Photodynamic therapy induces oxidation in breast and brain cancer cell lines

Sarah Ann Ziegler

University of Nevada, Las Vegas

Follow this and additional works at: https://digitalscholarship.unlv.edu/rtds

Repository Citation
https://digitalscholarship.unlv.edu/rtds/2030

This Thesis is brought to you for free and open access by Digital Scholarship@UNLV. It has been accepted for inclusion in UNLV Retrospective Theses & Dissertations by an authorized administrator of Digital Scholarship@UNLV. For more information, please contact digitalscholarship@unlv.edu.
PHOTODYNAMIC THERAPY INDUCES OXIDATION IN BREAST AND 
BRAIN CANCER CELL LINES

by

Sarah Ann Ziegler

Bachelor of Science
University of Nevada, Las Vegas
2003

A thesis submitted in partial fulfillment
of the requirements for the

Masters of Science Degree in Biochemistry
Department of Chemistry
College of Sciences

Graduate College
University of Nevada, Las Vegas
August 2006

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Copyright by Sarah Ann Ziegler 2006
All rights reserved
The Thesis prepared by
Sarah Ann Ziegler

Entitled
The Role of Oxidation in Photodynamic Therapy Induced Cytotoxicity
in Cancer

is approved in partial fulfillment of the requirements for the degree of
Masters of Science Degree in Biochemistry

Examination Committee Chair

Dean of the Graduate College
ABSTRACT

Photodynamic Therapy Induces Oxidation in Breast and Brain Cancer Cell Lines

by

Sarah Ann Ziegler

Dr. Stephen W. Carper, Examination Committee Chair
Professor of Chemistry
University of Nevada, Las Vegas

Photodynamic Therapy (PDT) is a cancer treatment modality that utilizes both a photosensitizing drug and light irradiation. To better understand how PDT induces cell death, four human breast cancer (DC4, DB46, MCF7 and MDA-MB-435) and two rat glioma (BT4C and F98) cell lines were treated with 635 nm light from a diode laser following incubation with either Photofrin® or aminoleuvulinic acid (ALA). Cellular responses were evaluated by: clonogenic survival, cell cycle distribution, fluorescent microscopy, protein oxidation and lipid oxidation assays. PDT was able to induce both apoptosis and necrosis as well as protein and lipid oxidation. Even though breast cancer cells were more sensitive to Photofrin® as compared to ALA-mediated PDT, there was less oxidation in the Photofrin® treated cells. While it appears that Photofrin® and ALA-mediated PDT caused cell death by two different mechanisms, bulk oxidation of either lipids or proteins was not correlative to cellular survival.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................................. iii
LIST OF FIGURES ...................................................................................................................... v
LIST OF TABLES ...................................................................................................................... vii
ABBREVIATIONS .................................................................................................................. viii
ACKNOWLEDGEMENTS ........................................................................................................ ix
CHAPTER 1 INTRODUCTION ................................................................................................ 1
CHAPTER 2 EXPERIMENTAL PROCEDURES ................................................................ 19
CHAPTER 3 RESULTS ........................................................................................................... 31
CHAPTER 4 DISCUSSION ..................................................................................................... 88
REFERENCES .......................................................................................................................... 101
VITA ........................................................................................................................................... 104
<table>
<thead>
<tr>
<th>Fig. 1.</th>
<th>Photosensitizer excitation</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 2.</td>
<td>The use of PDT to generate ROS</td>
<td>14</td>
</tr>
<tr>
<td>Fig. 3.</td>
<td>The heme biosynthetic pathway</td>
<td>15</td>
</tr>
<tr>
<td>Fig. 4.</td>
<td>$\alpha$-tocopherol's anti-oxidant function</td>
<td>16</td>
</tr>
<tr>
<td>Fig. 5.</td>
<td>The function of glutathione within the cell</td>
<td>17</td>
</tr>
<tr>
<td>Fig. 6.</td>
<td>BSO inhibition of glutathione formation</td>
<td>18</td>
</tr>
<tr>
<td>Fig. 7.</td>
<td>The quantification of lipid peroxidation</td>
<td>29</td>
</tr>
<tr>
<td>Fig. 8.</td>
<td>The quantification of reduced thiol residues</td>
<td>30</td>
</tr>
<tr>
<td>Fig. 9.</td>
<td>Clonogenic survival of MDA-MB-435 without light</td>
<td>46</td>
</tr>
<tr>
<td>Fig. 10.</td>
<td>Clonogenic survival of tumor cells exposed to light</td>
<td>47</td>
</tr>
<tr>
<td>Fig. 11.</td>
<td>Clonogenic survival of MDA-MB-435 cells with ALA-mediated PDT</td>
<td>48</td>
</tr>
<tr>
<td>Fig. 12.</td>
<td>Clonogenic survival of DB46 cells with ALA-mediated PDT</td>
<td>49</td>
</tr>
<tr>
<td>Fig. 13.</td>
<td>Clonogenic survival of DC4 cells with ALA-mediated PDT</td>
<td>50</td>
</tr>
<tr>
<td>Fig. 14.</td>
<td>Clonogenic survival of MCF7 cells with ALA-mediated PDT</td>
<td>51</td>
</tr>
<tr>
<td>Fig. 15.</td>
<td>Clonogenic survival of F98 cells with ALA-mediated PDT</td>
<td>52</td>
</tr>
<tr>
<td>Fig. 16.</td>
<td>Clonogenic survival of BT4C cells with ALA-mediated PDT</td>
<td>53</td>
</tr>
<tr>
<td>Fig. 17.</td>
<td>Clonogenic survival of MDA-MB-435 cells with Photofrin®-mediated PDT</td>
<td>54</td>
</tr>
<tr>
<td>Fig. 18.</td>
<td>Clonogenic survival of DB46 cells with Photofrin®-mediated PDT</td>
<td>55</td>
</tr>
<tr>
<td>Fig. 19.</td>
<td>Clonogenic survival of DC4 cells with Photofrin®-mediated PDT</td>
<td>56</td>
</tr>
<tr>
<td>Fig. 20.</td>
<td>Clonogenic survival of MCF7 cells with Photofrin®-mediated PDT</td>
<td>57</td>
</tr>
<tr>
<td>Fig. 21.</td>
<td>Flow cytometry of DC4 cells after ALA-mediated PDT</td>
<td>59</td>
</tr>
<tr>
<td>Fig. 22.</td>
<td>Fluorescence analysis of photosensitized cells</td>
<td>64</td>
</tr>
<tr>
<td>Fig. 23.</td>
<td>Fluorescence analysis of photosensitized cells at varying times</td>
<td>65</td>
</tr>
<tr>
<td>Fig. 24.</td>
<td>Attachment assay of MDA-MB-435 cells after ALA-mediated PDT</td>
<td>66</td>
</tr>
<tr>
<td>Fig. 25.</td>
<td>Attachment assay of MDA-MB-435 cells after Photofrin®-mediated PDT</td>
<td>67</td>
</tr>
<tr>
<td>Fig. 26.</td>
<td>Attachment assay of BT4C cells after ALA-mediated PDT</td>
<td>68</td>
</tr>
<tr>
<td>Fig. 27.</td>
<td>Fluorescent microscopy studies with ALA-mediated PDT</td>
<td>69</td>
</tr>
<tr>
<td>Fig. 28.</td>
<td>Fluorescent microscopy study of MDA-MB-435 cells</td>
<td>70</td>
</tr>
<tr>
<td>Fig. 29.</td>
<td>Protein oxidation after ALA-mediated PDT</td>
<td>71</td>
</tr>
<tr>
<td>Fig. 30.</td>
<td>Protein oxidation of MDA-MB-435 cells with BSO and vitamin E</td>
<td>72</td>
</tr>
<tr>
<td>Fig. 31.</td>
<td>Protein oxidation of MDA-MB-435 cells after PDT</td>
<td>73</td>
</tr>
<tr>
<td>Fig. 32.</td>
<td>Lipid peroxidation measured using HPLC</td>
<td>74</td>
</tr>
<tr>
<td>Fig. 33.</td>
<td>Fluorescent microscopy of MDA-MB-435 cells after PDT</td>
<td>75</td>
</tr>
<tr>
<td>Fig. 34.</td>
<td>Clonogenic survival of MDA-MB-435 cells with BSO and vitamin E</td>
<td>76</td>
</tr>
<tr>
<td>Fig. 35.</td>
<td>Clonogenic survival of DB46 cells with BSO and vitamin E</td>
<td>77</td>
</tr>
<tr>
<td>Fig. 36.</td>
<td>Clonogenic survival of DC4 cells with BSO and vitamin E</td>
<td>78</td>
</tr>
<tr>
<td>Fig. 37.</td>
<td>Clonogenic survival of MCF7 cells with BSO and vitamin E</td>
<td>79</td>
</tr>
</tbody>
</table>
Fig. 38. Clonogenic Survival of MDA-MB-435 with Photofrin®, BSO and vitamin E ................................................................. 80
Fig. 39. Clonogenic survival of DC4 cells with Photofrin® and vitamin E .......... 81
Fig. 40. Flow Cytometry Analysis of cell cycle distribution ................................ 82
Fig. 41. Fluorescence of MDA-MB-435 cells with Photofrin® and vitamin E ........ 83
Fig. 42. Oxidative sensitive probe with BT_4C cells ............................................. 84
Fig. 43. Oxidative sensitive probe with MCF7 cells ................................................. 85
Fig. 44. Oxidative sensitive probe with MDA-MB-435 cells treated with ALA mediated PDT .................................................................................................................. 86
Fig. 45. Oxidative sensitive probe with MDA-MB-435 cells treated with Photofrin® mediated PDT ........................................................... 87
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table I</td>
<td>Calculated EC$_{50}$ values for PDT</td>
<td>58</td>
</tr>
<tr>
<td>Table II</td>
<td>Flow Cytometry Analysis of MDA-MB-435 cells</td>
<td>60</td>
</tr>
<tr>
<td>Table III</td>
<td>Flow Cytometry Analysis of DB46 cells</td>
<td>61</td>
</tr>
<tr>
<td>Table IV</td>
<td>Flow Cytometry Analysis of DC4 cells</td>
<td>62</td>
</tr>
<tr>
<td>Table V</td>
<td>Flow Cytometry Analysis of MCF7 cells</td>
<td>63</td>
</tr>
<tr>
<td>Table VI</td>
<td>Summary of Results</td>
<td>100</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>ALA</td>
<td>5-aminoleuvilinic acid</td>
<td></td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 protein involved in apoptosis signaling</td>
<td></td>
</tr>
<tr>
<td>BSO</td>
<td>buthionine sulfoximine</td>
<td></td>
</tr>
<tr>
<td>CAD</td>
<td>caspase activated DNase</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Eagle Minimum Essential Medium</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5-ditosbis(2-nitro-benzoic acid), Ellman’s Reagent</td>
<td></td>
</tr>
<tr>
<td>EC_{50}</td>
<td>effective concentration (50%)</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
<td></td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
<td></td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
<td></td>
</tr>
<tr>
<td>Foscan</td>
<td>m-tetrahydroxy-phenylchlorin</td>
<td></td>
</tr>
<tr>
<td>G1 phase</td>
<td>gap 1 phase of the cell cycle</td>
<td></td>
</tr>
<tr>
<td>G2 phase</td>
<td>gap 2 phase of the cell cycle</td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione (reduced)</td>
<td></td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione (oxidized)</td>
<td></td>
</tr>
<tr>
<td>hsp</td>
<td>heat shock proteins</td>
<td></td>
</tr>
<tr>
<td>ICAD</td>
<td>inhibitor of caspase activated DNase</td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
<td></td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
<td></td>
</tr>
<tr>
<td>PDT</td>
<td>photodynamic therapy</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
<td></td>
</tr>
<tr>
<td>PpIX</td>
<td>protoporphyrin IX</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
<td></td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
<td></td>
</tr>
<tr>
<td>S phase</td>
<td>synthetic phase of the cell cycle</td>
<td></td>
</tr>
<tr>
<td>TBA</td>
<td>2-thiobarbituric acid</td>
<td></td>
</tr>
<tr>
<td>TEP</td>
<td>1,1,3,3-tetraethoxypropane</td>
<td></td>
</tr>
<tr>
<td>vitamin E</td>
<td>a-tocopherol</td>
<td></td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I am indebted to Dr. Stephen Carper for his unlimited amount of patience and his always helpful advice about science and life in general. He has helped me to become a better scientist. I would also like to thank Dr. Steen Madsen in his guidance with this project. His vast amount of wisdom in the area of PDT has helped me to become more efficient in my research. I am also grateful for the technical support that Casey Hall, Nicole Stevens, Kyler Elwell, Van Vo, Anu Elegbede, Shirley Shen and John Adebayo have provided. Undergraduates Becca Cox and Cherisse Loucks, did an amazing job with their research projects and the data they provided is included in this thesis. I also need to thank Dr. MaryKay Orgill and Grant Aguinaldo for their help in reviewing and editing this manuscript. I appreciate the time and effort that all my committee members have applied to my project. Finally, I would like to thank my family for their love, patience and support in my educational pursuits, especially my mother, my grandparents, my husband Jason and my daughter Gabrielle.
Despite more anti-cancer treatment advances, cancer is the second leading cause of death in the United States. More than one million new cases of cancer were diagnosed in 2005 alone (1). There are many treatment modalities currently available for cancer including surgery, chemotherapy, radiation, hormonal treatments and immunotherapy. These treatments have various side effects including pain, nausea, vomiting, hair loss, skin tenderness and fatigue. Unfortunately, with so many treatment options available, there is still the risk of cancer recurrence. Recurrent cancer is more difficult to treat due to gene mutations which cause resistance to anti-cancer therapies. Even though there are many new treatment options available, scientists have not eliminated the high mortality rate associated with cancer. Thus, it is of the utmost importance to develop more advanced and personalized treatment modalities.

Breast cancer was diagnosed in more than 200,000 women in the United States annually (2); furthermore breast cancer was the second most deadly form of cancer in women, with more than 40,000 fatalities reported in 2005(1). In the treatment of breast cancer, combination therapies are commonly used. Most women who are diagnosed with breast cancer will undergo a surgical resection to remove the tumor. In the majority of cases surgical resection is used in conjunction with focused radiation therapy to the tumor bed. Women who have breast cancer and are estrogen receptor (ER) positive or...
progesterone receptor (PR) positive may also be given anti-hormone treatments to retard the growth and spread of the cancer (3). Chemotherapy can also be administered in the form of a drug cocktail including multiple anti-cancer agents; however even with this complex treatment plan, breast cancer proves deadly for many women.

Unfortunately, many breast cancer patients develop recurrent tumors on the chest wall after preliminary treatments. Up to 5% of breast cancer patients will be affected by chest wall recurrence (4). Treatment of these recurrent sites include the same therapies used at the initial tumor location. The second round of treatment is sometimes unsuccessful, leaving physicians with few options. If these lesions are left untreated they can spread and ulcerate causing possible infection and bleeding (5). Many aggressive treatment modalities for chest wall recurrence can be fatal due to their ability to compromise the chest wall and the proximity of the lungs to the tumors (6). Chest wall recurrence is usually very painful and requires the patient to use analgesic pain medications. Since current treatments for chest wall lesions usually result in a recurrence of the tumors, a new treatment modality is needed. Photodynamic therapy has been studied for use with these tumor sites with promising results (7).

Photodynamic therapy (PDT) is an underutilized treatment modality and needs to be more aggressively studied in the U.S. The majority of publications on PDT research are of European and Asian origin. PDT has advantages over other treatment modalities in its ability to target specific tumor sites. PDT is administered in a two part treatment that involves the delivery of a photosensitizing drug followed by light irradiation. A photosensitizer is a chemical that absorbs light and causes a chemical or physical change in another molecule (8). By using both a photosensitizer and a laser light, the clinician is
able to specifically target tumor sites and induce cell death in a localized area (9). The photosensitizer uptake into the tumor cells happens preferentially to normal cells (10). This preferential uptake enhances PDT’s ability to target and localize damage to tumor cells. By combining a controlled light irradiation and preferential uptake of the photosensitizer into tumor cells, ubiquitous cell damage to normal cells can be minimized.

PDT works by harnessing energy from light and using it to induce cytotoxicity in tumor cells. The photosensitizing drug absorbs light energy within the cell and is elevated to an excited singlet state (Fig. 1, page 13). Subsequently, the photosensitizer is able to intersystem cross to an excited triplet state. Once in a triplet state, the photosensitizer can undergo two types of reactions to cause oxidative stress and/or damage (11). The Type I reaction, through the transfer of an electron or hydrogen, causes the formation of free radical species (Fig. 2, page 14). These free radicals can lead to the formation of superoxides. The Type II reaction is characterized by the formation of reactive oxygen species (ROS) through the excitation of oxygen from a triplet ground state to an energized singlet state. The reactive oxygen species and superoxides cause cell damage, leading to cell death.

Besides being able to absorb light and cause the formation of reactive oxygen species within the cell, many of the photosensitizers have intrinsic fluorescent properties. Fluorescence happens when the photosensitizer is able to absorb light energy and reach an excited singlet state. If this energy is rapidly released, fluorescence occurs by the emission of a photon. The light emission of the molecule is at a longer wavelength and a lower energy level than the light used to initially excite the photosensitizer. This
fluorescent property of the photosensitizers has been utilized by physicians to help in the diagnostic process of cancer therapy. By giving a patient a systemic photosensitizer dose and then exciting the photosensitizer with light, clinicians are able to visualize the whole tumor region with the naked eye. The photosensitizers' fluorescent properties also give scientists an easy way to quantify the amount of photosensitizer within the tumor cells.

But, like all treatments, there are possible side effects and problems with PDT treatment. After giving the patient a photosensitizer, the patient's whole body will become sensitized to light. Depending on the photosensitizer used this time may range from two days to a month or more, causing the patients to keep their skin covered at all times and wear protective eye wear. There is also the possibility of pain and burning near the treatment site or adverse side effects to the photosensitizer drug, but these side effects are rare.

One of the limitations of PDT is the availability of the light source to reach the tumor area. In order for PDT to be effective, the laser light must be able to reach the tumor location. Many researchers and physicians are exploring new ways to overcome this obstacle, but PDT may not be a good treatment option for all types of cancers. Another limitation of this treatment is the absorbance of the photosensitizer into the tumor cells. At the cellular level the photosensitizer must be intracellularly located to be effective in inducing cytotoxicity. This potentially could be a problem in treatment of brain cancer if the drug is not able to cross the blood brain barrier. It also limits researchers in the type of photosensitizer that can be used in PDT as it must be able to cross the plasma membrane.
Photosensitizing drugs must possess certain qualities to be effective for their use in PDT. Photosensitizing drugs can be taken up by the tumor cells inducing cytotoxic damage following light exposure. The drugs, or their metabolites, must also be able to absorb light in order to release energy to oxygen. It is also a good quality if the photosensitizer will be preferentially absorbed in tumor cells over normal cells. Many different photosensitizing drugs are being tested for their effectiveness in PDT.

The wavelength of light that is used in conjunction with PDT is also being optimized. Blue light has a higher amount of energy and therefore would be more efficient in causing cytotoxic damage. Many of the photosensitizers have absorbance peaks in the blue region rather than at longer wavelengths. But the problem with utilizing blue light is that it has a limited penetration depth through tissues. When treating tumor sites it is important to be able to penetrate through the skin to reach the basal level of the tumor bed. By using a red light at a longer wavelength, physicians are able to get a much deeper tissue penetration and are also able to treat tumors that are located under the skin without surgically exposing them. Red laser light at 635 nm is preferentially utilized to blue light in PDT in many settings because of its ability to penetrate tissues.

One drug that is being used in conjunction with PDT is 5-aminoleuvilinic acid (ALA). ALA is a pro-drug that increases the production of the photosensitizer, protoporphyrin IX (PpIX) within cells. This pro-drug has been FDA approved for treatment in cancer patients. ALA is a biosynthetic molecule that is one of the precursors in the heme pathway. ALA is a small molecule that is incorporated into the tumor cells. Once it is in the cell it is used to synthesize PpIX. PpIX is the photosensitizer utilized in ALA-mediated PDT. By extrinsically increasing the levels of ALA, the pathway can be
manipulated to produce a large concentration of PpIX within the cell. This molecular excess is preferentially found in tumor cells as compared to normal cells. By having an increased concentration of PpIX, the tumor cells become photosensitive and a target for PDT.

ALA-mediated PDT uses the heme biosynthetic pathway (Fig. 3, page 15). The metabolism of heme is tightly regulated within all cells under physiological conditions. ALA is synthesized in the mitochondria by the enzyme ALA synthase via a condensation of glycine and succinyl CoA. ALA synthase can be regulated by negative feed-back inhibition, and is the committed step in the anabolism of heme. ALA is transported out of the mitochondria to the cytosol where porphobilinogen, uroporphyrinogen III and coproporphyrinogen III are synthesized as the next three steps in the heme pathway. Coproporphyrinogen III is transported back into the mitochondria where through two steps, PpIX is synthesized. The final step in the heme pathway is the incorporation of ferrous iron into PpIX by the enzyme ferrocheletase to create heme within the mitochondria. Eight ALA molecules total are used in the formation of each PpIX.

One of the advantages of using ALA, is being a biosynthetic molecule, the body already has a system to metabolize ALA and PpIX. This results in a lower time of photosensitivity after PDT has been administered. Other photosensitizers that are not normally found within the body, have a much longer rate of excretion from the body and result in longer photosenitization after PDT treatment. ALA can also move across the blood brain barrier and therefore can be utilized in the treatment of gliomas.

Photofrin® is a FDA-approved commercially available proprietary photosenitizer that is produced by Axcan Pharma Inc. Photofrin® is a mixture of porphyrin ring systems
That exist as monomers, dimmers and oligomers. Due to similar chemical architectures, Photofrin® and PpIX both absorb light in the red region of the spectra around 635nm. Patients treated with Photofrin®-mediated PDT have a longer time frame of photosensitization due to the longer time needed to catabolize and excrete Photofrin®. This in vivo time frame can be as long as a month after PDT with Photofrin® (11).

There are many other photosensitizers that are being tested to be used with PDT. Another commercially available photosensitizer is Foscan (m-tetrahydroxy-phenylchlorin). Foscan is a porphyrin ring photosensitizer that localizes to the endoplasmic reticulum and golgi within the cell (12). Another type of photosensitizer that is currently under investigation is a beta-carboline derivative that directs the oxidative damage caused by PDT by intercalating into DNA (13). This mechanism provides a direct approach to cause DNA damage and induce cell death. Additionally, Acridine Orange is also a photosensitizer used in PDT, but is excited in the blue region (14).

Production of reactive oxygen species is one of the outcomes of PDT treatment. Reactive oxygen species can cause cellular damage to many different molecules within the cell including DNA, proteins and lipids. Oxidation of molecules can render them inactive. This can also be a localized event only occurring in one part of the cell or a total cell event. Oxidative stress can lead to cell death, if the damage is extensive and beyond the repair capabilities of the cell.

Proteins are targets for oxidative damage within the cell. Within proteins the side chains of the amino acids are sensitive to oxidative stress. Cysteine residues can be oxidized to form disulfide bonds. Other side chains that can easily be oxidized are found
on histidine, methionine, tryptophan and tyrosine. Tyrosine side chains can be oxidized to form dimers between two oxidized tyrosine residues (15). This can greatly affect the function of enzymes that rely on tyrosine residues within their active site.

Lipids are also targets for oxidation within cells. When reactive oxygen species react with arachidonic acid, prostaglandin endoperoxide is formed (16). Reduction of the prostaglandin endoperoxide, with the subsequent breakdown of the product will produce malondialdehyde (MDA). MDA is able to form adducts with DNA and is thought to be mutagenic in cells (17). The measurement of MDA as a way to quantitate the amount of oxidation within the cell is widely used (18).

Cells have intrinsic antioxidants to control the damage done by reactive oxygen species. Reactive oxygen species are synthesized under normal physiological conditions during electron transport chain reduction of oxygen and the oxidation of ATP. To control the harmful effects that reactive oxygen species induce, the cell contains antioxidant molecules. One of these is \( \alpha \)-tocopherol (vitamin E) which can be incorporated into the lipid membranes. \( \alpha \)-tocopherol’s mechanism of action is through the scavenging of free radicals (Fig. 4, page 16). The benzene ring of the \( \alpha \)-tocopherol is able to accept an electron to form a stable resonance structure. The levels of vitamin E in the cell can be manipulated by changing the concentration of vitamin E within the cellular media.

Another antioxidant within the cell is glutathione (GSH). GSH is a tripeptide molecule found in the cell consisting of glutamate, cysteine and glycine residues. GSH can reduce \( \text{H}_2\text{O}_2 \) molecules with the help of glutathione peroxidase, through the cysteine residue’s loss of a hydrogen atom (Fig. 5, page 17). When this occurs one molecule of \( \text{H}_2\text{O}_2 \) can be broken down into two water molecules. Two GSH molecules will make a
disulfide bond to form glutathione disulfide (GSSG). GSSG can then be recycled back to GSH by the enzyme glutathione reductase. Glutathione reductase uses one molecule of NADPH to regenerate GSH.

The synthesis of GSH is accomplished through two enzymes, γ-glutamylcysteine synthetase and glutathione synthetase (Fig. 6, page 18). These two enzymes link the glutamate residue and the cysteine residue through an isopeptide bond. The glycine residue is added last through a peptide bond to complete the molecule. One way to extrinsically regulate GSH within the cell, is by the application of buthionine sulfoximine (BSO). BSO is a γ-glutamylcysteine synthetase inhibitor that can reduce GSH levels within the cell by 75% (19).

There are two different cellular death pathways. Apoptosis is programmed cell death and is a tightly regulated process. Apoptosis can be signaled either extrinsically through a death receptor or intrinsically by cell stress. There are many different pathways that can lead to apoptosis. One of them is through the release of cytochrome c from the mitochondria. Cytochrome c release starts a cascade that signals for apoptosis, but itself needs to be signaled to be released. Cytochrome c once released from the mitochondria binds the Apaf-1 and leads to the cleavage of procaspase-9 to caspase 9. Caspase 9, Apaf-1 and cytochrome c bind together to form the apoptosome complex. The apoptosome complex can then lead to the cleavage and activation of caspase-3 which cleaves ICAD releasing CAD which migrates into the nucleus and starts DNA fragmentation. As apoptosis continues, proteins are cleaved by proteases and lose their function. The cellular contents are packaged and enzymes are rendered inactive.
Apoptosis is a slower more controlled type of cell death that leads to less stress on neighboring cells.

Necrosis is another type of cellular death. Necrosis is a faster type of cell death that is not programmed or controlled. Necrosis is much harder to study and identify because of its chaotic nature. Necrosis can be characterized by the cell rupture or loss of an intact cellular membrane. Necrosis is not signaled by caspase activation or Bax migration to the mitochondria as is apoptosis. Because necrosis is uncontrolled, it results in the release of the cellular contents into the cellular matrix. These contents contain proteases and the lysosomal contents and can cause damage to neighboring cells. When necrosis happens in the body, an immune response can be triggered causing inflammation. If necrosis occurs to a large amount of cells, it can cause damage to neighboring tissues that are otherwise healthy.

There are many hallmarks of apoptosis that can be used to characterize this type of cell death as compared to necrosis. One of these is that phosphatidyl serine, a cellular membrane component, can be found on the outside of the cellular membrane instead of only on the inner membrane side where it normally is found. Another hallmark of apoptosis is the activation of caspases. Caspases are unique to apoptosis. Some important caspases in apoptosis include caspase 3, 8 and 9. There are many more signs that can be used to determine what type of cell death the cells are undergoing. This includes DNA laddering which determines if DNA fragmentation has occurred. Also the ability of certain proteins to maintain their function can be tested including mitochondrial enzymes. The ability of the cell to preclude certain dyes, for example Hoechst, can additionally greatly facilitate the identification of the mode of cell death.
Heat shock proteins (hsp) are a family of proteins that are transcribed within the cell when the cell undergoes certain stresses (e.g., elevated temperature). Other stresses to the cell have also been identified as inducing the production of heat shock proteins, including many different types of cancer treatments (20, 21). Heat shock proteins are usually classified as chaperone proteins and can have protective effects for cells undergoing stress. This protection is an unwanted side effect to anti-cancer therapies and can help a cancer cell to survive. Understanding the function of hsp is important to cancer researchers because of their abilities to protect cancer cells.

Hsp27 is a heat shock protein that is 27kD in size. Hsp27 is found elevated in 34% of breast cancers (22). These 34% of breast cancers that are elevated in levels of Hsp27 will have enhanced resistance to chemotherapy. The function of Hsp27 is regulated by up to three phosphorylation events, furthermore, Hsp27 has been found to form dimers and oligomers within the cell. Hsp27 is also able to disrupt the apoptosis signaling within the cell by binding to cytochrome c and inhibiting the apoptosis signal (23, 24). There are still other roles that hsp27 plays within the cell, but the exact purpose has not been determined and is currently being studied in many laboratories.

To further study the role of hsp27 in the protection against PDT two cell lines were used in the studies reported here, that have been engineered to express different levels of hsp27. The DC4 and DB46 cells, from the parent cell line MDA-MB-231, have both been transfected with plasmids to regulate the constitutive levels of hsp27 (25). The DB46 cells produce an elevated level of hsp27, while the DC4 cells produce a normal level of hsp27 and are the control cell line for the DB46 cells. The DB46 cells produce a level of hsp27 that is equivalent to the DC4’s after a heat shock treatment (24).
A rat glioma model of brain cancer was also investigated to better understand the global ramifications of PDT. Malignant gliomas is difficult to treat in patients and the prognosis of patients diagnosed with a malignant glioma is poor. PDT has been shown to be a treatment for brain cancer patients and has improved the survival of these patients (26). Comparing a glioma model to that of a breast cancer model allows for the ability to compare two very different cellular types. Glial cells are highly specialized within the body. One thing that makes them unique is their lipid content. Glial cells contain lipids that are not found in other parts of the body. These include a class of lipids called sphingolipids. Sphingolipids play an important part in the formation of myelin sheaths.

**Hypothesis** - Both ALA and Photofrin®-mediated PDT will be effective in killing breast cancer cells in a dose dependent manner. This cytotoxicity will be induced through the formation of oxidized proteins and lipids. Both necrosis and apoptosis will be induced by both photosensitizers in a dose dependent manner. The switch between apoptosis and necrosis is dependent on the amount of lipid or protein oxidation. This model of either protein or lipid oxidation causing necrosis or apoptosis, will be universal, operating in a rat glioma model as well as a human breast cancer model.
Fig. 1. Photosensitizer excitation. When the photosensitizer is exposed to light, the light energy is absorbed and the photosensitizer is elevated to an excited state. The excited photosensitizer is also able to reach a triplet state through intersystem crossing. From there it can release energy through phosphorescence or can go on to create reactive oxygen species.
Type II Reaction (energy transfer)

Triplet State
Photosensitizer

Type I Reaction (electron/hydrogen transfer)
Free Radicals → Superoxides

Reactive Oxygen Species

Cytotoxicity

Fig. 2. The use of PDT to generate ROS. Two reactions cause the formation of reactive oxygen species within the cell.
Fig. 3. The heme biosynthetic pathway. Exogenous ALA application causes production of PpIX using the heme biosynthetic pathway.
Fig. 4. $\alpha$-tocopherol’s anti-oxidant function. $\alpha$-tocopherol scavenges free radicals to protect cells against oxidative stress.
Fig. 5. The function of glutathione within the cell. GSH functions as an anti-oxidant within the cell by reducing hydrogen peroxide to water. GSH is in very high concentrations within the cell to help protect the cell from oxidative damage.
Fig. 6. BSO inhibition of glutathione formation. The formation of glutathione is inhibited by buthionine sulfoximine.
CHAPTER 2

EXPERIMENTAL PROCEDURES

Chemicals Used - Minimum Essential Medium (MEM), Dulbecco's Eagle Minimum Essential Medium (DMEM), Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), trypsin-EDTA and G418 sulfate were purchased from Invitrogen, Inc (Carlsbad, CA). Buthionine-sulfoximine (BSO), α-tocopherol (vitamin E), 1,1,3,3-Tetraethoxypropane (TEP), Ellman's reagent (5,5-ditiosbis(2-nitro-benzoic acid)), 2-thiobarbituric acid (TBA) and 5-aminolevulinic acid was purchased from Sigma (St. Louis, MO). Photofrin® was a generous gift from AXCAN Pharma, Inc (QC, Canada). All reagents and enzymes used for flow cytometry were of analytical grade from Sigma (St. Louis, MO.) unless otherwise noted. The BODIPY C₃₃ (581/591) lipid oxidation probe (#D-3861) was purchased from Molecular Probes, Invitrogen, Inc (Carlsbad, CA). Bradford Assay Reagent (Coomassie Plus) was purchased from Pierce Biotechnology (Rockford, IL).

Tissue Culture - Two model systems for cancer were used in these experiments. The first system was a human breast cancer model system. There were four epithelial breast cancer cell lines used. The DC4 and DB46 breast cancer cells were genetically engineered from the parental line MDA-MB-231 as described by Carper et al (25). MDA-MB-231 cells are a human epithelial breast adenocarcinoma. To maintain the...
integrity of the transfection, the cells were grown in G418 sulfate. The MCF7 cells are a human epithelial breast adenocarcinoma which is caspase 3 negative. The MCF7 cells were obtained from American Type Culture Collection (ATCC), number HTB-22. MDA-MB-435 cells are a human breast ductal carcinoma also obtained from ATCC, number HTB-129.

The second model system used was a rat glioma model. Two rat glioma cell lines were used. The F98 cells are an undifferentiated malignant glioma derived from a CD Fisher rat fetus after exposure to ethyl-nitrosurea (ATCC). The BT4C cells are a glioma model derived from a BD-IX rat after exposure to ethyl-nitrosurea (27). Both cell lines were a generous gift from Dr. Steen Madsen.

MCF7, MDA-MB-435, DC4 and DB46 cells were grown in MEM supplemented with 10% FBS, 50 mM HEPES buffer (pH 7.4), Penicillin (100 U/mL) and Streptomycin (100 mg/mL). DC4 and DB46 cells were also grown in the presence of G418 sulfate (600 μg/mL). BT4C and F98 cells were grown in DMEM supplemented with 10% FBS, 50 mM HEPES buffer (pH 7.4), Penicillin (100 U/mL), and Streptomycin (100 mg/mL). All cell lines were maintained at 37°C, 100% humidity and CO2/air (5%/95%) atmosphere.

Vitamin E and BSO – The BSO stock was made at a concentration of 5 mM in PBS. Vitamin E was first dissolved in 95% ethanol and then diluted with PBS to a final concentration of 5 mM vitamin E in 25% ethanol. BSO and vitamin E were added to cell media 24 hours prior to irradiation at a concentration of 50 μM. When cells were washed
and re-suspended in media, free of serum, with the photosensitizer, BSO or vitamin E were added to the new media also (19, 28).

**Irradiation of Cells** - To perform PDT treatment on monolayered cells, a pretreatment of the photosensitizer is needed. A four hour pre-treatment with both ALA and Photofrin® was used. A final concentration of 1 mM of ALA and 2.5 μg/mL of Photofrin® was used unless otherwise indicated. These concentrations were determined through literature searches and empirical trials (29, 30). During pre-treatment, the cells were maintained in serum free media (0% FBS) to facilitate uptake of the photosensitizing agents into the cells and to maintain intracellular concentrations.

A 100 mM stock solution of ALA was prepared in 100 mM HEPES buffer (pH 7.5, with NaOH). Photofrin® was obtained from Axcan Pharma in a dehydrated form that had been normalized to a physiological pH by methods that were not disclosed. Photofrin® was resuspended with PBS at a stock concentration of 2.5 mg/mL in PBS.

Cells were seeded 48 hours prior to treatment, to obtain a non-confluent culture of dividing cells during treatment. To prepare the cells for pre-treatment, they were washed once with 5 mL of PBS and resuspended in 5 mL of serum-free medium. The stock solution of the photosensitizer was added to the serum-free media, to obtain a final concentration of 1 mM ALA or 2.5 μg/mL Photofrin®. Cells were allowed to incubate for four hours in the dark in a 37°C incubator. Following incubation, cells were irradiated with 635 nm light from a diode laser (High Power Devices, North Brunswick, NJ) at an irradiance of 35 mW/cm². Radiant exposures ranged from 0.5-20 J/cm² with irradiation times ranging from 14-571 seconds.
Fluorescence Studies - Tumor cells were incubated with the photosensitizer and serum-free medium at variable concentrations and time in the manner described above. After incubation, cells were washed with 5 mL of PBS and harvested with trypsin. The cells were re-suspended in 4 mL of media (10% FBS). The cell suspension was centrifuged at 1000 g for 5 minutes. The cell pellet was then resuspended in 5 mL of media. Fluorescence intensity was evaluated using a FACS Calibur cytometer. Cells were excited with a 488 nm laser and the fluorescence was measured using the FL-2 filter. The mean fluorescence of the total cell population was obtained after analyzing 2000 cells.

Clonogenic Survival - Clonogenic survival studies were done to identify the ability of ALA and Photofrin®-mediated PDT to inhibit cell proliferation. Following irradiation, cells were washed with 5 mL of PBS and harvested by trypsinization. After being resuspended in media, the cells were counted with a Coulter counter. A known number of cells were plated into 60 mm dishes. Cells were allowed to grow for 11-14 days and colonies containing more than 50 cells were counted by hand after staining with 0.5% crystal violet in 95% ethanol. The average number of colonies per 60 mm dish was 100. The number of colonies that formed in the experimental plates was normalized to the control group (cells incubated with the photosensitizer, but receiving no light treatment).

Flow cytometry with Propidium Iodide - Propidium iodide (PI) will stain DNA, but is not able to pass through an intact cellular membrane. PI is a fluorescent stain that is excited in the blue region and emits a red color. By first treating cells with a detergent and then
staining them with PI, we are able to quantify the amount of DNA per cell. The phase of the cell cycle can be determined by quantifying the amount of DNA. In the G1 phase of the cell cycle, the cells contain 1N amount of DNA. During the S phase, the cells are actively synthesizing DNA and have between a 1N and 2N amount of DNA. In the G2 phase of the cell cycle, the cell will contain a 2N amount of DNA. Apoptotic vesicles are classified by those containing less than 1N DNA and being smaller in size than whole cells.

Cells that were to be analyzed with PI for cell cycle distribution were washed with 5 mL of PBS. They were then harvested using trypsin and centrifuged for 5 minutes at 1000 g. The cell pellet was resuspended in 5 mL of PBS and the cells were centrifuged again at 1000 g for 5 minutes. The cell pellet was resuspended in 100 μL of PBS and then 1 mL of 70% ethanol at −20°C was added dropwise. The cell solution was stored at 4°C until the cells were analyzed. Ethanol-fixated cells were cleared of excess fixative by a single wash with 5 mL of cold PBS and centrifuged at 500 g for 5 minutes. The pellet was resuspended with 1.0% Triton X in PBS. A 1.0 mg/mL RNAsel solution was added and the mixture was incubated at room temperature for 10-15 minutes before staining with 100 μg/mL PI solution. The final PI concentration in the test mixture was 50 μg/mL. Cytometry acquisition was done using the BD FACS Calibur with the argon laser set at 488 nm on the linear Flow Channel 2 (FL-2) with Doublet Discriminatory Module and Threshold set on FL-2. Modfit 3.0 was used to assign distribution curves to data analyzed with PI and flow cytometry.
**Cell Attachment assay** - To study the immediate effects of PDT on cancer cells, cell attachment assays were performed within the first four hours after PDT treatment. Cells were seeded into flasks 48 hours prior to irradiation at a concentration of 200,000 cells per flask. After irradiation, cells were incubated at 37°C. After incubation, a 100 μL sample of the media was counted using a Beckman culture counter. The remaining media was discarded and the cells were washed with 5 mL of PBS. The cells were harvested using trypsin and a 100 μL sample of the attached cells was counted using the same culture counter. The total number of cells (attached and floating) in the control flask at time zero was used to normalize all other counts for that cell line.

**Fluorescent Microscopy** - To determine the amount of apoptosis and necrosis of the cells after PDT treatment, fluorescent microscopy was used. Hoechst 33342 is a molecule that stains DNA in apoptotic and necrotic cells. Hoechst stain is able to cross an intact plasma membrane, but healthy cells are able to pump the stain back into the extracellular space. Hoechst stain is a fluorescence dye that is excited in the UV region and emits a blue color. PI stain is also used with fluorescent microscopy. PI is able to stain the DNA of cells that do not have an intact cellular membrane. In order to characterize apoptotic cells, Hoechst positive staining without PI positive staining must be present. Necrotic cells are characterized by PI positive staining.

Approximately 10,000 cells per well were seeded on 4 chamber microscope slides, 48 hours prior to PDT treatment. Cells were irradiated as described above. After light irradiation, cells were washed with PBS and allowed to incubate in MEM supplemented with serum for a set period of time (24-48 hours). After incubation, cells were stained...
with 500 μL of Hoechst 33342 (2 μg/mL) and 500 μL of PI (10 μg/mL) for 15 minutes. In three separate fields, pictures were taken of the bright field, and then the same image was photographed with UV and blue excitation filter using a photometrics coolsnap CCD digital camera attached to Nikon TE-200U inverted fluorescent phase contrast microscope (Nikon, Inc. Melville, NY, USA). The total number of cells were counted in the bright field and this was compared to the number of cells staining positive for each dye. Cells staining positive for both Hoechst and PI were included only in the PI positive counts.

*Lipid peroxidation measurements with HPLC* - Lipid peroxidation was measured as an increase in intracellular MDA (31). MDA is the end product of fatty acid oxidation. By quantifying the amount of MDA in the cell, the amount of lipid peroxidation can be deduced. MDA can be measured by formation of an adduct with thiobarbutiric acid (TBA). TBA binds with MDA in a 2:1 ratio, forming a colorimetric product that absorbs at 532 nm (Fig. 7, page 29). To normalize the amount of MDA to the total amount of protein per sample, a Bradford assay was performed to quantify the total protein content. The standard used for this experiment was TEP. MDA is not stable in solution for long periods of time. TEP under high heat and acidic conditions will break down into MDA. TEP is an ideal standard for the TBA:MDA analysis.

Cells were seeded (800,000 per flask) 48 hours prior to irradiation. After incubation with the photosensitizer and prior to irradiation, the cells were washed with 5 mL of PBS and then resuspended in 2.5 mL of PBS. The cells were irradiated as described above. After irradiation the cells were scraped from the flask and a cell pellet was obtained by
centrifugation at 1000 g for 5 minutes. The pellet was resuspended in 0.75 mL of a 0.9% NaCl solution. The cells were then lysed open by freeze/thawing. There were two cycles of 20 minutes at -80°C followed by 5 minutes at 37°C. After lysing, 10 μL of the lysate was saved for protein quantification with Bradford reagent. To 650 μL of the remaining cell lysate, 250 μL of a 20% trichloracetic acid solution was added to precipitate the proteins. After a 10 minute incubation, 200 μL of a 0.67% solution of TBA was added and the sample was heated to 100°C for 20 minutes. After, heating the sample was centrifuged for 5 minutes at 12,000 rpm and a clear pink solution was obtained. Then 10 μL of this sample was then injected into the Water Separation Module 2695 HPLC. The samples were run at a 40% MeOH:60% 0.5 M Ammonium Acetate solution (pH 5.5) on a C18 column using a flow rate of 1 mL/min. The absorbance was measured to the linked Photodiode array 2996. The TBA:MDA adduct was measured as a peak at approximately 2 minutes at 532 nm. The area of the peak was quantified by Empower software (Waters, Milford, MA). The amount of MDA per sample was normalized to total protein content.

Protein oxidation using Ellman’s Reagent - There are many targets of oxidation in protein molecules. The thiol group of the amino acid cysteine is easily oxidized. The measurement of reduced thiol groups can be done using Ellman’s reagent (32). As the cysteine residues are oxidized, the amount of reduced thiol groups is decreased. Ellman’s reagent forms an adduct with reduced cysteine residues (Fig. 8, page 30). The by-product of this reaction is a thiol anion which absorbs at 405 nm. As cysteine residues are oxidized, the loss of color product is expected.
Cells were seeded 48 hours prior to irradiation at a concentration of 250,000 cells per flask. In order to measure thiol groups using Ellman’s reagent the cells were incubated with the photosensitizer as described above and prior to irradiation were washed with 5 mL of PBS and then covered in 1 mL of PBS. After the cells were irradiated, they were immediately placed in ice. The cells were scraped from the flasks and then were lysed by a freeze/thaw cycle. There were two cycles of 20 minutes at -80°C followed by 5 minutes at 37°C. After lysing, the cells were centrifuged for 5 minutes at 1000 g to pellet whole cells and large membrane fragments. The supernatant was then analyzed using a 0.04% solution of Ellman’s reagent (DNTB) in a 0.1 M tris buffer (pH 8.0). In a 96 well plate, 40 µL of cell lysate, 40 µL of a 0.1 M tris buffer containing 0.1 M glycine and 4 M EDTA (pH 8.0) and 10 µL of Ellman’s reagent were mixed. After incubating for 10 minutes at 37°C, the sample was measured using a Tecan plate reader at 405 nm. This measurement was then normalized to the total amount of protein in the sample as quantified with Bradford reagent.

**Oxidative Sensitive Lipid Probe** - The BODIPY 581/591 C11 probe (4,4-diflouro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) is a fatty acid analog that can be incorporated in cells. Before oxidation the probe has a red fluorescence (590 nm) when excited with a green light (530 nm). After oxidation the BODIPY probe has green fluorescence (535 nm) when excited with a blue light (485 nm). Using this probe we can visualize the location of oxidation within the cell and also measure the amount of lipid oxidation.
The BODIPY probe was first dissolved in DMSO and then MEM was added to a final concentration of 200 μM in 50% DMSO. Cells analyzed by fluorescent microscopy were seeded into welled slides at a concentration of 10,000 cells per well 48 hours prior to irradiation. After 4 hours incubation with the photosensitizer, the probe was added to the media at a final concentration of 10 μM. After a 15 minute incubation with the probe, the media was removed and PBS was added. The cells were irradiated and immediately after irradiation the cells were analyzed. Fluorescent microscopy pictures were taken after the chambers were removed from the slide and cover slips were placed over the cells. A 100X objective was used with a green filter to capture the red fluorescence pictures.

**Statistical Analysis** - Graph Pad Prism 4.0 (Graph Pad Software, San Diego, CA) was used to graph and run non-linear regressions on data. Graph Pad Quick Calcs (www.graphpad.com) was used for student's t test. P values less than 0.05 were considered significant. All graphical error bars are representative of standard error measurements.
Fig. 7. The quantification of lipid peroxidation. TBA forms a colorimetric product to MDA which allows for the quantification of lipid peroxidation.
5,5'-Disthiobis(2-nitrobenzoic acid) (DTNB, Ellman's Reagent) + Cysteine Residue within a peptide → Disulfide peptide product

Thiol anion (abs = 405nm)

Fig. 8. The quantification of reduced thiol residues. Ellman's reagent forms an adduct with cysteine residues resulting in a colored product.
CHAPTER 3

RESULTS

PDT is a two step treatment modality. Individually the photosensitizer or the light was not effective in killing tumor cells. ALA and Photofrin® toxicity was analyzed using the MDA-MB-435 cells (Fig. 9, page 46). Clonogenic survivals were conducted with both photosensitizers in the absence of light. ALA did not show a decrease in survival until 10 times the amount used in our PDT studies (10 mM). Photofrin® showed no toxicity at concentrations ranging from 0-15 μg/mL. The effect of the laser on the breast cancer cells was also studied (Fig. 10, page 47). The application of laser light alone did not show any significant dose effects on the MCF7 or MDA-MB-435 cells between 0 and 20 J/cm². This confirms that in order for PDT to be effective in killing tumor cells that both a light source and the photosensitizer must be present.

To better understand the effectiveness of PDT-induced cell death, clonogenic survivals were performed. ALA-mediated PDT was studied with all four breast cancer cell lines and the two glioma lines. Photofrin®-mediated PDT effectiveness was also assessed with the four breast cancer cell lines. All tumor lines exhibited a dose dependent response to PDT treatment. MDA-MB-435 cells were treated with ALA-mediated PDT and showed an EC₅₀ of 2.2 J/cm² (Fig. 11, page 48). The MDA-MB-435 cells reached an EC₉₀ around 4 J/cm². The DB46 cells had sensitivity similar to that of ALA-mediated PDT and had an EC₅₀ of 2.7 J/cm² (Fig. 12, page 49). Unlike the MDA-
MB-435 cells, the DB46 cells did not reach an EC$_{90}$ until a dose of 8 J/cm$^2$. The DC4 cells, which differ from the DB46 by lower levels of hsp27, were more sensitive than both the MDA-MB-435 and DB46 with an EC$_{50}$ value of 1.7 J/cm$^2$ (Fig. 13, page 50). At 4 J/cm$^2$, the DC4 cells reach an EC$_{90}$. The MCF7 cells were the most sensitive breast cancer cell line among those studied to ALA-mediated PDT (Fig. 14, page 51). The EC$_{50}$ value for MCF7 cells was 0.4 J/cm$^2$ and an EC$_{90}$ was reached before 2 J/cm$^2$ dose.

The two glioma lines studied were less sensitive to ALA-mediated PDT as compared to the breast cancer lines with the glioma EC$_{50}$ values being approximately 5 J/cm$^2$ greater than those of the breast cancer cells. This effect could be contributed to either a tissue or species difference. The F98 cells exhibited an EC$_{50}$ value of 4.4 J/cm$^2$ (Fig. 15, page 52). At a dose of 20 J/cm$^2$ the survival was still approximately 20%. The BT$_4$C were the least sensitive of the cell lines investigated with an EC$_{50}$ of 7.3 J/cm$^2$ (Fig. 16, page 53). There was 17% survival at the highest dose investigated (20 J/cm$^2$). Neither the F98 or the BT4C cells reached an EC$_{90}$ by 20 J/cm$^2$.

Photofrin®-mediated PDT efficacy was also investigated by clonogenic survival. MDA-MB-435 cells were treated with Photofrin®-mediated PDT and a clonogenic survival assay was performed (Fig. 17, page 54). The EC$_{50}$ value of the MDA-MB-435 cells treated with Photofrin®-mediated PDT was 0.7 J/cm$^2$. The MDA-MB-435 cells reached an EC$_{90}$ at 2 J/cm$^2$. The DB46 cells had similar sensitivity to Photofrin®-mediated PDT as the MDA-MB-435 cells (Fig. 18, page 55). The EC$_{50}$ value was the same as the MDA-MB-435 cells at 0.7 J/cm$^2$. The EC$_{90}$ for the DB46 cells was around 2 J/cm$^2$. The DC4 cells were slightly less sensitive to Photofrin®-mediated PDT than the DB46 cells (Fig. 19, page 56). The DC4 cells also reached an EC$_{90}$ at 2 J/cm$^2$. The DC4
cells have an EC$_{50}$ value of 0.8 J/cm$^2$. The MCF7 cells were again the most sensitive of the cell lines to Photofrin®-mediated PDT with an EC$_{50}$ value of 0.4 J/cm$^2$. The MCF7 cells reached an EC$_{90}$ value 1 J/cm$^2$. Table I (page 58) summarizes EC$_{50}$ values of all cell lines and PDT treatments investigated. The errors can be estimated by looking at the individual R$^2$ values of the non-linear regression lines on each graph. BT$_4$C and F98 cells were not investigated with Photofrin®-mediated PDT.

Flow cytometry analysis of the cell cycle distribution was performed on the four breast cancer lines with both ALA and Photofrin®-mediated PDT to confirm results seen with clonogenic survival assays. Approximately 2000 cells were analyzed per data point. As seen in Fig. 21 (page 59) the cells are graphically represented in a histogram format by the amount of DNA per each cell as quantified with PI. Modfit 3.0 was used to analyze the histogram and fit a standard curve to the data presented. The percent of cells per stage of the cell cycle is reported. As shown, the apoptosis peak, that is located pre-G1, increases as the dose of PDT increases. The data shown is typical of the data analyzed with all cell lines with both ALA and Photofrin®-mediated PDT.

MDA-MB-435 cells were analyzed with flow cytometry and the data is shown in Table II (page 60). Cells were harvested both at 24 and 48 hours post irradiation. The result from a single experiment is shown which is representative of both experiments. With ALA-mediated PDT, at the 24 hour time point the apoptosis peak increase as the light dose increases. This same trend is seen at the 48 hour time point, with the apoptosis peak increasing as the dose of ALA-mediated PDT. The 48 hour peaks are increased from the 24 peaks at the same dose. With ALA-mediated PDT there is no trend in the G1, S or G2 peak with dose. Photofrin®-mediated PDT was also investigated. There
was again a dose response effect observed with the apoptosis peak increasing with increasing dose both at the 24 and 48 hour time points. There is no observable difference between the 24 hour and 48 hour time points in the apoptosis peaks. There is also a trend in the decrease of the S peak in both the 24 and 48 hour time points.

The DB46 cells were also analyzed with flow cytometry at 24 and 48 hour time points (Table III, page 61). With ALA-mediated PDT, the DB46 cells showed a much lower dose dependent trend than the MDA-MD-435 cells with only 27% apoptosis as compared to 56% in the MDA-MB-435 cells. At the 24 time point the 4 J/cm$^2$ and the 20 J/cm$^2$ apoptosis peaks are similar at approximately 16%. At the 48 hour time point there is not a dose response until the 20 J/cm$^2$ dose. There is no discernible trend in the G1, S or G2 peak in either the 24 or 48 hour time point with the ALA-mediated PDT cells.

With Photofrin®-mediated PDT the DB46 cells again show a dose dependent trend with an increase in the apoptosis peak with an increase in dose at both 24 and 48 hour time points. The 24 and 48 hour time points again have very similar apoptosis peaks. The same trends are observed in the MDA-MB-435 cells where the S peak decreases as the dose of Photofrin®-mediated PDT increases.

The DC4 and MCF7 cells were also analyzed with flow cytometry with ALA and Photofrin®-mediated PDT (Table IV and Table V, pages 62-63). We see similar trends that were observed with the DB46 and MDA-MB 435 cells. Both time points and with both ALA and Photofrin®-mediated PDT there is a dose dependent response with an increase in the apoptosis peak. The DC4, 48 hour time point with Photofrin®-mediated PDT there is not a decrease in the S peak. Also in the MCF7 cells, at the 24 hour time point with Photofrin®-mediated PDT the S peak does not show a significant increase.
PpIX and Photofrin® have an inherent fluorescence. This property can be exploited to estimate the comparative concentration of photosensitizer within the cell. Fluorescent value of tumor cells after incubation with ALA or Photofrin® was quantified with the flow cytometer. Varying the concentration and the time of incubation with the photosensitizer was investigated. ALA concentrations were varied between 0 and 2.5 mM of ALA with an incubation time of four hours (Fig. 22A, page 64). All values were normalized to the zero point (data not shown). The MCF7 cells showed the highest amount of fluorescence of the cell lines investigated with a mean fluorescence per cell of 101 a.u. at 2.5 mM ALA. The MDA-MB-435 cells were the least fluorescent with a peak fluorescence per cell of 43 a.u. at 0.5 mM of ALA. All cell lines, DC4, DB46, MCF7, MDA-MB-435 and BT4C, reached a maximum fluorescence at approximately 1mM of ALA. Photofrin® concentrations were varied between 0 and 10 μg/mL with a four hour incubation (Fig. 22B, page 64). With the four cell lines investigated, MCF7, DC4, DB46 and MDA-MB-435, there was a dose dependent response with the increase of fluorescence per cell increasing with concentration of Photofrin®. The DB46 cells exhibited the highest fluorescence per cell with a peak fluorescence at 10 μg/mL of Photofrin® of 3700 a.u. The MDA-MB-435 cells were the least responsive to Photofrin® with a peak fluorescence per cell of 680 a.u. at 10 μg/mL of Photofrin®.

The incubation time of both ALA and Photofrin® was also investigated. Tumor cells were incubated with 1mM ALA for 0-5 hours (Fig 23A, page 65). The MCF7 cells, again, had the highest fluorescent values of the cell lines investigated and had a peak fluorescence of 585 a.u. at 4 hours. The MDA-MB-435 cells reached a plateau of fluorescence at 3 hours of 63 a.u. per cell. The DB46 cells reached a plateau earlier at 1
hour of 110 a.u. per cell. The DC4 cells exhibited an increase of fluorescence at every
time point with a peak fluorescence per cell of 218 a.u. at five hours. The BT4C cells
also had a dose dependent response with time. The BT4C peak fluorescence was 102 a.u.
at five hours. Photofrin® incubation was done at a concentration of 2.5 µg/mL with the
same time frame of 0-5 hours (Fig. 23B, page 65). With Photofrin® incubation all cell
lines exhibited a dose response with respect to time. The DB46 cells exhibited the
highest fluorescent response with the DC4 cells being not significantly different at the
highest fluorescent amount (p > 0.5). The DB46 fluorescent at five hours was 1000 a.u.
per cell. The MDA-MB-435 cells were again the least fluorescent of the cell lines
investigated with a peak fluorescence of 360 a.u. per cell at five hours.

Cell attachment assays were performed to investigate the immediate effects of
PDT treatment. Attached and floating cells were quantified at two and four hours after
PDT treatment. MDA-MB-435 cells were analyzed at 2 and 10 J/cm² dose of ALA-
mediated PDT (Fig. 24, page 66). The 2 J/cm² dose did not affect the attached cells as
compared to the control flasks. The 2 J/cm² dose did increase the amount of cells
floating in the media by a very small amount. The 10 J/cm² dose caused an increased
detachment of approximately 40% at the 4 hour time point, as confirmed in both the
attached cells and the floating cells. It is interesting to speculate if at time points greater
than 4 hours the number of cells detaching would continue to increase.

The attachment assay was also performed on MDA-MB-435 cells treated with
Photofrin®-mediated PDT (Fig. 25, page 67). These results differed from the ALA-
mediated PDT response. The 2 J/cm² dose appeared to be causing the detachment of
more cells than the higher dose of 10 J/cm². This assay was performed two times with

36
the same increased 2 J/cm$^2$ effect happening in both trials. At the four hour time point, with the 2 J/cm$^2$ dose, there is a decrease in attached cells of approximately 55% with an increase in floating cells of 35%. This may indicate that there is a loss of 20% of the cells due to necrosis. Also at the four hour time point, with the 10 J/cm$^2$ dose, there is a decrease in attached cells of 30% with an increase in floating cells of 40%. This does not make biological sense and warrants further investigation.

The rat glioma line, BT$_4$C cells were also analyzed with an attachment assay after treatment with ALA-mediated PDT (Fig. 26, page 68). BT$_4$C cells display a similar effect that was observed with the MDA-MB-435 cells. The detachment of cells is dose dependent and seems to have similar values at the two hour and four hour time point. The 10 J/cm$^2$ dose had a detachment of 30% with an increase in the floating cells of 5% at the four hour time point. This may again point to some loss of cells due to necrosis, but is not definitive. The 2 J/cm$^2$ dose had a smaller effect with the detachment of 10% of the cells and an increase in the floating cells of less than 5% at the four hour time point.

Fluorescent microscopy analysis was used to determine the amount of necrosis and apoptosis after PDT treatment. Four cell lines were investigated using ALA-mediated PDT 24 hours after treatment (Fig. 27, page 69). Apoptosis was measured by using Hoechst stain 33342 and normalizing the number of positive cells to the total number of cells in the bright field image (Fig. 27A, page 69). The amount of apoptosis that was observed was low (less than 25%), but did exhibit a dose dependent trend. The two brain cell lines, BT$_4$C and F98, exhibited higher amounts of apoptosis than the two breast cancer cell lines, MCF7 and MDA-MB-435. The F98 cells had the highest amount
of apoptosis, 22% at 10 J/cm$^2$. The MCF7 cells were very similar to the MDA-MB-435 cells and had the lowest amount of apoptosis, 5% at the 10 J/cm$^2$ dose. Necrosis was also measured using PI stain and normalizing the number of positive cells to the total number of cells in the bright field image (Fig. 27B, page 69). In all cell lines necrosis was dose dependent. The MDA-MB-435 cells had the highest amount of necrosis with 17% of the cells being necrotic. The BT$_4$C cells had the lowest amount of necrosis with less than 2% necrotic cells at the 10 J/cm$^2$ dose.

Fluorescent microscopy was also used to study the timeline of events after PDT treatment in the MDA-MB-43 cells (Fig. 28, page 70). ALA mediated-PDT was performed on MDA-MB-435 cells at 0, 2, 4, 10 and 20 J/cm$^2$ and microscopy images were obtained after both 24 and 48 hours (Fig. 28A, page 70). At the 2 and 4 J/cm$^2$ dose there is very little difference between the 24 and 48 hour time points. Both the apoptotic and necrotic values are less than 5% at both the 24 and 48 hour time point for 2 and 4 J/cm$^2$. At the 10 J/cm$^2$ dose there is an increase in the necrotic value and a decrease in the apoptotic value between the 24 hour and 48 hour time points. This might be indicating a shift from early to late apoptosis, since PI can also be indicative of late apoptotic events. At the 20 J/cm$^2$ dose there is a large decrease in the necrotic cells between the 24 and 48 hour time points. This might be indicative of the rupture of necrotic cells and the loss of total cell number between 24 and 48 hours.

Photofrin®-mediated PDT was also analyzed for a time course with fluorescent microscopy with MDA-MB-435 cells (Fig. 28B, page 70). In the Photofrin® treated cells there is a general decrease in necrotic cells and an increase in apoptotic cells between the 24 and 48 hour time points. At 24 hours, there is a large amount of necrosis present that
is dose dependent, with very little apoptosis. At the 48 hour time point the necrosis amounts have decreased and there is evidence of apoptosis at 2 and 4 J/cm\(^2\), between 5 and 10%. At 48 hours there is still a dose response of increased cell death with increased dose of Photofrin\(^\circledR\)-mediated PDT.

Oxidation is an important indicator of cell cytotoxicity with PDT treatments. Loss of reduced thiol groups was measured as an indicator of protein oxidation. Four cell lines were analyzed for protein oxidation with ALA-mediated PDT (Fig. 29, page 71). In all four cell lines, MDA-MB-435, MCF7, F98 and BT\(_4\)C, there was protein oxidation after ALA-mediated PDT treatment. There was not a strong dose dependent trend with the amount of oxidation and the dose of ALA-mediated PDT. The differences between the 2 J/cm\(^2\) and 10 J/cm\(^2\) amounts of protein oxidation were very little in all four cell lines. The brain cancer cell lines, BT\(_4\)C and F98, had a lower amount of protein oxidation than the breast cancer cell lines, MCF7 and MDA-MB-435. The MDA-MB-435 cells had the largest amount of oxidation at approximately 60% oxidation at 10 J/cm\(^2\). The F98 cells had the least amount of protein oxidation with less than 10% oxidized at 10 J/cm\(^2\).

Protein oxidation was also analyzed with the MDA-MB-435 cells with both ALA and Photofrin\(^\circledR\)-mediated PDT (Fig. 30, page 72). To compare oxidation amounts at equal survival levels, the protein oxidation was analyzed at the EC\(_{50}\) values of MDA-MB-435 with both ALA and Photofrin\(^\circledR\) treated cells. Also an EC\(_{90}\) value for each photosensitizer was analyzed. These values were 0, 0.5 and 2 J/cm\(^2\) for Photofrin\(^\circledR\)-mediated PDT and 0, 2.5 and 4 J/cm\(^2\) for ALA-mediated PDT. BSO and vitamin E were used to modulate the amount of anti-oxidants within the cell. With ALA treated MDA-
MB-435 cells there was no difference in protein oxidation between the 2.5 and 4J/cm\(^2\) (Fig. 30A, page 72). Interestingly the only significant difference that BSO and vitamin E played with the ALA-mediated PDT was that BSO slightly increased the amount of oxidation at the 4 J/cm\(^2\) dose. This is expected as BSO will decrease the amount of GSH within the cell. All treatment groups had approximately a 50% oxidation level. The Photofrin® treated cells showed a dose response with increased oxidation as dose increased (Fig. 30B, page 72). BSO did not significantly change the oxidation levels with Photofrin®-mediated PDT, but vitamin E did increase the amount of oxidation that occurred. The vitamin E treated cells had the highest amount of oxidation at 2 J/cm\(^2\) at approximately 50% oxidized. If protein oxidation was correlative of survival in PDT treatment we would expect to see similar oxidation levels at similar survival levels. This is not the case in comparing the ALA treated cells to the Photofrin® treated cells. The Photofrin® treated cells have a lower amount of oxidation at the EC\(_{50}\) values. At the EC\(_{90}\) values the Photofrin® and ALA treated cells do have similar protein oxidation levels.

In a comparison of similar doses of ALA and Photofrin®-mediated PDT, protein oxidation still does not exhibit a strong dose dependence (Fig. 31, page 73). Photofrin® has lower amounts of oxidation at 2 and 20 J/cm\(^2\). There is a significant difference of approximately 20% between both the ALA and Photofrin®-mediated PDT cells between the 2 and 20 J/cm\(^2\). The 4 J/cm\(^2\) Photofrin®-mediated PDT dose may not be representative, since it is lower than both the 2 and 10 J/cm\(^2\) dose.

Lipid peroxidation was analyzed to understand its role in cytotoxicity of MDA-MB-435 cells to ALA and Photofrin®-mediated PDT (Fig. 32, page 74). Lipid oxidation was quantified by measuring the amount of MDA with HPLC analysis using a
The ALA-mediated PDT treated cells showed a higher amount of lipid peroxidation at all doses. There was not a significant increase in lipid peroxidation until the 4 J/cm² dose in the ALA treated cells. The 10 and 20 J/cm² dose was similar at 0.003 μM/μg of MDA per protein in the ALA treated cells. The Photofrin® treated cells did not have a significant increase in lipid peroxidation until the 20 J/cm² dose (p > 0.05). This data suggests that bulk lipid peroxidation is not the only factor that plays a role in PDT cytotoxicity since there is a less than 10% cell survival of Photofrin® treated cells after a 4 J/cm² dose.

To better understand the effects of oxidation on cytotoxicity, BSO and vitamin E were used to modulate the levels of anti-oxidants within the cell. Fluorescent microscopy was used to analyze the effects of BSO and vitamin E on apoptosis and necrosis in MDA-MB-435 cells 24 hours after irradiation (Fig. 33A, page 75). Vitamin E had little effect on apoptosis or necrosis as compared to the ALA-mediated PDT treated control cells. With both the ALA treated cells and the ALA with vitamin E treated cells there was approximately 85% necrosis. The BSO treatment had two different effects on the MDA-MB-435 treated cells. At 0, 2 and 4 J/cm² it greatly increased the amount of necrosis and decreased the amount of apoptosis. This trend did not continue and at 20 J/cm² the BSO treated cells had lower amounts of necrosis. The Photofrin®-mediated PDT cells also were treated with BSO and vitamin E and analyzed with fluorescent microscopy (Fig. 33B, page 75). In all the Photofrin® treated MDA-MB-435 cells there was very little or no apoptosis present. At 2 J/cm², vitamin E protected the cells from necrosis by about 35%. At the same dose of 2 J/cm², the BSO may have sensitized the cells to the Photofrin® treatment. At 20 J/cm² the BSO treatment sensitized the cells by causing 12%
more necrosis. With the Photofrin®-mediated PDT, the effects seen with BSO and vitamin E are at low doses and in small quantities.

Clonogenic survivals were also performed with BSO and vitamin E on the breast cancer cell lines to understand the role of anti-oxidants in ALA-mediated PDT treatment. MDA-MB-435 cells responded to vitamin E and BSO as expected (Fig. 34, page 76). All three groups responded in a dose dependent manner. Vitamin E provided protection against ALA-mediated PDT at 1, 2.5 and 4 J/cm². BSO treatment of cells resulted in less clonogenic survival at 1 and 2.5 J/cm². At 10 J/cm² the ALA treatment, BSO and vitamin E treatment were similar at less than 5% survival. The DB46 cells did not have the expected result when treated with BSO and vitamin E (Fig. 35, page 77). The vitamin E treatment provided significant protection at all doses investigated between 0 and 20 J/cm². The BSO also protected the ALA treated DB46 cells at the 2 J/cm² dose, resulting in approximately 100% survival. At all other doses the BSO treatment did not seem to affect the ALA-mediated PDT survival response. The DC4 cells had an expected response to the BSO and vitamin E treatment (Fig. 36, page 78). The vitamin E provided protection to the ALA treated DC4 cells at 2 and 4 J/cm². The BSO also decreased the survival at 2 and 4 J/cm². At 8, 10 and 20 J/cm² there was less than 6% survival in the ALA, BSO and vitamin E treated cells. The MCF7 cells were exposed to ALA-mediated PDT, treated with BSO and had an increased sensitivity of 5% compared to controls at 2 J/cm² (Fig. 37, page 79). Vitamin E protected the MCF7 cells by increasing the survival by 10% as compared to the control group.

The role of vitamin E and BSO were also investigated with Photofrin®-mediated PDT MDA-MB-435 cells (Fig. 38, page 80). The no addition, BSO and vitamin E
groups respond in a dose dependent manner to the Photofrin®-mediated PDT. At the 0.5 J/cm² dose, there is lowered survival with the addition of BSO to the cells, but at 2 J/cm² the BSO treatment actually provides a significantly small amount of protection to the MDA-MB-435 cells. At 2 J/cm², vitamin E does provide protection to the cells against Photofrin®-mediated PDT cytotoxicity. At 4 and 10 J/cm² there is no significant difference between the no addition, BSO and vitamin E groups. Vitamin E was also shown to provide protection to DC4 cells against Photofrin®-mediated PDT (Fig. 39, page 81). At 2 and 4 J/cm² vitamin E provides significant protection against Photofrin®-mediated PDT. At 20 J/cm² there is no protection seen for vitamin E.

Vitamin E has been reported to both protect and sensitize cells to Photofrin®-mediated PDT. To investigate these effects further flow cytometry analysis was performed on DC4 cells treated with Photofrin®-mediated PDT and vitamin E (Fig. 40A, page 82). At the 24 hour point vitamin E has an increased apoptosis peak at 4 J/cm² as compared to the Photofrin® treated sample, but at 2 and 20 J/cm² there is a slight protective effect that vitamin E is exhibiting. At the 48 hour time point, at all doses investigated, vitamin E treated cells display more apoptosis than the Photofrin® treated cells. At both the 24 and 48 hour time points the cells responded in a dose dependent manner with increased apoptosis peaks at higher doses. DB46 cells were also analyzed using flow cytometry with Photofrin®-mediated PDT and vitamin E (Fig. 40B, page 82). The DB46 cells display similar trends to the DC4 cells. At the 24 hour time point, vitamin E has similar apoptosis peaks at the 2 and 4 J/cm² doses and a slightly lower peak at the 20 J/cm² dose. At the 48 hour time point, vitamin E has a much higher apoptosis peak than the Photofrin® treated cells at the 2 and 20 J/cm² dose, but has a lower peak at
the 4 J/cm² dose. From this data, it may be concluded that vitamin E was not having a protective effect against Photofrin®-mediated PDT and may be sensitizing cells in some way to Photofrin®-mediated PDT.

To investigate the effect that vitamin E has on breast cancer cells with Photofrin®, the uptake of Photofrin® into the cell was investigated (Fig. 41, page 83). Both Photofrin® and vitamin E are lipophilic molecules. Perhaps vitamin E may be facilitating the uptake of Photofrin® into the cell. By measuring Photofrin® fluorescence, it was determined that vitamin E did not have any effect on the amount of uptake of Photofrin® into the cell. The amount of fluorescence with Photofrin® alone and Photofrin® with vitamin E was not different.

The localization of the oxidative stress of ALA and Photofrin®-mediated PDT was investigated. The lipid oxidative probe, BODIPY C₁₁, was utilized with fluorescent microscopy. This was done to identify the intracellular localization of the oxidative stress. BT₄C cells were incubated with the oxidative probe and treated with ALA-mediated PDT (Fig. 42, page 84). When looking at the fluorescent images we see a general shift from red to green, confirming the oxidation of lipids is observed. In the 4 J/cm² image, there are areas of red and green, but it is unclear as to the intracellular areas involved (Fig. 42D, page 84). In the 10 J/cm² image, everything appears to be green fluorescent, signifying that both the cellular membrane and intracellular organelles have been oxidized (Fig. 42F, page 84). The MCF7 cells have a very similar pattern after ALA-mediated PDT treatment (Fig. 43, page 85). In the 4 J/cm² fluorescent picture there appears to be a very punctuate pattern of oxidation, but without a secondary stain it is unclear as to the identification of the organelles involved (Fig. 43D, page 85). The 10
J/cm² fluorescent image, again shows cells that are completely green, indicating that both the cellular membrane and intracellular membranes were oxidized (Fig. 43F, page 85).

The MDA-MB-435 cells do not show the expected pattern of red to green fluorescence as increased doses of ALA-mediated PDT occurred (Fig. 44, page 86). The 4 J/cm² image is much greener than the 10 J/cm² image (Fig. 44D and 44F, page 86). There is also a punctuate pattern in the 10 J/cm² fluorescent image. The MDA-MB-435 cells were also analyzed after Photofrin®-mediated PDT (Fig. 45, page 87). In this set of images it is easy to see the red to green shift as oxidation occurs. In the 10 J/cm² image, everything within the cell appears green (Fig. 45F, page 87). Unfortunately, without a secondary stain, this data is inconclusive as to the target of oxidation within the cell. A secondary probe and confocal microscopy would be needed to better identify the localization affects within the cell. Lower doses of PDT may also be helpful in identifying any intracellular targets.
Fig. 9. Clonogenic survival of MDA-MB-435 without light. Photosensitizers alone do not cause toxicity at experimental doses. Clonogenic survival of MDA-MB-435 cells with photosensitizers without light irradiation was performed. Vertical line represents the doses of photosensitizers used in ALA (A) and Photofrin® (B) mediated PDT experiments. Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. Values were normalized to the 0 point, which did not receive any photosensitizer.
Fig. 10. Clonogenic survival of tumor cells exposed to light. Light irradiation alone does not cause toxicity at experimental doses. Clonogenic survival of MDA-MB-435 and MCF7 cells was performed with light irradiation, but without photosensitizers. Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. Values were normalized to the 0 point, which did not receive any light irradiation.
Fig. 11. Clonogenic survival of MDA-MB-435 cells with ALA-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC$_{50}$ for the MDA-MB-435 cells with ALA-mediated PDT. $EC_{50} = 2.2$ J/cm$^2$

Graph shown is a combination of five experiments. In each experiment, each data point was done in triplicate. $R^2=0.9925$ for the non-linear regression.
Fig. 12. Clonogenic survival of DB46 cells with ALA-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC\textsubscript{50} for the DB46 cells with ALA-mediated PDT. EC\textsubscript{50} = 2.7 J/cm\textsuperscript{2} Graph shown is a combination of four experiments. In each experiment, each data point was done in triplicate. R\textsuperscript{2}=0.9725 for the non-linear regression.
Fig. 13. Clonogenic survival of DC4 cells with ALA-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC\textsubscript{50} for the DC4 cells with ALA-mediated PDT. EC\textsubscript{50} = 1.7 J/cm\textsuperscript{2} Graph shown is a combination of four experiments. In each experiment, each data point was done in triplicate. R\textsuperscript{2}=0.9974 for the non-linear regression.
Fig. 14. Clonogenic survival of MCF7 cells with ALA-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC$_{50}$ for the MCF7 cells with ALA-mediated PDT. EC$_{50}$ = 0.4 J/cm$^2$ Graph shown is a combination of four experiments. In each experiment, each data point was done in triplicate. $R^2=0.9738$ for the non-linear regression.
Fig. 15. Clonogenic survival of F98 cells with ALA-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC50 for the F98 cells with ALA-mediated PDT. EC50 = 4.4 J/cm² Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. R²=0.9040 for the non-linear regression.
Fig. 16. Clonogenic survival of BT4C cells with ALA-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC50 for the BT4C cells with ALA-mediated PDT. EC50 = 7.3 J/cm² Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. R²=0.9477 for the non-linear regression.
Fig. 17. Clonogenic survival of MDA-MB-435 cells with Photofrin®-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC$_{50}$ for the MDA-MB-435 cells with Photofrin®-mediated PDT. EC$_{50}$ = 0.7 J/cm$^2$. Graph shown is a combination of four experiments. In each experiment, each data point was done in triplicate. $R^2=0.9755$ for the non-linear regression.
Fig. 18. Clonogenic survival of DB46 cells with Photofrin®-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC$_{50}$ for the DB46 cells with Photofrin®-mediated PDT. EC$_{50} = 0.7$ J/cm$^2$. Graph shown is a combination of three experiments. In each experiment, each data point was done in triplicate. $R^2=0.9814$ for the non-linear regression.
Fig. 19. Clonogenic survival of DC4 cells with Photofrin®-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC$_{50}$ for the DC4 cells with Photofrin®-mediated PDT. EC$_{50}$ = 0.8 J/cm$^2$ Graph shown is a combination of five experiments. In each experiment, each data point was done in triplicate. $R^2$=0.9896 for the non-linear regression.
Fig. 20. Clonogenic survival of MCF7 cells with Photofrin®-mediated PDT. Non-linear regression fit by Graph Pad Prism 4.0 software. Vertical line signifies the location of the EC₅₀ for the MCF7 cells with Photofrin®-mediated PDT. EC₅₀ = 0.4 J/cm². Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. R²=0.9981 for the non-linear regression.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>ALA-mediated PDT</th>
<th>Photofrin®-mediated PDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MD-435</td>
<td>Breast</td>
<td>2.2</td>
<td>0.7</td>
</tr>
<tr>
<td>DB46</td>
<td>Breast</td>
<td>2.7</td>
<td>0.7</td>
</tr>
<tr>
<td>DC4</td>
<td>Breast</td>
<td>1.7</td>
<td>0.8</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>F98</td>
<td>Glioma</td>
<td>4.4</td>
<td>nd</td>
</tr>
<tr>
<td>BT4C</td>
<td>Glioma</td>
<td>7.3</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd = not determined
Fig. 21. Flow cytometry of DC4 cells after ALA-mediated PDT. Cells were treated with ALA-mediated PDT and 24 hours later were fixed for staining with PI. Data is representative of multiple experiments with DB46, MCF7 and MDA-MB-435 with both ALA and Photofrin®-mediated PDT.
<table>
<thead>
<tr>
<th>Dose (J/cm^2)</th>
<th>G1 (%)</th>
<th>G2 (%)</th>
<th>S (%)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>24 hours after ALA-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>31.97</td>
<td>21.85</td>
<td>46.18</td>
<td>0.14</td>
</tr>
<tr>
<td>2</td>
<td>28.20</td>
<td>43.20</td>
<td>28.61</td>
<td>1.57</td>
</tr>
<tr>
<td>4</td>
<td>31.91</td>
<td>18.16</td>
<td>49.93</td>
<td>10.44</td>
</tr>
<tr>
<td>20</td>
<td>29.92</td>
<td>23.26</td>
<td>46.82</td>
<td>25.99</td>
</tr>
<tr>
<td><strong>48 hours after ALA-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>40.37</td>
<td>22.77</td>
<td>36.86</td>
<td>1.58</td>
</tr>
<tr>
<td>2</td>
<td>33.19</td>
<td>24.87</td>
<td>41.93</td>
<td>2.20</td>
</tr>
<tr>
<td>4</td>
<td>25.35</td>
<td>24.53</td>
<td>50.12</td>
<td>13.55</td>
</tr>
<tr>
<td>20</td>
<td>50.64</td>
<td>35.61</td>
<td>13.75</td>
<td>56.39</td>
</tr>
<tr>
<td><strong>24 hours after Photofrin®-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>40.83</td>
<td>19.92</td>
<td>39.25</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>26.31</td>
<td>31.64</td>
<td>42.05</td>
<td>1.11</td>
</tr>
<tr>
<td>4</td>
<td>32.50</td>
<td>24.98</td>
<td>42.52</td>
<td>5.80</td>
</tr>
<tr>
<td>20</td>
<td>47.98</td>
<td>41.30</td>
<td>10.71</td>
<td>30.93</td>
</tr>
<tr>
<td><strong>48 hours after Photofrin®-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>40.62</td>
<td>23.46</td>
<td>35.92</td>
<td>2.06</td>
</tr>
<tr>
<td>2</td>
<td>31.82</td>
<td>21.72</td>
<td>46.46</td>
<td>5.06</td>
</tr>
<tr>
<td>4</td>
<td>32.46</td>
<td>21.19</td>
<td>46.35</td>
<td>6.47</td>
</tr>
<tr>
<td>20</td>
<td>57.67</td>
<td>29.52</td>
<td>12.80</td>
<td>29.86</td>
</tr>
<tr>
<td>Dose (J/cm²)</td>
<td>G1 (%)</td>
<td>G2 (%)</td>
<td>S (%)</td>
<td>Apoptosis (%)</td>
</tr>
<tr>
<td>-------------</td>
<td>--------</td>
<td>--------</td>
<td>-------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>24 hours after ALA-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>44.64</td>
<td>20.31</td>
<td>35.05</td>
<td>2.94</td>
</tr>
<tr>
<td>2</td>
<td>47.44</td>
<td>18.19</td>
<td>34.36</td>
<td>1.68</td>
</tr>
<tr>
<td>4</td>
<td>27.30</td>
<td>24.08</td>
<td>48.62</td>
<td>17.10</td>
</tr>
<tr>
<td>20</td>
<td>37.97</td>
<td>19.73</td>
<td>42.30</td>
<td>14.58</td>
</tr>
<tr>
<td><strong>48 hours after ALA-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>44.34</td>
<td>20.32</td>
<td>35.34</td>
<td>6.47</td>
</tr>
<tr>
<td>2</td>
<td>35.50</td>
<td>26.39</td>
<td>38.11</td>
<td>3.69</td>
</tr>
<tr>
<td>4</td>
<td>28.28</td>
<td>31.38</td>
<td>40.35</td>
<td>4.00</td>
</tr>
<tr>
<td>20</td>
<td>39.57</td>
<td>43.18</td>
<td>17.25</td>
<td>27.27</td>
</tr>
<tr>
<td><strong>24 hours after Photofrin®-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>31.65</td>
<td>20.46</td>
<td>47.89</td>
<td>0.37</td>
</tr>
<tr>
<td>2</td>
<td>32.29</td>
<td>25.38</td>
<td>42.32</td>
<td>4.70</td>
</tr>
<tr>
<td>4</td>
<td>30.32</td>
<td>32.32</td>
<td>38.26</td>
<td>9.12</td>
</tr>
<tr>
<td>20</td>
<td>52.43</td>
<td>42.04</td>
<td>5.53</td>
<td>35.27</td>
</tr>
<tr>
<td><strong>48 hours after Photofrin®-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>43.34</td>
<td>17.23</td>
<td>39.43</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>35.02</td>
<td>21.54</td>
<td>43.44</td>
<td>3.21</td>
</tr>
<tr>
<td>4</td>
<td>31.79</td>
<td>19.05</td>
<td>49.17</td>
<td>14.27</td>
</tr>
<tr>
<td>20</td>
<td>52.79</td>
<td>36.79</td>
<td>10.42</td>
<td>34.82</td>
</tr>
</tbody>
</table>
Table IV
Flow Cytometry Analysis of DC4 cells

<table>
<thead>
<tr>
<th>Dose (J/cm²)</th>
<th>G1 (%)</th>
<th>G2 (%)</th>
<th>S (%)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>24 hours after ALA-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>15.92</td>
<td>27.58</td>
<td>56.50</td>
<td>0.31</td>
</tr>
<tr>
<td>2</td>
<td>17.01</td>
<td>21.40</td>
<td>61.59</td>
<td>7.75</td>
</tr>
<tr>
<td>4</td>
<td>16.99</td>
<td>11.13</td>
<td>71.88</td>
<td>6.51</td>
</tr>
<tr>
<td>20</td>
<td>34.81</td>
<td>6.33</td>
<td>58.87</td>
<td>12.29</td>
</tr>
<tr>
<td><strong>48 hours after ALA-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>15.24</td>
<td>26.73</td>
<td>58.03</td>
<td>2.78</td>
</tr>
<tr>
<td>2</td>
<td>25.97</td>
<td>17.29</td>
<td>56.74</td>
<td>5.56</td>
</tr>
<tr>
<td>4</td>
<td>18.66</td>
<td>20.33</td>
<td>61.01</td>
<td>11.21</td>
</tr>
<tr>
<td>20</td>
<td>24.53</td>
<td>21.73</td>
<td>53.74</td>
<td>12.97</td>
</tr>
<tr>
<td><strong>24 hours after Photofrin®-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>23.51</td>
<td>24.41</td>
<td>52.08</td>
<td>0.37</td>
</tr>
<tr>
<td>2</td>
<td>18.87</td>
<td>28.36</td>
<td>52.76</td>
<td>9.82</td>
</tr>
<tr>
<td>4</td>
<td>22.17</td>
<td>24.08</td>
<td>52.74</td>
<td>10.76</td>
</tr>
<tr>
<td>20</td>
<td>50.08</td>
<td>34.11</td>
<td>15.81</td>
<td>37.82</td>
</tr>
<tr>
<td><strong>48 hours after Photofrin®-mediated PDT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>33.67</td>
<td>21.24</td>
<td>45.09</td>
<td>0.58</td>
</tr>
<tr>
<td>2</td>
<td>24.85</td>
<td>24.60</td>
<td>50.55</td>
<td>9.48</td>
</tr>
<tr>
<td>4</td>
<td>27.45</td>
<td>25.56</td>
<td>46.99</td>
<td>11.54</td>
</tr>
<tr>
<td>20</td>
<td>25.86</td>
<td>18.07</td>
<td>56.07</td>
<td>20.00</td>
</tr>
<tr>
<td>Dose (J/cm²)</td>
<td>G1 (%)</td>
<td>G2 (%)</td>
<td>S (%)</td>
<td>Apoptosis (%)</td>
</tr>
<tr>
<td>--------------</td>
<td>--------</td>
<td>--------</td>
<td>-------</td>
<td>---------------</td>
</tr>
<tr>
<td>24 hours after ALA-mediated PDT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>43.61</td>
<td>13.71</td>
<td>42.68</td>
<td>8.92</td>
</tr>
<tr>
<td>2</td>
<td>59.61</td>
<td>15.15</td>
<td>25.23</td>
<td>10.95</td>
</tr>
<tr>
<td>4</td>
<td>35.63</td>
<td>0.31</td>
<td>64.06</td>
<td>20.34</td>
</tr>
<tr>
<td>20</td>
<td>31.63</td>
<td>8.35</td>
<td>60.03</td>
<td>42.44</td>
</tr>
<tr>
<td>48 hours after ALA-mediated PDT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>26.23</td>
<td>0</td>
<td>73.77</td>
<td>1.63</td>
</tr>
<tr>
<td>2</td>
<td>34.61</td>
<td>19.3</td>
<td>46.09</td>
<td>1.78</td>
</tr>
<tr>
<td>4</td>
<td>28.14</td>
<td>28.05</td>
<td>43.81</td>
<td>5.66</td>
</tr>
<tr>
<td>20</td>
<td>23.44</td>
<td>30.72</td>
<td>45.85</td>
<td>22.14</td>
</tr>
<tr>
<td>24 hours after Photofrin®-mediated PDT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>39.14</td>
<td>14.07</td>
<td>46.79</td>
<td>11.88</td>
</tr>
<tr>
<td>2</td>
<td>36.72</td>
<td>7.49</td>
<td>55.79</td>
<td>20.06</td>
</tr>
<tr>
<td>4</td>
<td>35.01</td>
<td>9.28</td>
<td>55.72</td>
<td>16.68</td>
</tr>
<tr>
<td>20</td>
<td>39.3</td>
<td>0.63</td>
<td>60.07</td>
<td>54.18</td>
</tr>
<tr>
<td>48 hours after Photofrin®-mediated PDT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>48.5</td>
<td>8.64</td>
<td>42.86</td>
<td>10.19</td>
</tr>
<tr>
<td>2</td>
<td>40.13</td>
<td>17.05</td>
<td>42.82</td>
<td>17.54</td>
</tr>
<tr>
<td>4</td>
<td>38.47</td>
<td>21.89</td>
<td>39.64</td>
<td>31.6</td>
</tr>
<tr>
<td>20</td>
<td>63.37</td>
<td>24.32</td>
<td>12.31</td>
<td>45.75</td>
</tr>
</tbody>
</table>
Fig. 22. Fluorescence analysis of photosensitized cells. The amount of photosensitizer fluorescence per cell was determined using flow cytometry. Measurements were done after four hours of incubation. Varying concentrations of ALA (A) and Photofrin® (B) were investigated. Results are an average of three experiments with each experiment done in duplicate.
Fig. 23. Fluorescence analysis of photosensitized cells at varying times. Fluorescence of photosensitized tumor cells was investigated with varying times. ALA (A) was used at a concentration of 1 mM. Photofrin® (B) was investigated at a concentration of 2.5 µg/mL. All values were normalized to the zero point (not shown). Values are an average of 2 separate experiments, each experiment was done in duplicate.
Fig. 24. Attachment assay of MDA-MB-435 cells after ALA-mediated PDT. ALA-mediated PDT induces detachment and necrosis of MDA-MB-435 cells. The number of attached cells (A) and the number of cells found in the media (B) was normalized to total (attached plus floating cells) found in the control flasks at time zero. Each data point was done in duplicate. Data is representative of two experiments.
Fig. 25. Attachment assay of MDA-MB-435 cells after Photofrin®-mediated PDT. Photofrin®-mediated PDT causes detachment and necrosis in MDA-MB-435 cells. The number of attached cells (A) and the number of cells found in the media (B) was normalized to total (attached plus floating cells) found in the control flasks at time zero. Each data point was done in duplicate. Data is representative of two experiments.
Fig. 26. Attachment assay of BT4C cells after ALA-mediated PDT. ALA-mediated PDT induces detachment and necrosis on BT4C cells. The number of attached cells (A) and the number of cells found in the media (B) was normalized to total (attached plus floating cells) found in the control flasks at time zero. Each data point was done in duplicate. Data is representative of two experiments.
Fig. 27. Fluorescent microscopy studies with ALA-mediated PDT. Breast cancer cells (MDA-MB-435 and MCF7) and brain cancer cells (F98 and BT4C) were treated with ALA-mediated PDT and analyzed with fluorescent microscopy 48 hours after treatment. Hoechst stain is an indicator of apoptosis and PI is an indicator of necrosis. The number of cells were counted in the bright field image and the cells that were positive for staining were normalized to the total number of cells.
Fig. 28. Fluorescent microscopy study of MDA-MB-435 cells. 24 and 48 hours after treatment MDA-MB-435 cells were analyzed with Hoechst and PI stain to identify apoptotic and necrotic cells. The total number of cells in the bright field was used to normalize the number of cells positive for each stain. Data is an average of two experiments with three pictures taken in each well.
Fig. 29. Protein oxidation after ALA-mediated PDT. ALA-mediated PDT causes more thiol oxidation in breast cancer cell lines than in brain cancer cell lines. The number of reduced thiol groups was measured using Ellman’s reagent immediately after ALA-mediated PDT treatment. The values were normalized to the 0 point, which received ALA, but no light treatment. Values are a combination of at least 2 experiments. * = p < 0.05
Fig. 30. Protein oxidation of MDA-MB-435 cells with BSO and vitamin E. Photofrin® has less thiol oxidation at equal survival levels. 2.5 and 0.5 J/cm² are the approximate EC50 values for ALA (A) and Photofrin® (B) mediated PDT, respectively. At 4 and 2 J/cm² there is less than 90% survival for ALA and Photofrin®-mediated PDT, respectively. The number of reduced thiol groups was measured using Ellman’s reagent immediately after PDT treatment. The values were normalized to the 0 point, which received photosensitizer, but no light treatment. Both BSO and vitamin E samples were pre-treated for 24 hours with the drugs before PDT. Values are a combination of at least 2 experiments. * = p< 0.05
Fig. 31. Protein oxidation of MDA-MB-435 cells after PDT. Thiol oxidation is not dose dependent with ALA or Photofrin®-mediated PDT. MDA-MB-435 cells were treated with both Photofrin® and ALA-mediated PDT. The number of reduced thiol groups was measured using Ellman's reagent immediately after ALA or Photofrin®-mediated PDT treatment. The values were normalized to the 0 point, which received the photosensitizer, but no light treatment. Values are a combination of at least 2 experiments. * = p <0.05
Fig. 32. Lipid peroxidation measured using HPLC. MDA-MB-435 cells were analyzed immediately after PDT treatment. Quantification of MDA was normalized to total protein content. Control values were subtracted from each data point. Data is a combination of three separate experiments.
Fig. 33. Fluorescent microscopy of MDA-MB-435 cells after PDT. MDA-MB-435 cells were analyzed with Hoechst and PI stain 24 hours after PDT to identify apoptotic and necrotic cells. The total number of cells in the bright field was used to normalize the number of cells positive for each stain. Data is an average of two experiments with three pictures taken in each well.
Fig. 34. Clonogenic survival of MDA-MB-435 cells with BSO and vitamin E. MDA-MB-435 cells were treated with ALA-mediated PDT and clonogenic survival assays were performed. Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. BSO and vitamin E were applied 24 hours prior to irradiation at a concentration of 50 μM. * = p <0.05
Fig. 35. Clonogenic survival of DB46 cells with BSO and vitamin E. DB46 cells were treated with ALA-mediated PDT and clonogenic survival assays were performed. Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. BSO and vitamin E were applied 24 hours prior to irradiation at a concentration of 50 μM. * = p <0.05
Fig. 36. Clonogenic survival of DC4 cells with BSO and vitamin E. DC4 cells were treated with ALA-mediated PDT and clonogenic survival assays were performed. Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. BSO and vitamin E were applied 24 hours prior to irradiation at a concentration of 50 μM. * = p <0.05
Fig. 37. Clonogenic survival of MCF7 cells with BSO and vitamin E. MCF7 cells were treated with ALA-mediated PDT and clonogenic survival assays were performed. Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. BSO and vitamin E were applied 24 hours prior to irradiation at a concentration of 50 μM. * = p <0.05
Fig. 38. Clonogenic Survival of MDA-MB-435 with Photofrin®, BSO and vitamin E. MDA-MB-435 cells were treated with Photofrin®-mediated PDT and clonogenic survival assays were performed. Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. BSO and vitamin E were applied 24 hours prior to irradiation at a concentration of 50 µM. * = p <0.05
Fig. 39. Clonogenic survival of DC4 cells with Photofrin® and vitamin E. DC4 cells were treated with Photofrin®-mediated PDT and clonogenic survival assays were performed. Graph shown is a combination of two experiments. In each experiment, each data point was done in triplicate. Vitamin E was applied 24 hours prior to irradiation at a concentration of 50 μM. * = p <0.05
Fig. 40. Flow Cytometry Analysis of cell cycle distribution. DC4 (A) and DB46 (B) cells were treated with Photofrin®-mediated PDT and vitamin E. PI was used to stain the DNA of the cells. Vitamin E was applied to cells 24 hours prior to irradiation at a concentration of 50 μM. Cells were harvested at 24 and 48 hours after irradiation.
Fig. 41. Fluorescence of MDA-MB-435 cells with Photofrin® and vitamin E. MDA-MB 435 cells were treated with 0 or 2.5 μg/mL of Photofrin®. Vitamin E was applied 24 hours prior to analyzing cells at a concentration of 50 μM. Cell fluorescence was measured using flow cytometry.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Fig. 42. Oxidative sensitive probe with BT$_4$C cells. BODIPY C$_{11}$ probe was loaded into cells 15 minutes before irradiation. BT$_4$C cells were treated with ALA-mediated PDT and then analyzed. The probe fluorescence was measured with the fluorescent microscope and images were taken with a blue and green filter. The change from red to green fluorescence signifies oxidation. A, C and E are the phase contrast images of B, D and F respectively. B, D and F are the fluorescent images taken and merged to give the red and green fluorescent image. A and B are the control images receiving no light irradiation. C and D are the 4 J/cm$^2$ images and E and F are the 10 J/cm$^2$ images. Pictures are representative of three shots taken per treatment.
Fig. 43. Oxidative sensitive probe with MCF7 cells. BODIPY C_{11} probe was loaded into cells 15 minutes before irradiation. MCF7 cells were treated with ALA-mediated PDT and then analyzed. The probe fluorescence was measured with the fluorescent microscope and images were taken with a blue and green filter. The change from red to green fluorescence signifies oxidation. A, C and E are the phase contrast images of B, D and F respectively. B, D and F are the fluorescent images taken and merged to give the red and green fluorescent image. A and B are the control images receiving no light irradiation. C and D are the 4 J/cm^2 images and E and F are the 10 J/cm^2 images. Pictures are representative of three shots taken per treatment.
Fig. 44. Oxidative sensitive probe with MDA-MB-435 cells treated with ALA-mediated PDT. BODIPY C_{11} probe was loaded into cells 15 minutes before irradiation. The probe fluorescence was measured with the fluorescent microscope and images were taken with a blue and green filter. The change from red to green fluorescence signifies oxidation. A, C and E are the phase contrast images of B, D and F respectively. B, D and F are the fluorescent images taken and merged to give the red and green fluorescent image. A and B are the control images receiving no light irradiation. C and D are the 4 J/cm^2 images and E and F are the 10 J/cm^2 images. Pictures are representative of three shots taken per treatment.
Fig. 45. Oxidative sensitive probe with MDA-MB-435 cells treated with Photofrin®-mediated PDT. BODIPY C_{11} probe was loaded into cells 15 minutes before irradiation. The probe fluorescence was measured with the fluorescent microscope and images were taken with a blue and green filter. The change from red to green fluorescence signifies oxidation. A, C and E are the phase contrast images of B, D and F respectively. B, D and F are the fluorescent images taken and merged to give the red and green fluorescent image. A and B are the control images receiving no light irradiation. C and D are the 4 J/cm^2 images and E and F are the 10 J/cm^2 images. Pictures are representative of three shots taken per treatment.
CHAPTER 4

DISCUSSION

In these studies, it has been shown that ALA and Photofrin®-mediated PDT are effective in inducing cell death (both apoptosis and necrosis) in rat glioma cell lines and human breast cancer cell lines. Oxidation of proteins and lipids was detected with both photosensitizers. Photofrin® and ALA-mediated PDT have each been shown to cause cell cytotoxicity by two different mechanisms. A summary of these differences is presented in Table VI (page 100).

The effectiveness of PDT is dependent on both light irradiance and photosensitizer application (Fig 9, page 46) which is in good agreement with published results. In these studies, breast cancer cell lines are more sensitive to Photofrin® than to ALA-mediated PDT (Table I, page 58). For the four breast cancer cell lines studied the EC$_{50}$ values were significantly lower for the Photofrin®-mediated PDT than the ALA-mediated PDT. Not only is the EC$_{50}$ lower in the Photofrin® treated cells, but they reach an EC$_{90}$ at lower doses of light than the ALA treated cells. The observation that Photofrin® is more lethal than ALA is supported by the flow cytometry analysis of the breast cancer cells (Table II-IV, pages 60-63). The trend that the amount of apoptotic vesicles was higher in the Photofrin® treated cells than the ALA treated cells is observed. The comparison that Photofrin® is more effective than ALA-mediated PDT has been reported earlier by other groups (33, 34). This comparison of the two photosensitizers is
not clinically relevant because in vivo ALA and Photofrin® target different types of tissues. Photofrin® targets vasculature within the tumor where ALA targets the tumor cells specifically (35, 36). Since the two photosensitizers have different effects within the patient, it may not be clinically relevant that Photofrin® is more effective against breast cancer cell lines in vitro.

In these studies the two rat glioma cell lines compared to the four breast cancer cell lines were shown to be more resistant to ALA-mediated PDT. This can be seen by examining the EC\textsubscript{50} values of the BT\textsubscript{4}C and the F98, (Table 1, page 58) which occur at higher doses than the breast cancer cell lines investigated. Also, when examining the clonogenic survival results, the glioma cell lines did not reach an EC\textsubscript{90} value of 20 J/cm\textsuperscript{2}. This is different than the breast cancer cell lines which did reach EC\textsubscript{90} values before 20 J/cm\textsuperscript{2}. This difference between the two types of cell lines could be either a tissue or species specific difference or both. There are intrinsic differences when comparing two different species models. It is also true that gliomas are resistant to treatment with conventional therapies (37).

The fluorescence properties of photosensitizers have been utilized in imaging of tumor locations and can aid in diagnostic practices (38). The relative amount of fluorescence of ALA induced PpIX and Photofrin® can also allow us to estimate the concentration of photosensitizer within the cell. When comparing PpIX and Photofrin® fluorescence levels, it is observed that Photofrin® has a 10 times higher amount of fluorescence per cell. This is most likely due to the intrinsic fluorescence of Photofrin®. When looking at the overall trends of ALA and Photofrin® fluorescence by varying concentrations, it is seen that in most of the cell lines there is a maximum fluorescence
that is obtained at 1 mM ALA (Fig. 22, page 64). With Photofrin®, a maximum fluorescence is not reached by a concentration of 10 μg/mL. This could indicate that with increasing the concentration of ALA above 1 mM will not change the concentration of PpIX within the cell. The same trends that were seen with varying concentrations are also seen when time is varied with both ALA and Photofrin® (Fig. 23, page 65). After three hours of incubation with ALA most of the cell lines reach a maximal concentration of PpIX and incubation for longer times would not increase that level. With Photofrin®, there is no maximal concentration reached by five hours and incubation for longer times might produce a higher concentration of Photofrin®. Unfortunately, the fluorescence levels are not comparable between the concentration and time variance experiments. The MCF7 cells incubated with ALA have fluorescence of 100 a.u. in Fig. 22 and 600 a.u. in Fig. 23 under the same incubation conditions. These variations in fluorescence are seen with all cell lines and both photosensitizers and deserve further investigation.

When analyzing the ALA induced PpIX fluorescence, the MCF7 cells have the highest fluorescence levels which correlate to the MCF7 cells being the most sensitive to ALA-mediated PDT as seen by the low EC₅₀ value. It has been reported that there is a correlation with increased PpIX concentrations within the cell and higher effectiveness of tumor cell line killing which is consistent with the data reported here (39). It is also important to note that MCF7 cells are the only caspase 3 deficient cell line that was investigated. This lack of caspase 3, increased PpIX production or another factor not yet determined may be leading to higher levels of cell death. The other four cell lines investigated had relatively similar levels of PpIX fluorescence. PpIX production is not the only factor that leads to cell sensitivity since the BT4C cells, which were the least
sensitive to ALA-mediated PDT, showed a similar fluorescence level with the MDA-MB-435, DC4 and DB46 cells.

Photofrin® fluorescence does not correlate with clonogenic survival results. The MCF7 cells were again the most sensitive to the Photofrin®-mediated PDT in the clonogenic survival, but had the second lowest Photofrin® fluorescence. The DC4, DB46 and MDA-MB-435 cells all had similar EC50 values, but the DC4 and DB46 cells showed higher amounts of fluorescence as compared to the MDA-MB-435. From this data it can be concluded that PpIX production may have an effect on sensitivity of the four cells to ALA-mediated PDT, but there are other factors that affect sensitivity. With Photofrin®-mediated PDT, Photofrin® concentration within the cell does not have a direct correlation with sensitivity. Other studies have reported that the amount of photosensitizer within the cell does not always correlate to cell survival (40). It may be hypothesized that there is a threshold amount of Photofrin® that must be incorporated into the cell to achieve sensitivity, but concentrations above that amount do not effect sensitivity. In the studies reported here it may be assumed that the threshold level of Photofrin® was obtained by a concentration of 2.5 µg/mL of Photofrin® since fluorescence levels of Photofrin® are not correlative with survival.

It has been reported that both apoptosis and necrosis occurs after PDT induced cytotoxicity (41, 42). It has also been reported that two different apoptotic pathways are induced during ALA-mediated PDT (42, 43). In the studies reported here, earlier reports are confirmed and show a timeline of two types of cell death occurring which appears to be dose and photosensitizer dependent. The exact molecular mechanism that causes cell death has not been reported for either ALA or Photofrin®-mediated PDT. There are
many speculations on the molecular mechanism, but a specific pathway has not been confirmed. Because of this, ALA and Photofrin® induced mechanisms of cell death have not been compared to each other extensively in the literature. Here it is reported that ALA and Photofrin®-mediated PDT have two different timelines of cell death that may indicate two different molecular mechanisms.

With ALA-mediated PDT it was observed that there was a fast type of cell death which is characteristic of necrosis and a slow type of cell death characteristic of apoptosis. This was confirmed with fluorescent microscopy, flow cytometry and attachment assays. With Photofrin®-mediated PDT, cell death is mostly necrotic and happens more quickly than the ALA-mediated PDT induced cell death. When looking at fluorescent microscopy results, the ALA-mediated PDT cells have early necrotic events seen at the 24 hour time point and that decrease by the 48 hour time point (Fig. 28, page 70). There is the presence of apoptotic cells both at 24 and 48 hours. With Photofrin®-mediated PDT fluorescent microscopy results indicate that there are high amounts of necrosis at all light doses at the 24 hour time point with a decrease in the amount of necrosis at the 48 hour time point. A small amount of apoptosis can only be observed at low doses at the 48 hour time point.

The fact that there are two types of cell death is also supported by the data from the attachment assays and flow cytometry. In the attachment assays, there was a detachment and loss of cells within four hours with both ALA and Photofrin®-mediated PDT (Fig. 24-26, pages 66-68). Loss of cells is characteristic of necrotic cell death. Flow cytometry results also confirm that Photofrin® and ALA-mediated PDT are using two different mechanisms of cell death. With ALA-mediated PDT there was an increase
in the number of apoptotic cells between the 24 and 48 hour time points with all cell lines. With the Photofrin®-mediated PDT there was a decrease or no change in the number of apoptotic cells between the 24 and 48 hour time points. Flow cytometry also showed a general decrease in the S phase of the cell cycle as the dose of Photofrin®-mediated PDT was increased. This may indicate a block in the cell cycle that is not evident with ALA mediated-PDT. This trend was not observed in the ALA-mediated PDT cells.

Another observation that can be made from these studies was that the rat glioma cell lines have a different response to ALA-mediated PDT than the breast cancer cell lines. It has been reported that brain tissue will undergo both apoptosis and necrosis after PDT treatment (44). It is also logical to expect differences in cell death with two tissues that are as different as brain and breast tissue. Brain tissue is unique in the lipids that are found within the cell. Lipids play an important role in ALA-mediated PDT induced apoptosis, which could explain these differences (45). In our studies we see much higher levels of apoptosis in brain cells treated with ALA-mediated PDT than the breast cancer cells treated. This is actually beneficial in the clinical setting as apoptosis does not induce inflammation and does not cause cell death to adjoining cells as necrosis does (33).

Protein oxidation has been reported to occur after PDT treatment in different types of cell systems, but not always in a dose dependent response (15, 45, 46). The data presented here supports these earlier findings. It was observed that both ALA and Photofrin®-mediated PDT caused protein oxidation through the loss of reduced thiol groups. The protein oxidation was not correlative to survival levels within the cell lines.
or between the two photosensitizers. The data that is correlative to cell survival is that
the two brain cell lines, BT_4_C and F98, had the least amount of protein oxidation and had
the highest EC_{50} values of cells treated with ALA-mediated PDT. Photofrin® has been
shown to be more effective than ALA-mediated PDT in killing the MDA-MB-435 cells,
yet it had lower amounts of protein oxidation at both equal survival and equal doses.
This again points to two different types of cell death between the Photofrin® and ALA-
mediated PDT treated cells. Because the protein oxidation is not dose dependent with
survival, apoptosis levels or necrosis levels, it suggests that a bulk oxidation of protein is
not the key to the switch between apoptosis and necrosis within the cell.

Lipid peroxidation has also been reported in conjunction with PDT induced
cytotoxicity (47). However, it has been suggested though that lipid peroxidation is not
the general mechanism that produces cell death in PDT treated cells (40, 48). The data
reported here supports these earlier findings. There is an increase in lipid peroxidation
after PDT treatment with both ALA and Photofrin®-mediated PDT. Increased lipid
peroxidation does not reach a significantly higher level than control until higher doses of
light irradiation. This is not correlative to survival data, since there is no increase in lipid
peroxidation above basal level until after the EC_{50} dose in both ALA and Photofrin®-
mediated PDT cells. Also the ALA treated cells show a higher amount of lipid
peroxidation as compared to the Photofrin® treated cells. This is not correlative with the
observed effects in the clonogenic survivals. There is not a clear correlation of lipid
peroxidation and apoptosis. Lipid peroxidation has been reported to cause apoptosis,
which our studies do not support (41). This could be due to a targeted analysis by Kriska

94

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
et al. of mitochondrial lipids as compared to the studies done in this manuscript where the whole cell was analyzed (41).

The application of antioxidants and PDT have been investigated in a number of studies since they can play an important role in the oxidative stress a cell undergoes. Vitamin E and vitamin C have been shown to protect cells against PDT (49, 13). It has also been shown by a single research group that Foscan-mediated PDT can be enhanced by vitamin E application (48, 50). The data presented here supports both of these findings. Vitamin E was shown to protect cells against ALA and Photofrin®-mediated PDT in the clonogenic survival assays. But in other assays vitamin E was shown to sensitize cells to Photofrin®-mediated PDT. This was evident when looking at protein oxidation, fluorescent microscopy and flow cytometry. It was first thought that this was a function of increased Photofrin® concentration within the cell. This was not the case, as fluorescence studies were done. It was undetermined as to the reason for both protection and sensitization that vitamin E had during PDT treatments.

It has been reported that BSO can enhance Photofrin®-mediated PDT by lowering GSH levels within the cell (51). The effects BSO has in conjunction with ALA-mediated PDT have not been reported. In our studies we have shown that BSO selectively sensitizes cells to ALA-mediated PDT. It would be expected that by lowering the levels of GSH within the cell that there would be increased oxidative stress. In Photofrin®-mediated PDT cells there was increased protein oxidation with application of BSO, but not in cells treated with ALA-mediated PDT. BSO sensitized the breast cancer cells, MCF7, DC4 and DB46, to ALA-mediated PDT, but did not in the MDA-MB-435 cells.
These findings confirm that the mechanism of cell death is not as simple as changes in bulk levels of oxidation, changing the sensitivity of tumor cells to PDT.

Since the total amount of lipid or protein oxidation within the cell does not seem to be correlative with cell survival, it was hypothesized that a target or set of targets may be the key to PDT induced cell death. It has been reported that ALA induced PpIX and Photofrin® localize in different areas of the cell, which may lead to different targets of oxidation. PpIX localization within the cell has been said to be mitochondrial bound (41, 52). Photofrin® is highly lipophillic, associates with high density lipoproteins, and can be found in the cellular, endoplasmic reticulum and nuclear membranes (9, 53). To confirm the different localization patterns of the photosensitizers, fluorescent microscopy was used with a fluorescent probe to determine lipid oxidation patterns. Unfortunately, with the data obtained, it was not possible to conclusively determine any localization patterns within the cellular organelles with Photofrin® or ALA-mediated PDT. Fluorescence studies with the photosensitizers alone may help to determine the location of the photosensitizer within the cell. Also the use of a secondary dye specific to organelles within the cell may be useful in determining the localization patterns of the photosensitizers.

ALA-mediated PDT induced cytotoxicity has been investigated by many different research groups. Shahzidi et al. showed that ALA-mediated PDT caused the loss of membrane potential of the mitochondria, the release of cytochrome c and the activation of caspase 3 and caspase 9 (54). At this time, there have been no conclusive reports about the role of caspase 8 in cell death induced by ALA-mediated PDT. There have been investigations that have shown that along with mitochondrial changes within the
cell, the disruption of the actin filaments leads to necrosis in ALA-mediated PDT treated cells (55). Finlan et al. reported that ALA-mediated PDT induced DNA damage, but did not induce the activation of p53 (56). It has also been reported that ALA-mediated PDT induces the production of Hsp70 in response to the stress of PDT (57). These studies show that ALA-mediated PDT induces cell death that is characteristic of apoptosis through mitochondrial signaling.

Photofrin®-mediated PDT induces cell death that is similar to that of ALA-mediated PDT. Wu et al. showed the activation of caspase 3 within Photofrin® treated cells, but not an activation in caspase 8 (53). Unlike the ALA-mediated PDT cells, Photofrin®-treated PDT cells showed an induction of the p53 pathway (58, 59). Takahira et al. demonstrated that DNA ladder formation occurred after Photofrin®-mediated PDT along with the activation of caspase 3 and caspase 9 and concluded that mitochondrial damage was important to apoptosis activation (60). The induction of Hsp70 after Photofrin®-mediated PDT has also been reported (61, 62). Overall, Photofrin®-mediated PDT treated cells shows the general hallmarks of apoptosis which is similar to the ALA-mediated PDT treated cells.

Even though in our studies we have seen distinct differences in the type of cell death in ALA and Photofrin®-mediated PDT treated cells, this is not necessarily what has been reported earlier. Unfortunately there have not been many studies conducted that compare Photofrin® to ALA with the same cell line and same treatment conditions. When comparing the mechanism of ALA and Photofrin® it is important to have similar experimental conditions, since incubation times, laser conditions and cell lines all can give unique results. One of the strengths of the studies reported here is that similar
treatment conditions have been used. This allows for the direct comparison of ALA and Photofrin®. Another problem with earlier reports is that the same hallmarks of cell death have not been reported on for both photosensitizers. Caspase 8 for instance has been reported to not be activated in Photofrin®-mediated PDT, but there are no reports on its effect with ALA-mediated PDT. In the studies reported here by using the same assays for both ALA and Photofrin® treated cells a direct comparison can be made between the two photosensitizers.

Summary - There is now preliminary data to show that Photofrin® and ALA-mediated PDT are inducing cell death by two different types of molecular mechanisms. While both ALA and Photofrin®-mediated PDT induce apoptosis, the data presented in this study indicate they do so by two unique mechanisms. There is also evidence that it is not just the amount of bulk lipid or protein oxidation that determines whether apoptosis, necrosis or cell survival will occur after PDT treatment. A specific set of targets may be the key to understanding the mechanism behind cell cytotoxicity after PDT.

To further study the molecular mechanism it may be important to look at the last set of molecules that can be oxidized, DNA. Photosensitizers have been shown to bind to DNA within the nucleus (13, 63). The key to the difference in molecular mechanism between apoptosis and necrosis with ALA and Photofrin®-mediated PDT may be due to DNA binding properties of the photosensitizer or location of the oxidation of the DNA. This could also explain the decrease in the S phase peak when Photofrin®-mediated PDT is used. Looking at DNA binding and oxidation with PDT may be the key to the molecular mechanism.
Also, further studies should look at the role of the membrane components with PDT. Looking at the specific lipids within the cells that get targeted for oxidation during PDT might also prove useful. Glial cells have different lipid content as compared to other tissues, and this may be the reason for decreased sensitivity in this cell type. Vitamin E is also a lipophilic molecule that associates with cellular membranes. The results presented here do not conclusively explain vitamin E’s role in PDT. Photofrin® associates with lipoproteins within the membrane (9). These important factors may also point to an important membrane component of PDT-induced cytotoxicity. By modulating the lipid content within the cell, it may become clear how to increase the effectiveness of PDT (64).

Finally, to improve the clinical application of PDT, it is important to understand how combination therapies affect PDT effectiveness. Breast cancer patients today are not given a single therapy. Multiple therapies are used and most breast cancer patients will receive chemotherapy in addition to other treatments. Understanding the effects of PDT with other chemotherapeutic agents is important. It may be found that synergistic combinations exist along with negative combinations. Not only should these be done at the cellular level, but in vivo work should also be carried out. Photofrin® and ALA work differently in vivo than what is seen at the cellular level. Having this knowledge will help physicians incorporate PDT into the normal regimen of cancer therapies.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>ALA-mediated PDT</th>
<th>Photofrin®-mediated PDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival</td>
<td>Less Lethal</td>
<td>More Lethal</td>
</tr>
<tr>
<td>Auto Fluorescence</td>
<td>Less</td>
<td>More</td>
</tr>
<tr>
<td>Cell Counts</td>
<td>Less Detachment</td>
<td>More Detachment</td>
</tr>
<tr>
<td>Fluorescent Micro.</td>
<td>More Apoptosis</td>
<td>More Necrosis</td>
</tr>
<tr>
<td>Protein Oxidation</td>
<td>More</td>
<td>Less</td>
</tr>
<tr>
<td>Lipid Peroxidation</td>
<td>More</td>
<td>Less</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>Less Apoptosis</td>
<td>More Apoptosis</td>
</tr>
<tr>
<td>BSO Survival</td>
<td>Sensitivity</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>Vitamin E Survival</td>
<td>Protection</td>
<td>Protection</td>
</tr>
</tbody>
</table>

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
REFERENCES


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
VITA
Graduate College
University of Nevada, Las Vegas
Sarah Ann Ziegler

Local Address:
3070 Casey Dr Unit 201
Las Vegas, NV 89120

Degrees:
Bachelor of Science, Biochemistry, 2003
University of Nevada, Las Vegas

Publications:


Thesis Title:
Photodynamic Therapy Induces Oxidation in Breast and Brain Cancer Cell Lines

Thesis Examination Committee:
Chairperson, Stephen Carper, Ph.D.
Committee Member, Steen Madsen, Ph.D.
Committee Member, Bryan Spangelo, Ph.D.
Graduate College Representative, Susan Meacham, Ph.D.