basal level. $[^{32}P]$-labeled cDNA probes generated from poly (A)+ RNA from HT29 and HT29-P14 cells were hybridized to a cDNA microarray containing 234 known human “stress” genes. After a series of high stringency washes, hybridization patterns were analyzed by autoradiography followed by densitometric scanning of the membranes. The upward arrow represents the localization within the array of the sole gene significantly altered as in the PDT resistance HT29-P14 cells. This gene located at K5 is the human Heat shock protein of 27kDa (Hsp27). The relative expression levels of specific cDNA's were assessed by first normalizing with a wide range of housekeeping genes. Results are representative of 2 separate hybridization experiments.
Figure 2. Northern Blot analysis of Hsp27 levels in HT29 and HT29-P14 cells at basal level. 25ug of total RNA was collected and run on an agarose gel and transferred to a nylon membrane. Hsp27 expression was determined using a radiolabeled Hsp27 DNA probe, and a GAPDH probe was used to serve as a loading control. The blots were analyzed by autoradiography followed by densitometric scanning. Hsp27 mRNA levels were determined to be approximately 24 fold higher in the resistant HT29-P14 cells. Results are representative of 2 separate Northern blot experiments in duplicate.
Northern blot analysis of Hsp27
Figure 3. Western blot analysis of Hsp27 levels in HT29 and HT29-P14 cells at basal level.

Both cell lines were plated and let grow overnight under two conditions, either at low confluence of 40% (left) or at high confluence of 90% (right). 50ug of protein from each sample was run on a 10% SDS-PAGE gel, transferred to a membrane and incubated with Hsp27 mouse monoclonal primary antibodies. The bands were visualized using a HRP conjugated secondary antibody and ECL+. At both levels of confluence, Hsp27 expression is significantly higher in the resistant HT29-P14 variant by approximately 10-15 fold. Results are representative of 2 separate Western blot experiments in duplicate.
CHAPTER 4

PRELIMINARY DATA
Hsp60 is Not Inducible by Photofrin®-Mediated Photodynamic Therapy in T24 Bladder Carcinoma Cells
INTRODUCTION

Photodynamic therapy employs the localized delivery of light in the presence of oxygen to activate photosensitizing dyes which are preferentially retained by tumors (Moan et al., 1982; Levy, 1994). Singlet oxygen has been proposed as the cytotoxic agent responsible for the cellular damage, which occurs at membranes including mitochondria and other cellular organelles (Weishaupt et al., 1976; Wilson et al., 1997).

The oxidative stress associated with PDT increases the expression of a number of stress proteins such as various Heat shock proteins (Hsp) (Gomer et al., 1991; Gomer et al., 1996). These proteins are ubiquitously expressed and are induced as a stress response following exposure to numerous adverse environmental factors (Schlesinger, 1990). Hsp60 is an important molecular chaperone, which binds to nascent or denatured proteins and mediates their proper folding and/or secretion from the cells (Ellis, 1993; Gupta, 1995). It is found in the mitochondrial compartment and also secretory granules and plasma membrane (Soltys & Gupta, 1996). Since, our sensitizer, Photofrin® (PII), localizes to the mitochondria by 18-24 hours (Wilson and Singh, 1997), much of the damage caused will be in proximity to Hsp60 and the proteins that Hsp60 folds.

We have previously generated and used Photofrin®-induced resistant cells to PDT in both murine (Singh et al., 1991) and human cancer cells (Singh et al. 2001) and identified Hsp60 as a potential mediator of this resistance based on greater induction of Hsp60 in the resistant variants relative to their parental cells following PDT or Photofrin® incubation alone. (Hanlon et al., submitted).
In this study, we use a T24 bladder cancer cell model consisting of parental T24 cells, a GRP78 overexpressing clone (A4) and an empty vector control (pcDNA) to further study the role of Hsp60 in PDT. This model is of interest and relevance to PDT because we have also shown increased survival after PDT in the transfected cells (A4) when compared to the parental or vector-transfected cells with a ten to twenty fold decrease in sensitivity at the highest light dose tested (Hanlon et al., unpublished). The purpose of this study was to examine Hsp60 expression after Photofrin® incubation or PDT in the parental as well as GRP78 overexpressing T24 cells.

MATERIALS AND METHODS

Photosensitizer. Photofrin was provided by Quadra Logics Phototherapeutics, Inc. (Vancouver, British Columbia, Canada) and was reconstituted in 5% dextrose in water at 2.5 mg/ml and stored at -20°C. The stock solution was diluted to the appropriate concentration in media immediately before use.

Cells and Culture Conditions. All cell lines (T24 and its clones) were grown as a monolayer in M199 media supplemented with 10% fetal bovine serum, 1% Antibiotic (Gibco-BRL, Burlington, Ontario, Canada). The generation of these stable GRP78 overexpressing clones is described elsewhere (Werstuck et al., 2001). All cell lines were routinely trypsinized with 0.05% trypsin/0.53 mM EDTA three times a week and kept in a humidified atmosphere with 5% CO₂.

In vitro Treatment. Prior to PDT treatment, 1x10⁶ cells/ml were seeded in 100-mm plastic culture dishes and allowed to adhere overnight. Cells were incubated for 18 hrs
with a constant concentration of Photofrin (6 μg/ml) at 37°C. Following incubation, the media containing photosensitizer was removed and replaced with fresh media immediately prior to differing lengths of light exposure (1-7 min.) and irradiated as described previously (Singh et al., 1991). Plates were returned to the incubator for 0-24 hrs. All procedures after plating of the cells were carried out in ambient light. At the end of the incubation period plates were washed in cold PBS, trypsinized and the cells used immediately for flow cytometry. Cells used for electrophoresis were lysed directly on the plate and frozen until ready to be run.

**Flow Cytometry.** Protocol is similar to published protocols (Chant et al., 1995). Cells were trypsinized washed with cold phosphate buffered saline (PBS) and fixed in freshly prepared PBS supplemented with 4% paraformaldehyde and 2% bovine serum albumin (BSA) for 10 min. After permeabilizing the cells with 0.25% saponin (Sigma Chemical Co., Mississauga, Ontario, Canada) for 5 min the samples were labeled using rabbit polyclonal Hsp60 antibody raised against human recombinant Hsp60 (1:30) (Soltys and Gupta, 1996). This primary antibody was suspended in the permeabilizing solution as described above, a Goat anti-rabbit fluorescein isothiocyanate (FITC) secondary antibody in PBS (1:400) (Gibco-BRL, Burlington, Ontario, Canada) for 1 hr. Cells were then washed 3 times in cold PBS and analyzed using the EPICS IV flow cytometer (Coulter Electronics, Haleigh, CA).

**Electrophoresis and Immunoblotting.** Cell were lysed on the plate, on ice for 30 min in 500 μl of lysis buffer (150mM NaCl, 100mM Tris-HCl, 1% Noniodet P-40 (v/v) (pH
supplemented with fresh 1mM dithiothreitol, 20 μg/ml leupeptin and 40 μg/ml aprotinin. Insoluble material was pelleted at 15,000 rpm for 10 min at 4° C and the supernatant was removed and stored at -80° C. The protein concentration of the samples was determined according to the Bio-Rad protein assay (Bradford, 1976) (Bio-Rad, Burlington, Ontario, Canada) and 10μg of protein were resolved on 0.75-mm thick 7.5% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to Hybond-C nitrocellulose membrane (Amersham, B’aie d’Urfe, Quebec, Canada) and non-specific binding was blocked overnight with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 7.5% skim milk. The membranes were probed with mouse monoclonal antibodies anti-Hsp60 or anti-β-actin (StessGen, Vancouver, BC Canada). The membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Burlington, Ontario, Canada) for 1 hour at room temperature and the protein bands were visualized by ECL (Amersham Pharmacia Biotech, B’aie d’Urfe, Quebec, Canada).

**Immunohistochemistry.** Approximately 10,000 cells were plated on a sterilized coverslip and allowed to adhere overnight. PDT treatment was carried out as above and cells were examined 6-8 hours post PDT. First, in ambient light, the cells were incubated in pre-warmed media containing 50 nM Mitotracker red (Molecular Probes, Oregon, USA) for 15 min. They were then washed twice, fixed and washed again. Permabilization and incubation were also carried out as described above but the more stable secondary antibody goat anti-rabbit ALEXA (Molecular Probes, Oregon, USA) at 1:1000 was used to overcome the quick quenching associated with FITC antibodies. Cells were viewed
under confocal as well as fluorescent microscopes and pictures were taken of the various conditions in addition to detailed analysis of mitochondrial and Hsp60 localization within cells.

RESULTS AND DISCUSSION

Hsp60 expression is not altered by GRP78 overexpression

The T24 cell line was used to examine the effect of GRP78 overexpression on Hsp60 expression at basal levels as well as after PDT. Hsp60 expression was determined by both Western blot analysis and flow cytometry in the T24 parental cells, the A4 overexpressing clone which has levels of GRP78 expression approximately two fold that of the parental and finally the pcDNA cells, which serve as an empty vector transfection control. Hsp60 levels were analyzed concurrently with GRP78 levels and were found to be similar in all three cell lines regardless of GRP78 expression. This was repeated three times in duplicate and as a result, we concluded that Hsp60 expression was not altered by changes in GRP78 expression. As always with cell culture work, these findings may be cell line specific and further work is necessary before generalized conclusions may be made. Hsp60 localization, as determined by immunohistochemistry co-localization studies employing the mitochondrial specific dye Mitotracker® revealed similar patterns of mitochondrial staining in all cells tested.

Hsp60 expression is not altered by Photofrin® incubation or PDT in T24 cells

Similar to our work with the HT29 model of PDT sensitive and resistant cells, we examined PDT expression in response to PDT in T24 cells and both their GRP78
overexpressing clone and empty vector clone. In the HT29 and HT29-P14 cells, incubation alone or PDT increased Hsp60 expression in a temporal and dose dependent manner. However, in the T24 cells time course experiments at low and high doses as well as dose response experiments to study Hsp60 levels failed to show any induction of Hsp60 in any of the T24 cells under all conditions tested. These experiments have been performed repeatedly by both Western blot analysis and flow cytometry. While our HT29 cells are colorectal cells, these T24 cells are from a bladder cancer and it has been previously shown that cells from different tissue can respond differently to PDT up to and including the mechanism of death (Moore et al. 1997; Schuitmaker et al., 1996). This finding suggests that Hsp60 induction in response to Photofrin® may be cell line specific. If this is indeed the case, it does not negate the circumstantial evidence that Hsp60 may play a role in the increased resistance of RIF-8A and HT29-P14 cells to PDT. It does however suggest that this induction of Hsp60 may be cell line specific and hence is not a generalizable mechanism of PDT resistance or even a generalizable response to Photofrin incubation. Again, more work, using different cell lines must be performed in order fully understand both the response of Hsp60 to Photofrin or PDT as well as any role it may have in mediating PDT sensitivity.
Figure 1. Representative Western Blot Analysis time course experiment for Hsp60 expression in T24 cells. Cells were incubated with 5ug/ml of Photofrin II for 18 hours. All drug was removed and fresh media added prior to a constant 5-minute irradiation in a light box. Cell lysates were then harvested at 6, 12, 18, and 24 hours post PDT. Equal volumes of whole cell lysates were then separated and transferred to be probed with Hsp60 primary antibody and a HRP tagged secondary antibody. Hsp60 expression was then measured using autoradiography and image quant software. Hsp60 expression was not altered by either PDT or Photofrin II incubation in these cells at any of the times or doses tested. This experiment was done in triplicate with similar results.
Time in Hours: 0 6 12 18 24

D D D D D

Hsp60
GRP78 Protein Expression is Similar in HT29 and
HT29-P14 PDT Treated Cells
INTRODUCTION

The Endoplasmic Reticulum chaperone, Glucose Related Protein, GRP78 binds transiently to nascent, secretory and transmembrane proteins and more permanently to damaged or abnormally folded proteins in the ER. It is known to be induced by anoxia, glucose starvation, alterations in intracellular calcium, inhibitors of glycosylation and various other cellular insults (Gething et al., 1994; Morris et al., 1997). Numerous conditions that cause the induction of GRP's such a glucose starvation or incubation with A23187 (a calcium ionophore) or 2-deoxyglucose are correlated with cellular resistance to various attacks including doxorubicin, and tumour necrosis factor (Shen et al., 1987; Shen et al 1989). Additionally, indirect evidence for the protective function of GRP78 to PDT was found by Gomer et al. (1991). They showed that incubation with A23187, which is known to induce GRP78 caused cellular resistance to PDT in a radiation induced fibrosarcoma cell line. Finally, we have direct evidence that GRP78 overexpression causes PDT resistance through stable transfection experiments using T24 Bladder cancer cells. These findings support a hypothesis that GRP78 can modulate sensitivity to cellular stresses and may have a protective function during and after stress where protein processing in the endoplasmic reticulum is disturbed. This current study was performed to examine the expression of GRP78 protein in the PDT sensitive HT29 and PDT induced resistant HT29-P14 cells to determine if it is involved in the increased resistance of the HT29-P14 cells.
MATERIALS AND METHODS

Cells and Culture Conditions. Both cell lines (HT29 and HT29-P14) were grown as a monolayer in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 1% Antibiotic (Gibco-BRL, Burlington, Ontario, Canada). All cell lines were routinely trypsinized with 0.05% trypsin/0.53 mM EDTA three times a week and kept in a humidified atmosphere with 5% CO₂.

Photosensitizer. Photofrin was provided by Quadra Logics Phototherapeutics, Inc. (Vancouver, British Columbia, Canada) and was reconstituted in 5% dextrose in water at 2.5 mg/ml and stored at -20°C. The stock solution was diluted to the appropriate concentration in media immediately before use.

In vitro Treatment. Prior to PDT treatment, 1x10⁶ cells/ml were seeded in 100-mm plastic culture dishes and allowed to adhere overnight. Cells were incubated for 18 hrs with increasing concentrations of Photofrin (2.5-30μg/ml) at 37°C. Following incubation, the media containing photosensitizer was removed and replaced with fresh media immediately prior to light exposure and irradiated as described previously (Singh et al., 1991; Singh et al., 2001). Plates were returned to the incubator for 0-24 hrs. All procedures after plating of the cells were carried out in ambient light. At the end of the incubation period plates were washed in cold PBS, trypsinized and the cells used immediately for flow cytometry. Cells used for electrophoresis were lysed directly on the plate and frozen until ready to be run.
Electrophoresis and Immunoblotting. Cell were lysed on the plate, on ice for 30 min in 500 μl of lysis buffer (150mM NaCl, 100mM Tris-HCl, 1% Noniodet P-40 (v/v) (pH 8.0)) supplemented with fresh 1mM dithiothreitol, 20 μg/ml leupeptin and 40 μg/ml aprotinin. Insoluble material was pelleted at 15 000 rpm for 10 min at 4° C and the supernatant was removed and stored at -80° C. The protein concentration of the samples was determined according to the Bio-Rad protein assay (Bradford, 1976) (Bio-Rad, Burlington, Ontario, Canada) and 50μg of protein were resolved on 1.0-mm thick 7.5% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to Hybond-C nitrocellulose membrane (Amersham, B’aie d’Urfe, Quebec, Canada) and non-specific binding was blocked overnight with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 7.5% skim milk. The membranes were probed with mouse monoclonal antibodies anti-K-Del and anti-β-actin (StessGen, Vancouver, BC Canada). The membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Burlington, Ontario, Canada) for 1 hour at room temperature and ECL (Amersham Pharmacia Biotech, B’aie D’Urfe, Quebec, Canada), visualized the protein bands.

Immunohistochemistry. Approximately 10,000 cells were plated on a sterilized coverslip and allowed to adhere overnight. PDT treatment was carried out as above and cells were examined 6-8 hours post PDT. First, in ambient light, the cells were incubated in pre-warmed media containing the endoplasmic reticulum stain 50 nM ER Blue (Molecular Probes, Oregon, USA) for 15 min. They were then washed twice, fixed and washed again. Permabilization and incubation were also carried out as described above but the
more stable secondary antibody rabbit anti mouse ALEXA (Molecular Probes, Oregon, USA) at 1:1000 was used to overcome the quick quenching associated with FITC antibodies. Cells were viewed under fluorescent microscopes and pictures were taken of the various conditions in addition to analysis of the endoplasmic reticulum and GRP78 localization within cells.

RESULTS AND DISCUSSION

GRP78 Expression is Similar in HT29 and HT29-P14 Cells

The expression of the Glucose Related Protein, GRP78 was examined by Western blot analysis at basal level, as well as at three hour intervals after PDT at various doses. GRP78 expression was found to be similar in HT29 and HT29-P14 cells at basal level. Both cell lines showed a temporal and dose dependent response to PDT but no induction following Photofrin® incubation alone. The time and degree of this induction was again similar between the parental and PDT resistant cells. Finally, immunohistochemical analysis confirmed localization to the endoplasmic reticulum in all cells tested and no gross morphological changes could be detected in the induced resistant cells.

This data strongly suggests that GRP78 expression is not a factor in the increased resistance of HT29-P14 cells. Based on the similar levels of expression and localization under all conditions tested. However it should be noted that Hsp’s (including the GRP’s) are known to undergo post-translational modification and perform a diverse range of functions (Sarto et al., 2000; Gething, 1999). As a result of these considerations, the above data of similar expression and localization are therefore not sufficient to exclude GRP78 as a factor in the resistance observed in the HT29-P14 cells in this model.
Figure 2: GRP78 protein content for HT29 and HT29-P14 cells exposed to increasing doses of Photofrin II both with and without light activation. Mean fluorescence units are determined using FITC labeled GRP78 antibodies and flow cytometry. Each data point is the average of 3 experiments and SE.
GRP78 Expression in HT29 and HT29-P14 cells
18 hours post PDT
SUMMARY AND CONCLUSIONS
Over the last decade, Photodynamic Therapy has become an increasingly more accepted and popular choice for some solid tumours. Despite this increased approval and use, the mechanism(s) by which PDT exerts its cytotoxic effect as well as the mechanism(s) by which some cells survive this insult remains unclear.

We have demonstrated that some cells, namely RIF and HT29 can attain a degree of induced resistance. Although this resistance is generally no more than two fold, the phenomena is clinically relevant and an important tool for understanding mechanisms of action by, and response to PDT.

As other groups have previously noted, PDT exerts a significant oxidative stress on the cells and causes various so-called stress responses including the induction of various early response genes (Luna et al., 1994) as well as various Heat shock proteins (Hsp) including the Glucose Related Proteins (GRP) (Gomer et al., 1991, 1996, 1996).

We have hypothesized that these various stress response pathways elicited by PDT are important mediators of PDT sensitivity and may be involved in PDT resistance. Specifically we have hypothesized that the mitochondrial stress protein (Hsp60) is involved in the resistance observed in the HT29-P14 cells. This was based on evidence that the mitochondria are primary targets of porphyrin-mediated PDT (Hisazumi et al 1984, Wilson et al., 1997) The preliminary evidence for mitochondrial changes observed in HT29-P14 cells and the localization of Hsp60 to the mitochondrial matrix.

We examined the level of Hsp60 protein expression following either Photofrin® incubation or PDT by flow cytometry. Significant differences were found between the two cells where the resistant HT29-P14 cells displayed higher Hsp60 content per cell as
well as significantly higher levels of dose-dependent and temporal induction as a result of incubation alone. Surprisingly, there was no significant increase in expression in either cell line following photosensitization, this suggests that the presence of the drug is responsible for the initial increase in expression. Increased induction of Hsp60 may protect the HT29-P14 cells from the subsequent damage caused by photoactivation. While this correlation of increased induction by Hsp60 and PDT resistance now exists in both a murine and human tumour model, more direct experiments are required before definitive conclusions about the role of Hsp60 in PDT may be drawn. It must, however be acknowledged that Hsp60 is not induced in all cells in response to PDT as we have shown in the preliminary data in a T24 bladder cancer cells which may negate the importance of Hsp60 as a generalizable mediator of PDT sensitivity.

We also hypothesized that overexpression of GRP78 protein causes cellular resistance to PDT. This was based on evidence that the calcium ionophore A23187, which causes GRP78 induction, caused resistance to PDT (Gomer et al., 1991). Additionally, GRP78 is a chaperone protein which would be required to refold the proteins damaged by PDT and is localized in the endoplasmic reticulum where Photofrin® is also known to be found. (Afonso et al., 1999).

We examined the sensitivity towards PDT by colony forming assay in parental T24 bladder cancer cells, a GRP78 overexpressing clone (A4) and an empty vector control (pcDNA). The GRP78 overexpressing clone was found to be 10-20 fold more resistant to PDT at the highest doses tested relative to the parental cells or empty vector control. While the overexpressing clone had a two-fold increase in GRP78 expression at basal levels, the levels of all three cell lines were comparable after each was induced in a
temporal and dose dependent manner by PDT. No induction was found in any of the cell lines by incubation alone. This finding is direct evidence implicating elevated levels of GRP78 and increased resistance to PDT. Taken together, these findings are also suggestive that the levels of GRP78 expression at the time of photoactivation and the initial insult are important in determining PDT sensitivity however more experiments are necessary to confirm this hypothesis. Studies of GRP78 levels in the HT29 model did not show any difference in GRP78 expression before or after PDT between the two cells, suggesting that GRP78 expression is not involved in the increased resistance of HT29-P14 cells.

The most deadly stage of tumorgenesis is when tumour cells become resistant to therapy and can metastasize. This progression is a multistep process stemming from the accumulation of genetic events that render the tumour more motile and invasive as well as resistant to apoptosis or other forms of drug induced killing. These characteristics of cancers, including drug sensitivity can differ in each patient and can change over time or as a result of repeated treatment. In an attempt to understand the changes associated with the increased resistance in our HT29-P14 cells, we employed cDNA microarray technology to analyze the expression profile of 234 human “stress” genes simultaneously. This enabled us to screen for changes in expression which could be correlated with the change in PDT sensitivity.

Microarray analysis revealed that the repeated PDT treatments which resulted in the PDT resistant variant did not significantly alter the “stress” gene profile of these cells. 168 of the 169 analyzable genes did not have a significant (> 5 fold) change in gene expression. The only exception was Hsp27 which was found to be expressed at levels
20-fold higher in the resistant variant. This finding was confirmed by Northern blot analysis and shown to be maintained at the protein level by Western blot analysis. This finding implicates Hsp27 overexpression as a possible mechanism of the increased resistance in HT29-P14 cells. This protein has been shown to inhibit apoptosis, increase GSH levels, act as a chaperone and stabilize actin and microfilaments under times of stress; each of these mechanisms could contribute to increased PDT resistance and the significance of this overexpression is currently being examined in our laboratory.

As demonstrated by the data, a degree of induced resistance is possible to PDT. This induced resistance is correlated with changes in the expression of at least two Heat shock proteins, Hsp60 and Hsp27. While the significance of these alterations still remains unclear, this work provides some insight and is the basis for further investigation. In addition to studies documenting expression of these proteins, proteonomic based studies will soon offer a much more precise and efficient way to probe protein expression and modification resulting from diseases and characterize changes in cellular sensitivity to various treatments. It should be noted that variations in protein expression as well as variations in the presence and in the amount of post-translational modifications, such as phosphorylation, or oligomerization have been observed to influence the structure and function of Hsps, especially Hsp27. Since many post-translational modifications are not well characterized (Sarto et al., 2000), some of the functions and effects of Hsp’s await better explanation before we can fully understand the complex role of these proteins in cancer and cancer therapy. It is only when this understanding is complete, that the exact role of Hsp’s in PDT and other cancer therapies can be fully understood.
FUTURE DIRECTIONS
(1) Hsp60

The expression of Hsp60 in response to Photofrin incubation should be examined in various other cell lines to determine whether or not Hsp60 induction is cell type specific. Studies with stable transfection of Hsp60 sense or antisense still remains a possibility, however microarray analysis has shown that many ‘stress’ genes are differentially expressed in the resistant cells at ratios greater than that of Hsp60. The exact mechanism of how the presence of the Photofrin drug causes Hsp60 induction also remains a mystery and an interesting avenue of investigation. Finally, Hsp60 expression in the other HT29 resistant variants should be examined.

(2) GRP78

We have shown GRP78 overexpression to cause cellular resistance to PDT and hypothesized that the initial higher levels are what confer sensitivity. While the data suggests that the levels of GRP78 at time of photosensitization are what determine survival, other experiments are necessary to confirm this hypothesis. PDT in the presence of transcription inhibitors as well as using a clone with a reporter gene such as luciferase (instead of GRP78) are potential experiments for confirming this hypothesis and confirm that the CMV promoter is not involved in the stress response. Dr Austin and his group have already confirmed that this CMV promoter is not activated by various other oxidative stresses (Austin, pers. comm.). Studies of GRP78 expression in the other HT29 resistant variants may also be worth considering.
(3) Microarray and Hsp27

This component of the thesis has undoubtedly been the most ‘data rich’. That not withstanding, further analysis of the data with more technically advanced software programs may reveal patterns or clustering that were not detectable in the original analysis. Such software will soon be altruistically available on such sites as ‘microarrays.org’ and will facilitate this analysis. Moreover these “stress” cDNA membranes can still be used and should be re probed to test the expression profile of HT29 and HT29-P14 shortly after PDT in order to look for differences in the stress response to PDT itself.

Some of the future directions of the Hsp27 work are already underway. These experiments include: post incubation/PDT time course and dose response experiments, immunohistochemical analysis and co-localization studies (with actin etc.), stable transfection experiments using sense and antisense Hsp27, and finally analysis of phosphorylation and oligomerization status in both cells before, during and after PDT. Combined these experiments should help give us a more complete understanding of the role/importance of Hsp27 overexpression in the PDT resistant HT29-P14 cells.
REFERENCES


APPENDIX
Appendix 1 (From Chapter 1). Survival curves for HT29 (■) and HT29-P14 (x) cells exposed to increasing doses of Photofrin as determined by colony forming assays. Cells were incubated for 18 h with Photofrin II in the dark then washed and fresh media was added. Each data point is the average of a representative experiment done in triplicate and it’s SD.
CFA FOR HT29 AND HT29-P14 cells
Photofrin Incubation (No Light)
Appendix 2. (From Chapter 1): Doubling times of HT29 cells and their HT29-P14 variants were determined using a Heoscht assay. Known numbers of each cell line were used to generate standard curves and growth rate was determined by harvesting cells every 24 hours for 4 days. Data repeated in triplicate ±SD. Experiment was repeated three times.
Growth rate - Doubling Time

Cell Type

Doubling time (Hours)

- HT29-P14
- HT29
Appendix 3 (From Chapter 1): To study drug uptake dynamics HT29 cells and their HT29-P14 variants were incubated in two concentrations of Photofrin II for 18 hours. Flow cytometry was then used to measure the mean florescence per cell for 10 000 cells. Cells without drug were used to determine auto-fluorescence. Data repeated in triplicate ±SE. Experiment was repeated three times.
Drug uptake after 18 hours incubation with Photofrin

Drug Concentration (ug/uL)

Mean Fluorescence Units
Appendix 4 (From Chapter 1): Representative Western Blot Analysis of time course experiment for HT29-P14 cells. Cells were incubated with 10ug/ml of Photofrin II for 18 hours. All drug was removed and fresh media added prior to a constant 5-minute irradiation in a light box. Cell lysates were then harvested at 2, 4, 6, 8, and 24 hours post PDT. Equal volumes of whole cell lysates were then separated and transferred to be probed with Hsp60 primary antibody and a HRP tagged secondary antibody. Hsp60 expression was then measured using autoradiography and image quant software. Hsp60 expression was found to be significantly elevated and greatest in HT29-P14 cells at 6-8 hours post PDT. This experiment was done in triplicate with similar results.
Appendix 5 (From Chapter 1): Representative Western Blot Analysis of dose response experiment for HT29-P14 cells. Cells were incubated with varying doses (7.5-15ug/ml) of Photofrin II for 18 hours. All drug was removed and fresh media added prior to a constant 5-minute irradiation in a light box except those cells in the Drug-No Light and No Drug-No Light condition. Cell lysates were then harvested at 6 hours post incubation. Equal volumes of whole cell lysates were then separated and transferred to be probed with Hsp60 primary antibody and a HRP tagged secondary antibody. Hsp60 expression was then measured using autoradiography and image quant software. Hsp60 expression was found to increase with increasing levels of drug regardless the presence or absence of light. This experiment was done in triplicate for HT29-P14 cells with similar results.
Appendix 6 (From Chapter 2): Survival curves for T24 cells and their various clones (pcDNA & four GRP78 overexpressors) exposed to increasing doses of Photofrin II as determined by colony forming assays. Cells were incubated for 18 h with Photofrin II in the dark then washed and fresh media was added prior to 5 minutes of irradiation in a light box. From these colony-forming experiments A4 was found to be the most significantly resistant clone and was thus used for further analysis. Each data point is the average of a representative experiment done in triplicate and it’s SD. The downward arrow represents zero surviving colonies.
CFA T24 cells: Parental, pcDNA and Overexpressing clones

![Graph showing photofrin concentration vs percent survival for different clones.](image-url)
Appendix 7 (From Chapter 2): Representative Western Blot Analysis of GRP78 basal level experiments for T24 cells, pcDNA cells and the overexpressing clone A4. Cells were plated and allowed to adhere overnight. Cell lysates were then harvested and equal volumes of whole cell lysates were then separated and transferred to be probed with a K-Del primary antibody (recognizes both GRP78 and GRP94 proteins) and a HRP tagged secondary antibody. GRP78 expression was then measured using autoradiography and image quant software using actin and GRP94 as loading controls. GRP78 expression was found to be approximately 1.5-2 fold higher in the A4 clone than either the parental or empty vector control. This experiment was done repeatedly with similar results, however over time the degree of overexpression continued to decrease.
Appendix 8 (From Chapter 2): Representative Western Blot Analysis of time course experiment for T24 cells GRP78 overexpressing clone (A4). Cells were incubated with 4µg/ml of Photofrin II for 18 hours. All drug was removed and fresh media added prior to a constant 5-minute irradiation in a light box. Cell lysates were then harvested at 4, 8, 12, 16, 20, 24 and 30 hours post PDT. Equal volumes of whole cell lysates were then separated and transferred to be probed with a K-Del primary antibody (recognizes both GRP78 and GRP94 proteins) and a HRP tagged secondary antibody. GRP78 expression was then measured using autoradiography and image quant software using actin as a loading control. GRP78 expression was significantly induced and greatest in these T24 clone cells at 12-20 hours post PDT. PDT did not alter GRP94 expression in these cells. This experiment was repeated twice with similar results.
Time in Hours: 0 4 8 12 16 20 24 30
A4 D D D D D D D D

Time Course

GRP94
GRP78
Appendix 9 (From Chapter 3): Representative Western Blot Analysis of dose response experiment for pcDNA and A4 GRP78 overexpressing cells. Cells were incubated with varying doses (2-4ug/ml) of Photofrin II for 18 hours. All drug was removed and fresh media added prior to a constant 5-minute irradiation in a light box except those cells in the Drug-No Light and No Drug-No Light condition. Cell lysates were then harvested at 16 hours post incubation. Equal volumes of whole cell lysates were separated and transferred to be probed with a K-Del primary antibody and a HRP tagged secondary antibody. GRP78 expression was then measured using autoradiography and image quant software. GRP78 expression was found to increase with increasing levels of drug in the PDT condition but no induction was observed in the absence of light. Again GRP94 was not induced at any dose. Experiment was repeated and also quantified by flow cytometry with similar results.
## Dose Response

<table>
<thead>
<tr>
<th></th>
<th>A4</th>
<th>pcDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDNL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2DL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4DL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4DNL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDNL</td>
<td></td>
<td>GRP94</td>
</tr>
<tr>
<td>2DL</td>
<td></td>
<td>GRP78</td>
</tr>
<tr>
<td>4DL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4DNL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix 10 (From Chapter 3): Quantification of significant results for HT29 and HT29-P14 microarray analysis in tabular form. One gene (Hsp27) was found to be significantly (>5 fold) differentially expressed. One other gene was found to be expressed differentially at a level of greater than two fold; this gene is known both at glutathione peroxidase-gastrointestinal (GSH-PX-GI) and also glutathione peroxidase-related protein 2 (GSH-RP2). For completion, the relative levels of Hsp60 and GRP78 at the mRNA level are also included. For each gene the expression has been normalized to one for HT29 and the relative HT29-P14 value is then expressed accordingly.
HT 29 and HT29-P14 Microarray Analysis: Relative Expression

<table>
<thead>
<tr>
<th>GENE (position)</th>
<th>HT29</th>
<th>HT29-P14</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Significantly Altered Expression (&gt; 5 fold)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp27 (K5)</td>
<td>1</td>
<td>21.7</td>
</tr>
<tr>
<td><strong>Altered Expression (&gt;2.5 fold)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase-gastrointestinal (GSHPX-GI)</td>
<td>1</td>
<td>0.335</td>
</tr>
<tr>
<td>AKA Glutathione peroxidase-related protein (GPRP) (E23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Also of Interest</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp60 (L7)</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>GRP78 (M6)</td>
<td>1</td>
<td>1.1</td>
</tr>
</tbody>
</table>