DRUG RESISTANCE IN D. DISCOIDEUM: ISOLATION OF 4-NITROQUINOLINE 1-OXIDE RESISTANT MUTANTS

A Thesis presented to the Faculty of the Graduate School
University of Missouri-Columbia

In Partial Fulfillment
Of the Requirements for the Degree

Masters of Arts

by

ANDREW L. STEGNER

Dr. Stephen Alexander and Dr. Hannah Alexander
Thesis Supervisors

JULY 2005
The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

DRUG RESISTANCE IN D. DISCOIDEUM: ISOLATION OF 4-NITROQUINOLINE 1-OXIDE RESISTANT MUTANTS

Presented by Andrew L. Stegner

a candidate for the degree of Masters of Arts

and hereby certify that in their opinion it is worthy of acceptance.

[Signatures]

[Signatures]
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisors Drs. Stephen and Hannah Alexander for their guidance, support, time, and instruction during my time at the University of Missouri. I would also like to thank Dr. Dudley McCaw for his advice and support with my research and thesis preparation.

Thank you to all the Alexander lab members past and present, it was an honor and privilege to work with you all. Special thanks to "the girls" Dr. Junxia Min, Priya Sridevi and Bandhana Katoch, who were always cheerful, and willing to help in any way possible.

I would like to thank my family for their support and encouragement in my academic pursuits. I am especially grateful to my brother Greg and his wife Jackie for their support and understanding over the years.

Lastly but not least I would like to give unending thanks to my beautiful and soon to be wife Shelly for her love, patience and support.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS................................................................. ii

TABLE OF CONTENTS....................................................................... iii

LIST OF FIGURES........................................................................... iv

Chapters

1. INTRODUCTION................................................................. 1
   Cancer Basics.............................................................................. 1
   Chemotherapy.............................................................................. 4
   *Dictyostelium* as a model organism........................................ 6
   The Sphingolipid metabolic pathway........................................ 8

2. 4-Nitroquinoline 1-oxide...................................................... 15
   Introduction................................................................................. 15
   Materials and Methods................................................................. 19
   Results........................................................................................ 25
   Discussion.................................................................................... 35

APPENDIX

1. Proteomics of Nipple Aspirate Fluid................................. 44

REFERENCES............................................................................... 53
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Life cycle of <em>Dictyostelium discoideum</em></td>
<td>7</td>
</tr>
<tr>
<td>2.</td>
<td>Sphingolipid metabolic pathway</td>
<td>10</td>
</tr>
<tr>
<td>3.</td>
<td>Ceramide/Sphingosine-1-Phosphate rheostat model</td>
<td>11</td>
</tr>
<tr>
<td>4.</td>
<td>4NQO metabolic pathway</td>
<td>16</td>
</tr>
<tr>
<td>5.</td>
<td>4NQO DNA adduct formation</td>
<td>17</td>
</tr>
<tr>
<td>6.</td>
<td>4NQO Ax4 killing curve</td>
<td>26</td>
</tr>
<tr>
<td>7.</td>
<td>3B8B mutant 4NQO screen for resistance</td>
<td>28</td>
</tr>
<tr>
<td>8.</td>
<td>6E3A mutant 4NQO screen for resistance</td>
<td>30</td>
</tr>
<tr>
<td>9.</td>
<td>Restriction Enzyme Mediated Integration</td>
<td>32</td>
</tr>
<tr>
<td>10.</td>
<td>6E3A and 3B8B inverse PCR</td>
<td>34</td>
</tr>
<tr>
<td>11.</td>
<td>6E3A REMI insertion</td>
<td>36</td>
</tr>
<tr>
<td>12.</td>
<td>6E3A alignment</td>
<td>37</td>
</tr>
</tbody>
</table>
Cancer is the uncontrolled growth of abnormal cells in the body. Cancer affects one in every three persons in developed countries, and is a major cause of death in underdeveloped nations. Cancer is the second leading cause of death in the United States, at approximately half a million people per year. Many recent developments, including timely and accurate diagnosis, selective surgery, radiation therapy, hormone therapy, biological therapy, and chemotherapeutic drugs have increased the effectiveness of cancer treatment and decreased deaths. Ongoing investigation in areas of cell biology, genetics, proteomics, and biotechnology are investigating causes and mechanisms of this disease.

Cancer Basics

A tumor is an abnormal growth of tissue resulting from uncontrolled, progressive multiplication of cells (neoplasms), which serves no physiological function, and can arise from any tissue in the body [1, 2]. There are a variety of tumors and each has different properties that facilitate their development. Cancer is the term which grossly refers to more than 100 forms of the disease [2]. A non-life threatening tumor that does not spread
A spreading tumor is malignant and the spreading of malignant tumor cells throughout the body is metastasis [1].

Cancer is complex and there are many variables that contribute to carcinogenesis and its development such as: an inherited germline mutation, mutations incurred during normal cellular metabolism and environmental factors including radiation, chemicals, or introduction of new DNA sequences by viruses. Most cancers result from a number of mutated genes, affecting intricate pathways within the cell. [2, 3].

Malignant transformation is acquired through the accumulation of mutations in the cellular genes. Mutations can result in the alteration of a genes' function or expression. In many cases these genes are known as proto-oncogenes or tumor suppressors genes. When proto-oncogenes are mutated to carcinogenic oncogenes cell growth is stimulated. Proto-oncogenes encode genetic components that regulate many cellular signaling pathways controlling positive growth signals. Some of these affected pathways are: cell division, phosphorylation of proteins, growth promoters, and surface receptors [2].

Tumor suppressors encode genetic components that regulate negative growth signals in the cell. Mutation of a tumor suppressor gene inactivates its function and can affect: secreted inhibitory substances, their receptors, negative regulators of intracellular growth stimulatory signaling pathways and proteins that shut down progression through the cell cycle [2].
These positive and negative growth regulatory signaling pathways ultimately lead to control of the cell cycle. The two major components that control the cell cycle are cyclins and cyclin dependent kinases. These enzymes control entrance into the stages of the cell cycle. Normal cells go through the cell cycle stage, and ultimately die. Cancer cells circumvent the normal cell cycle controls and become neoplastic [2].

If caught early enough, or if the tumor is benign, surgical removal may cure the cancer. However, surgery cannot eradicate all forms of the disease because many times metastasis has occurred in which cancer cells from a primary site spread to other organs. If metastatic, the primary tumor will keep developing and will eventually develop until it no longer has nourishment from blood vessels to sustain more growth. At this point a process called angiogenesis occurs, in which the cells secrete proteins that induce the growth of new blood vessels. The next step of metastatic cancer is it needs to break out of its primary site or organ. To do this the cells will express enzymes that will dissolve the encompassing matrix and result in the migration of cancer cells through the blood stream leading to the formation of a metastatic tumor at multiple sites in the body, making surgery ineffective.
Chemotherapy

Chemotherapy is a cancer treatment that uses drugs to block and/or promote different functions involved in cell growth and replication. Chemotherapy is a whole body treatment delivered via the blood. Chemotherapy is one of the most widely used treatments for cancer and can be quite successful in treating some forms of metastatic cancer such as acute childhood leukemia, testicular cancer, and Hodgkin's Disease [4]. Many forms of cancer can be controlled for long periods of time with chemotherapy, prolonging the life expectancy of the patient. It can also be useful in providing symptomatic relief for patients with other types of cancer. Often chemotherapy is one of the only available treatments for patients. Chemotherapy can be used by itself or in conjunction with other treatments. For instance after surgical removal of a tumor chemotherapeutic drugs can be used to destroy the remaining cancer cells in the body. This type of therapy is referred to as adjuvant chemotherapy. Other forms of combined therapy use chemotherapeutic drugs before surgery or incorporate radiation therapy into the treatment [4].

Chemotherapy has disadvantages. Chemotherapy affects all parts of the body and therefore patients may experience side effects. There is a fine line between reducing a tumor size and causing toxicity in the body, which can have adverse effects on normal organs and lead to other health problems. Each cancer is different and every individual is affected differently by the therapy and it cannot be predicted accurately who or how each person will react to a treatment [4].
Today there are many approaches being studied to improve the desired affects and reduce the undesirable side effects of chemotherapy. These are: new drugs, new combinations of drugs, new delivery techniques, new approaches for targeting drugs more specifically at the cancer cells, drugs to reduce side effects, hematopoietic stem cell transplantation and agents that overcome multidrug resistance.

The development of drug resistant tumor cell populations restricts chemotherapeutic efficiencies. Some tumors are intrinsically resistant to anti-tumor drugs, while in others resistance is selected for during the course of therapy [5]. Not only can the cells become resistant to the administered drug but they can also become cross-resistant to other drugs that operate differently [6]. Resistance to chemotherapy is believed to cause treatment failure in over 90% of the patients with metastatic cancer [6]. By understanding the mechanism of drug resistance and discovering novel targets for pharmacological, molecular, and even genetic intervention, it may become possible to increase the usefulness of existing drugs.

Model organisms are used in research to make studies easier and fruitful. Model organisms share similar basic biological systems, allowing what has been learned from a model organism to be applied to other organisms such as humans. Model organisms have characteristics such as: quick dividing time, short life cycles, smaller genomes, and well developed genetic and biological techniques. Examples of commonly used model organisms are: yeast, *D. discoideum*, *Caenorhabditis elgans*, Zebrafish and mouse.
**Dictyostelium as a model organism**

*Dictyostelium discoideum* cells feed on bacteria or can be maintained on liquid media and divides mitotically until the food is consumed. Starvation induces groups of cells to collect into aggregates and form fruiting bodies, consisting of a ball of resistant spores resting on the top of a stalk. This characteristic of the life cycle alternating between vegetative growth and multicellular development is one of the most interesting features of *Dictyostelium*, making it an attractive model to use for the study of cell and developmental biology. Genes needed for development generally are not needed during mitotic growth, and these genes can be mutated without affecting the viability of the growing organism. Starvation induces expression of a variety of new genes, some of which are necessary for the synthesis and detection of cAMP [7, 8].

As the starved amoebae release cAMP, the nearby cells detect it and begin to chemotax towards the signal as they too start releasing their own cAMP to signal other cells [7]. Groups of approximately 100,000 cells come together. As the cells aggregate closer they become adhesive and will differentiate based on the position of the cells in the cell cycle at the time of starvation. It is believed that cells in S and early G2 phases of the cell cycle become prestalk cells, while cells in the rest of the cell cycle will become prespore cells [9]. About 20% of the aggregated cells differentiate into prestalk
Figure 1 Life cycle of *Dictyostelium discoideum*. The diagram shows the sequence of morphological changes leading to the formation of a mature fruiting body, and alternative pathway of slug migration [8].
cells, and 80% differentiate into prespore cells. The aggregates further develop into mature fruiting bodies where the prestalk cells die and become vacuolated and the prespore cells become environmentally resistant spores, which can be activated by a food source which initiates germination and cell division [7] (Figure 1).

*Dictyostelium* cells are haploid, making recessive mutations more observable. The genome is 34 Mb and consists of six chromosomes ranging in size from 4Mb to 7Mb [7, 10]. The genome consists of approximately 15,000 genes. Many of the genes are homologous to higher eukaryotic organisms. Introns are generally small, consisting of approximately <200 bp. Most genes have only one small intron although there have been genes found with multiple introns or no introns. The genome is highly A/T rich (~78%), with intergeneic regions reaching greater than 95% [10]. *Dictyostelium* cells have a doubling time of about 12 hours. And the cultures are easily maintained. There are a number of well developed genetic techniques including homologous recombination and insertional mutagenesis that increase the genetic usefulness of *Dictyostelium* [7, 10].

The sphingolipid metabolic pathway

Previous work has used *Dictyostelium discoideum* to study chemotherapy drugs with the aim of better understanding how they function and to combat the problem of drug resistance. One drug of particular interest is the platinum based drug cisplatin *[cis-diamminedichloroplatinum (II)]*. Cisplatin is widely used in treatment of small-cell and non-small-cell lung cancer, non-Hodgkin’s lymphoma, testicular cancer, ovarian cancer,
head and neck cancer, esophageal cancer and bladder cancer, among others. Cisplatin treatment is often limited due to resistance of the tumors to the drug [11].

Using Restriction Enzyme Mediated Integration (REMI), an insertional mutagenesis technique, six novel genes involved in resistance to cisplatin were identified [12]. One of the genes was sphingosine-1-phosphate (S-1-P) lyase (sglA), which encodes an enzyme that catalyzes the last step in the sphingomyelin degradation pathway, converting S-1-P to phosphoethanolamine and hexadecanal (Figure 2). The S-1-P lyase null mutant (sglAΔ) exhibited dramatic phenotypic changes in its growth and development indicating that S-1-P plays a central regulatory role in D. discoideum development [13, 14]. S-1-P lyase has also been shown to play important roles in cell function and development in other systems as well, such as yeast [15], Caenorhabditis elgans [16], Drosophila melanogaster [17], and mouse cells [18].

Sphingolipids are emerging as important components in signal transduction. Ceramide and S-1-P are major components of the sphingomyelin degradation pathway. S-1-P has been shown to be an important regulatory molecule in cell function and development and in the regulation of cell fate. The balance between S-1-P and ceramide is a crucial determinant, with proportionally greater levels of S-1-P resulting in cell proliferation, while proportionally greater levels of ceramide resulting in cell death (Figure 3) [19]. It has been shown that manipulating the sphingolipid pathway at various
Figure 2 Sphingolipid metabolic pathway.
Figure 3 Ceramide/Sphingosine-1-Phosphate rheostat model. In the sphingolipid metabolic pathway, increased levels of ceramide lead to cell death while increased levels of S-1-P lead to cell proliferation.
points can result in increased sensitivity to cisplatin and has the potential for increasing the clinical usefulness of this important drug [20, 21].

Three strains of *Dictyostelium* that stably overexpress S-1-P lyase were generated. The strains had an expression-dependent increase in the sensitivity to cisplatin. The overexpressor strains also had a reduced growth rate as compared to the parent cells. The increased sensitivity of the overexpressors to cisplatin was also observed with the cisplatin analog carboplatin. The S-1-P lyase overexpressor was treated with other drugs such as: doxorubicin, 5-flourouracil, and etoposide. These drugs did not have a significant effect on the cells, indicating that the sphingolipid metabolic pathway is not part of a global genotoxic stress response [20].

Mutant cells were treated with cisplatin and D-erythro-N,N,dimethylsphinogine (DMS), a known inhibitor of sphingosine kinase [20]. Sphingosine kinase is the enzyme that synthesizes S-1-P from sphingosine and ATP, thus increasing the S-1-P and/or sphingosine levels in the cell [20, 21]. This treatment mimicked the *sglA* overexpressor (*sglAOE*) phenotype and resulted in increased sensitivity to cisplatin in both parent and mutant cells, most possibly due to the decreased level of S-1-P. The increased sensitivity was partially reversed by the addition of a sphingosine kinase activator 8-Br-cAMP or exogenous S-1-P to the mutant cells [20].

Two sphingosine kinase genes in *D. discoideum* were identified, *sgkA* and *sgkB*, both of which are homologous to those of other species including the human Sphk1 and
Sphk2 enzymes. Using homologous recombination we have constructed null strains for
sgkA, sgkB, and a sgkA/sgkB double null strain, as well as a sgkA overexpressor in
Dictyostelium. The deletion of the sphingosine kinase genes affects growth rate and
sensitivity to cisplatin, with the double null mutant being more sensitive than either of the
single null mutants. The SgkA overexpressor mutant showed increase resistance to
cisplatin as compared to the parental strain. Addition of exogenous S-1-P to sphingosine
kinase null mutants reversed the increased sensitivity to cisplatin, whereas the increased
resistance of the sphingosine kinase overexpressor mutant was reversed by the addition of
DMS. Similar results were shown with the platinum-based drug carboplatin but not
with doxorubicin, 5-fluorouracil, or etoposide [21].

The Dictyostelium sphingolipid pathway genes are homologous to that of
mammals, thus making this an ideal organism to study this pathway and apply to humans.
As described above the pathway can be manipulated either pharmacologically or
genetically to increase drug sensitivity. Cisplatin is normally administered in the
maximum allowable dose, thus additional co-treatments of DMS or other sphingosine
kinase inhibitors could prove to be useful combinations with cisplatin to increase anti-
tumor activity [20, 21].

In this dissertation I will discuss my involvement in using a genetic approach in
which the model organism Dictyostelium discoideum was used to study anti-cancer drugs
and the molecular pathways affected by these drugs. There is also an appendix
describing proteomics and how we applied it to discovering biomarkers for the detection of breast cancer.
CHAPTER 2

4-NITROQUINOLINE 1-OXIDE

The drug 4-nitroquinoline 1-oxide (4NQO) was first synthesized in 1942. It results from a variety of industrial processes that present a potential source of contamination to humans such as coal and tar production. Early experiments showed that this compound could destroy abdominal tumors in mice. However, the carcinogenicity of 4NQO was quickly established in mice with skin misting and subcutaneous injections resulting in sarcomas. Further experimentation has lead to the classifying of this compound as an ultimate carcinogen and DNA damaging agent [22-25].

4NQO is light sensitive. It produces both DNA strand breaks (like ionizing radiation) and alkali-stable DNA lesions (like UV light) [26]. 4NQO is metabolized by a series of enzymes (Figure 4) [27-31] which leads to the formation of DNA adducts (Figure 5) [32, 33] which are mutagenic [23, 34-37]. 4NQO could also be toxic and/or mutagenic by redox cycling to generate reactive oxygen species [38-40].

While 4NQO bulky base DNA damage almost completely mimics UV DNA damage, parallel responses between 4NQO and UV light are not always observed [41]. In most organisms including bacteria, fungi and mammals, mutants that are sensitive to UV
Figure 4 4NQO metabolic pathway. A) 4NQO is first metabolized into B) 4-hydroxyaminoquinoline 1-oxide (HAQO). This component apparently is further activated by a reaction with seryl-tRNA-synthetase in which 4-hydroxyaminoquinoline 1-oxide instead of tRNA is acylated by the seryl-AMP enzyme complex and then to C) 4-aminoquinoine 1-oxide to become carcinogenic. This complex then introduces quinoline groups into DNA [30, 33].
Figure 4 The major DNA adducts of 4-Nitroquinoline 1-oxide (4NQO). Adducts at C-8 of guanine but also at the exocyclic N2 of guanine and N6 of adenine have been identified. The N2 adduct appears to be the major lesion that accounts for 50 to 80% of all quinoline base adducts, depending on the superhelicity of the target. 4NQO treatment can also result in the formation of 8-hydroxyguanine and lead to a significant amount of strand breakage, probably indicative of the formation of unstable adducts.
light and γ-irradiation are also sensitive to 4NQO. However, *D. discoideum radC* mutants that were selected for sensitivity to UV and γ-irradiation were not also sensitive to 4NQO. In contrast, *D. discoideum* mutants selected for sensitivity to 4NQO generally showed cross-sensitivity to UV and γ-irradiation, but one mutant was uniquely sensitive to 4NQO. This implies the existence of genes or pathways that specifically control the cells response to 4NQO [42].

Cisplatin also produces bulky DNA lesions [26, 30, 33]. Thus understanding the way 4NQO functions may help to understand how cisplatin and other drugs that function in similar ways operate. The goal of the project was to use the model organism *Dictyostelium* to identify genes that are associated with the resistance of 4NQO to better understand the anticancer properties of DNA damaging drugs.

REMI is a process used to randomly insert linearized plasmid DNA into the genome of the organism, thus generating mutations by gene disruption [43]. Cells that contain the integrated plasmid DNA can then be screened for mutations that may affect a number of cellular or developmental processes [44]. We used REMI to identify individual genes that have a direct link to 4NQO resistance.
MATERIALS AND METHODS

Strains and conditions for growth and development

The *Dictyostelium* parental strain Ax4 was stored as desiccated spores on silica gel at 4°C. REMI strains were stored as cells in horse serum contained % DMSO at -80°C. New stocks were plated onto SM agar plates containing the bacterial food source *Klebsiella aerogenes* [45]. After the strains grew on the plates, spores of the fruiting bodies were picked and placed into 2 ml of sterile HL-5 medium and incubated at 22°C for 2 days. The 2 ml of culture were added to 8 ml of HL-5 media in a flask and grown axenically at 22°C with shaking. Cells were passaged when they reached a density of 2-3 X 10⁶ cells/ml (mid-log phase).

Determining the concentration of 4NQO required for toxicity in *Dictyostelium*

Forty ml of a cell culture (approximately 2 x 10⁶ cells/ml) were harvested by centrifugation at 3500 RPM at room temperature for 10 minutes. The cell pellets were washed 2 times at room temperature saline solution (SS) (10 mM NaCl, 10 mM KCl, 2.7 mM CaCl₂) buffer and diluted to 2 x 10⁶ cells/ml in SS buffer. Three ml of the culture was transferred to UV sterilized glass scintillation vials (20ml) wrapped in aluminum foil. The cell cultures were treated to the concentrations of 0, 5, 10, 15, and 20 µg/ml of
4NQO (Acros Organics, Pittsburg, PA) using a 2.5mg/ml 4NQO/Dimethyl Sulfoxide (DMSO, Sigma, Saint Louis, MO) stock solution. The cultures were treated with 4NQO, shaking at 200 RPM at 22°C for 1 and 3 hours.

After 1 and 3 hours 100 µl of each culture was diluted 1:2 with SS buffer in a 96 well plate. The cultures were diluted serially 1:3, 8 times. Fifteen µl of the last 6 dilutions were plated with 20 µl of *K. aerogenes* suspension on 24 well plates filled with 2 ml of SM agar/well. After plating the 24 well SM agar plate was rotated by hand for 2 minutes to distribute the cells and *K. aerogenes* evenly. The plates were left at room temperature until dried, and incubated at 22°C for 2 days. After the 2 day incubation the plates were digitally scanned twice a day until the *Dictyostelium* plaques ran together and/or a plaque count could no longer be obtained [46]. The scanned plates were later analyzed and plaques counted in order to calculate the 4NQO killing curve of the *Dictyostelium* strain Ax4. Survival is calculated as percent over untreated culture.

### Screening REMI libraries for 4NQO resistant mutants

Five previously created REMI mutant libraries were used to screen for 4NQO mutants. Each REMI mutant library was screened on a 96 well plate with each well containing 100 µl of the REMI culture at 2 X 10^4 cell/ml, giving a total of 2000 cells/well. The 96 well plates were incubated at 22°C overnight.
4NQO was added to the 96 well plates. A 10 mg/ml 4NQO/DMSO solution was diluted to create a 15 µg/ml 4NQO solution in SS buffer. The media was aspirated from each well of the 96 well plates. Two hundred µl of 15 µg/ml 4NQO solution was added to each well, and a 15 µg/ml DMSO solution in SS buffer was used as a control. The 96 well plates were placed into a box wrapped in aluminum foil and incubated at 22ºC for 2 hours. After 2 hours the 4NQO solution was aspirated from each well and 200 µl of HL-5 media was added, and incubated at 22ºC. Each plate was examined daily under a microscope (Nikon TMS) for two weeks to identify any putative 4NQO resistant *Dictyostelium* mutants.

The putative 4NQO resistant *Dictyostelium* mutants were identified by colony formation on the bottom of the 96 well plates. Positive wells varied from a confluent well to smaller colonies. After the putative mutants were identified, 10 µl were transferred into 2 ml of HL-5 media in a well of a 24 well plate. The 24 well plates were incubated at 22ºC in the presence of constant light and observed daily under the microscope (Nikon TMS).

When the cultures in the 24 well plates reached confluence, 50 µl of cell culture was taken and plated with 250 µl of *K. aerogenes* suspension onto SM agar plates. Without sterilizing the spreader, two additional plates containing 250 µl *K. aerogenes* were also spread, to dilute the cells to form single plaques. The plates were incubated at 22ºC with light for three days. Three days later, single plaques from the SM plates were picked and streaked onto SM agar plates with *K. aerogenes*. The streaked plates were
incubated at 22°C with light for 3 days, after which cells were transferred to 2 ml of HL-5 media in a test tube and incubated at 22°C with light for 2 days. After 2 days the 2 ml of culture were brought up to 10 ml of HL-5 and grown at 22°C with shaking and the cultures were maintained between 5 X 10^4 cells/ml to 2-3 X 10^6 cells/ml.

**Screening putative 4NQO resistant *Dictyostelium* mutants for confirmed 4NQO resistance (method 1)**

Using Nunc (Rochester, NY) white 96 well plates 90 µl of 5 X 10^4 cells/ml of the putative 4NQO resistant *Dictyostelium* mutants and the Ax4 parental control were treated with 200 µl of 2.5, 5, and 10 µg/ml 4NQO concentrations. The plates were incubated at 22°C for 24 hours, in a vented aluminum foil covered box. After the 24 hour incubation the cell viability was assayed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to manufacturer instructions. The plates were read using a Veritas microplate reader (Turner BioSystems). Viability was calculated as percent survival over untreated cultures.

**Screening putative 4NQO resistant *Dictyostelium* mutants for confirmed 4NQO resistance (method 2)**

Using aluminum foil covered 125 ml flasks, 10 ml of the putative 4NQO resistant mutants were treated with 2.5, 5, and 10 µg/ml 4NQO concentrations. The drug treated
cultures were shaken at 22°C and assayed for viability on white 96 well plates using the Promega CellTiter-Glo Luminescent Cell Viability Assay protocol at zero, 2, 6, 24, and 48 hours after adding 4NQO. The 0, 2, and 6 hour time point was diluted 1:20, the 24 hours time point was diluted 1:50, and the 48 hour time point was diluted 1:100. The dilutions were assayed according to the CellTiter-Glo Luminescent Cell Viability Assay in white 96 well plates (Nunc). The plates were read using the Veritas microplate reader.

**Genomic DNA isolation**

The *Dictyostelium* 4NQO resistant mutant cultures were grown to 2 x 10^6 cells/ml, washed in SS buffer, harvested and stored in pellets of 1 x 10^8 cells/pellet. Using DNAzol (Molecular Research Center Inc.) genomic DNA was isolated, and analyzed on a 0.8% agarose gel to confirm presence of genomic DNA.

**Inverse PCR and sequencing**

DNA from 4NQO resistant *Dictyostelium* cells was digested with the restriction enzyme *AluI* overnight at 37°C. The following day, the digest product was adjusted to 400 µl with sterile water, and 400 µl of 1:1 phenol:chloroform was added. The samples were mixed well and centrifuged at room temperature for 10 minutes at 1300 RPM. The aqueous phase was transferred to a fresh tube. One tenth volume of 3M sodium acetate pH 5.0 (i.e. 40 µl) and 2 volumes of 100% EtOH (i.e. 800µl) was added to the aqueous phase, and incubated at -20°C for 20 minutes. The sample was centrifuged again at 1300
RPM for 10 minutes and washed with 70% ethanol. After the digested product was cleaned, it was ligated in a total volume of 200 µl at 16°C overnight.

The next day the ligated product was prepared the same way as after digestion, and inverse PCR was performed using PBSR1 primer 339 (5'-GATGCTACACAATTAGGC-3', Integrated DNA Technologies, Inc.) and Alu1 site primer 347 (5'-ATGCCGCATAGTTAAGCCAG-3', Integrated DNA Technologies, Inc.). The PCR program was: 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 48°C for 1 minute, 68°C for 5 minutes, followed by 68°C for 10 minutes and 4°C.

Identification of genes

The sequences were analyzed against the Dictyostelium database using blast searches to identify which genes were disrupted (http://dictybase.org).
RESULTS

Determining the concentration of 4NQO required for toxicity in

*Dictyostelium*

In order to determine a concentration of 4NQO to be used for selecting 4NQO resistant mutants from the REMI library, it was crucial to develop a survival curve for the *Dictyostelium* parental strain Ax4 treated with a range of 4NQO concentrations. Bronner has previously established a survival curve treating wild-type *Dictyostelium* with 4NQO [42], thus giving the basic parameters to develop 4NQO treatment. Both growth stage and cell concentration affect the sensitivity to 4NQO [42]. Thus the *Dictyostelium* cells were washed two times with SS buffer and adjusted to a standard cell count before 4NQO treatment. However, any time longer than three hours without media could result in the *Dictyostelium* initiating into development, the stage of the *Dictyostelium* life cycle that is activated when the organism is starved. Strain Ax4 was treated with 2.5, 5, 10,15, and 20 μg/ml 4NQO for 1 and 3 hours (Figure 6). At 3 hours 15 μg/ml 4NQO caused 100% killing, thus 15 μg/ml 4NQO was used to screen the REMI mutant library for 4NQO resistant mutants.
Figure 6 4NQO Ax4 strain survival curve. Log phase cells were assayed using plaque assays [46]. Dictyostelium Ax4 strain were treated with 0, 5, 10, 15, and 20 µg/ml 4NQO for 1 hour, represented by the closed diamonds and 3 hours, represented by the closed squares. Viability was reduced to zero at 3 hours with 15 µg/ml 4NQO treatment (indicated by the broken line).
Screening REMI libraries for 4NQO resistant mutants

The 6E3A and 3B8B putative 4NQO resistant mutant colonies were identified in the initial REMI 4NQO drug screen. These mutants were grown up into an HL-5 culture and treated with 4NQO to confirm 4NQO resistance.

Re-screening putative 4NQO resistant Dictyostelium mutants (Method 1)

Using this screen, one mutant, 3B8B, was confirmed to be more resistant to 4NQO than the parent Ax4 cells (Figure 7). The 3B8B mutant 4NQO resistance is more apparent as the 4NQO concentration increased as compared to the parental Ax4 strain. The 2.5 μg/ml 4NQO concentration at 24 hours had the least effect resulting in a 1.5 fold increase in percent survival of the 3B8B culture as compared to Ax4. At a 5 μg/ml 4NQO concentration the 3B8B mutant culture had a two-fold increase, and at the 10 μg/ml 4NQO concentration had a 4.5 fold increase in survival as compared to Ax4. The 10 μg/ml 4NQO concentration was the highest concentration used in order not to completely kill off the Ax4 cell line.
Figure 7 3B8B mutant 4NQO screen for resistance. Survival curves of parental Ax4 and mutant 3B8B cells following 24 hours of treatment with 2.5, 5.0, 10 µg/ml 4NQO.
Screening putative 4NQO resistant *Dictyostelium* mutants for confirmed 4NQO resistance (Method 2)

Many putative 4NQO mutants were screened using Method 1. However one putative mutant in particular, 6E3A, had given varying results using the first method. Thus Method 2 was developed to further evaluate 6E3A. In the previous method only the 24 hour time point was assayed. In the second method the cellular response to 4NQO treatment at time points 0, 6, 24 and 48 hours were assayed. Using 125 ml flasks with 10 ml of volume instead of a 200 µl of volume, as used in Method 1, decreased dilution errors. As the time points increased it became necessary to dilute the cultures in order to get an accurate reading using the CellTiter-Glo Luminescent Cell Viability Assay because the untreated Ax4 control cells, which divide every 12 hours, produce a reading beyond the linear range.

Method 2 identified the 6E3A mutant as being more resistant to 4NQO than the parental Ax4 cells (Figure 8). At 24 hours of drug treatment there is a 2.5 fold and 3.2 fold increase at the 2.5 and 5.0 µg/ml 4NQO concentrations, respectively. An unpredicted reading resulted in a negative percent viability at the 10 µg/ml 4NQO concentration. We suspect that this reading is due to the ATP in the media. The CellTiter-Glo Luminescent Cell Viability Assay functions by determining the amount of ATP present. Thus the assay may have been producing a background reading created by the ATP in the media. The assay resulting in negative viability may be caused by the HL-5 media ATP
Figure 8  6E3A mutant 4NQO screen for resistance.  A) Survival curve of parental Ax4 and mutant 6E3A following 24 hours of treatment with 2.5, 5.0, and 10 µg/ml 4NQO.  B) Survival curve of parental Ax4 and mutant 6E3A following 48 hours of treatment with 2.5, and 5.0 µg/ml 4NQO.
becoming degraded over time, resulting in negative viability if the HL-5 media background is subtracted.

The 48 hour time point showed a 2 fold increase in the 2.5 µg/ml 4NQO concentration and a 3.8 fold increase in the 5 µg/ml 4NQO concentration in 6E3A mutant as compared to the parental Ax4 strain (Figure 8).

**Identification of Mutant Genes**

In the first step of REMI, a linearized plasmid is electroporated into the *Dictyostelium* genome cells with restriction enzymes that will produce compatible sticky ends enabling the linearized plasmid to incorporate into the *Dictyostelium* genome. Our REMI libraries were created with the restriction enzyme *DpnII* (Figure 9). The inserted plasmid contains the blasticidin resistance (Bsr) gene for selection, which allows for isolation of cells that successfully integrated the plasmid into their genome [44]. The REMI mutants were further screened for resistance to 4NQO.

The genomic DNA for each putative *Dictyostelium* 4NQO resistant mutant was digested with the restriction enzyme *AluI*, a four base pair cutter (AGCT). *AluI* produces a mean genomic DNA fragment length of 1050 base pairs in *Dictyostelium*. The REMI insertion plasmid contains a known *AluI* site. Thus, theoretically when digested with *AluI* a product of approximately 1Kb will be produced. The digest will also lead to an
Figure 9  Schematic presentation of REMI and inverse PCR.
interrupted gene fragment containing a known sequence from the Bsr cassette. We have designed DNA primers that anneal to the 5’ end of the Bsr cassette and are used for the sequencing of the disrupted genes [47].

The digest was then ligated and inverse PCR was used to amplify the plasmid. Inverse PCR used primers that would anneal with the Bsr cassette flanking the insertion site, PBSR1 primer 339 (5'-GATGCTACACAATTAGGC-3’), and the Alul site primer 347 5'-ATGCCGCATAGTTAAGCCAG-3’). Thus the plasmid containing the cassette inserted in the Dictyostelium genome would be amplified. The PCR product was analyzed on a 1.5% agarose gel. The 6E3A cells gave a 1.3 Kb band and the 3B8B cells gave a 0.6 kb and a 0.4 kb band (Figure 10). After the band identification was made, the remaining PCR product was run on a 1.5% low melt agarose gel and the band was eluted. The flanking ends of the REMI vector were sequenced, with the same primers as were used for inverse PCR. Sequencing was performed by the University of Missouri DNA Core Facility.

After sequencing it was found that the two PCR bands had the same insert. After digestion with Alul another segment of DNA produced from the digest ligated into the circularized DNA thus producing a larger PCR product. Furthermore, it is an unlikely event to ever obtain two REMI inserts for reasons unknown. It has previously been shown using Southern blotting that REMI results in only one insert. Thus, to further confirm that indeed there is only one REMI insert a Southern blot can be done.
Figure 10  **6E3A and 3B8B inverse PCR products**, for A) 6E3A with a 1.3 Kb fragment and B) 3B8B with 0.6 Kb and 0.4 Kb fragments.
Only small amounts of DNA sequence flanking the integration site are needed to compare the sequences against the *Dictyostelium* database to identify which genes were disrupted. Blast results show that the 6E3A mutant had a REMI insert in the retrotransposon gene JC1V_0_00607 on chromosome 1 (Figure 11) (Figure 12). In the 3B8B mutant the insert was between two genes JC1V2_0_00842 (S-adenosylmethionine-dependent methyltransferase activity gene) and JC1V2_0_00841 (retrotransposon) on chromosome two.

**DISCUSSION**

Many studies have used 4NQO as a carcinogen to induce cancer. While it is well known that 4NQO has tumor producing effects it is not known how it operates. The aim of this study was to use genetics to attempt to identify how 4NQO operates. The REMI insert led to the disruption of a retrotransposon and an insertion in an intergenic region, creating mutants that demonstrated resistance to the 4NQO.

Many genomes are composed of a considerable number of repetitive elements. The *Dictyostelium* genome is composed of 10% repetitive elements [10, 48]. Many of these sequences are mobile and thus called transposable elements (TE) or transposons [49, 50]. Retrotransposons are a class of TEs that propagate themselves through reverse transcriptase, an enzyme that can synthesize a DNA strand from RNA [50, 51].
Figure 11 Schematic of 6E3A REMI insertion. The 6E3A mutant had a REMI disruption in the retrotransposon JC1V_0_00607 on chromosome 1. After digestion with AluI and ligation, the 1.3 Kb circularized DNA was formed.
Figure 12  6E3A mutant alignment with retrotransposon (JC1V2_0_00607) on chromosome 1.
Thus a retrotransposon is amplified during transposition [49], and they apparently encode functions needed for transposition [52].

It is interesting that the disruption of a retrotransposon resulted in resistance to 4NQO in *Dictyostelium*. Transposable elements appear to have no obvious function [49, 53]. TEs have been termed "selfish DNA," or DNA that only seems to exist and has no known phenotype [49, 50]. However, studies have shown that a transposition event can cause spontaneous mutations and lead to rearrangements in the host genome [54, 55], thus giving the genome a source of variability [10].

Werner syndrome (WS) is a rare autosomal recessive disease whose phenotype mimics premature aging. A fibroblast cell line from WS patients has an unusual increased sensitivity to 4NQO but not to γ-radiation or to hydrogen peroxide. When the WS fibroblasts were fused with control fibroblasts, the resulting hybrid cell line was resistant to 4NQO demonstrating the recessive nature of the 4NQO sensitive phenotype [56]. The study did not identify the underlying cause for the increased sensitivity of the WS fibroblasts to 4NQO [56].

One theory why the WS fibroblast have increased sensitivity to 4NQO is that the 4NQO adducts form at junctions between B- and Z-forms of DNA. Studies with plasmid DNA substrates show that 4NQO adducts are preferentially formed at junctions between B- and Z-form DNA [57]. Because of the helicase and nuclease activities of the WS protein, [58-63] the 4NQO adducts are normally repaired in B-Z junctions through the
nucleotide excision repair (NER) pathway, thus limiting potential of un repaired 4NQO damage to alter gene expression or promote recombination [56]. In WS cells the helicase activity is missing, leading to increased sensitivity.

4NQO has been found not to react with Z-DNA [57]. Z-DNA is widely dispersed in the human genome, and Z-DNA may play a physiological role in the expression of genes and/or genetic recombination [64]. Furthermore, Tx1, a transposable element in Xenopus laevis, has been found to potentially form left handed Z-form DNA [52]. Thus, it is plausible that disrupting a TE could release Z-form DNA, forming a typical DNA double helix enabling 4NQO DNA adducts to form. These newly formed 4NQO DNA adducts could in turn disrupt gene expression that regulate cellular pathways. Certain pathways have previously been found that can be manipulated by certain enzymes to select for drug sensitivity [20, 21]. Thus, it is possible the retrotransposon disrupted in the 6E3A mutant, could have disrupted a pathway in this way. This disrupted pathway would in some way be linked with providing 4NQO resistance. Furthermore, RNA quantification or Northern blotting could be used to identify gene regulation downstream of the retrotransposon.

Discovering a 4NQO resistant human WS fibroblast cell line is exciting in itself that human cells are capable of becoming resistant to 4NQO, a drug known mainly for its carcinogenic and mutagenic effects. A drawback that limits the study in this cell line is that cells of Werner Syndrome (WS) patients have a number of abnormal characteristics [65-67], which make this cell line difficult to study the effects of 4NQO. Some of these
abnormal characteristics include: Reduced DNA replication, premature replicative senescence [68-77], abnormal cell cycle characteristics [78], unusually large numbers of chromosomal breaks and translocations [79, 80], increased rates of spontaneous mutations [81, 82], increased deletion mutations [66, 83], and increased rate of homologous recombination [84]. The use of Dictyostelium with its many molecular and genetic means of investigation will prove to be a much better organism to mediate the future study of 4NQO resistance.

Northern hybridization analysis studies have shown, that there is an increase in transcription of the Ty retrotransposon in yeast when exposed to 4NQO or UV light [85]. It is know that Ty element regulation in yeast is carefully maintained, and the protein level expressed by Ty elements is very low in yeast [86]. It is not understood how the 4NQO or UV treatment is involved in this increased transcription of the Ty retrotransposon, however one theory is that host factors within the yeast genome needed for transcription and transposition may have been altered rather than directly affecting the retrotransposon [87]. It is not understood what cellular functions are affected by increased transcription of the Ty element it yeast [85]. This study is interesting in that it provides link between retrotransposons and cellular pathways that operate through 4NQO treatment. It would be interesting to look further into the expression levels of retrotransposons in the wild type D. discoideum as compared to the 6E3A and 3B8B mutants. Could it be retrotransposons do play an unknown role in the regulation of cellular pathways?
In the 3B8B 4NQO mutant, the REMI vector inserted into an intergenic region between an S-adenosylmethionine-dependent methyltransferase activity gene, and a retrotransposon. These genes were not directly affected due to a direct insertion, however if the insert disrupted a gene regulatory mechanism such as a promoter then the expression of genes downstream of the REMI insert may have been affected. The S-adenosylmethionine-dependent methyltransferase gene lies downstream of the REMI insert and thus, its expression may have been altered. It is interesting to note S-adenosylmethionine-dependent methyltransferase, has a role in DNA methylation.

DNA methylation is the chemical modification that adds methyl (CH$_3$) groups onto cytosine bases. The methylation process is achieved by DNA methyltransferases that catalyze the addition of a methyl group from S-adenosylmethionine to the 5'-carbon position of cytosine, which leads to the formation of a thymidine base. This process of creating a point mutation of a cytosine to a thymidine goes unrecognized as mutated by the cell since thymidine is a naturally occurring base [88]. This DNA methylation process only occurs on CpG base pairs [88-90].

DNA methylation plays an important role in regulating transcription and chromatin structure, however most CpG are unmethylated. The CpG base pairs tend to form islands of CpG repeats, mainly in promoter regions of genes. Active transcription in the promoter region seems to keep the CpG base pair islands unmethylated [90]. However, hypermethylation of these CpG promoter regions can cause inappropriate gene
silencing [88, 91], which could lead to cancer. About 50% of cancers are known to undergo methylation-associated silencing [89].

The S-adenosylmethionine-dependent methyltransferase gene was not a disrupted gene found in the 4NQO resistant screen, however it would be interesting to find out if the expression of the S-adenosylmethionine-dependent methyltransferase gene was affected by the interruption with a quantitative assay. Both hypomethylation and hypermethylation have been associated with epigenetic causes of malignancies, through gene silencing [88]. Thus, it is plausible that disrupting a promoter of the S-adenosylmethionine-dependent methyltransferase gene may have affected DNA methylation in the 3B8B mutant causing silencing of a gene which led to an increased resistance to 4NQO treatment by the 3B8B mutant.

It is interesting in itself that Dictyostelium 4NQO resistant mutants exist, as it is very rare to identify resistance to this drug in any organism. In this project I used an insertional mutagenesis technique with the model organism Dictyostelium to identify genes associated with the resistance to the carcinogenic compound 4NQO. The REMI insertion led to a disruption of a retrotransposon and an insertion in an intergenic region between S-adenosylmethionine-dependent methyltransferase activity gene, and a retrotransposon. These results in themselves require further investigation in order to incorporate a broader understanding of how this drug functions. Future studies involving this drug will in turn result in a better understanding of how this drug operates and eventually reveal a cellular signaling pathway involved in its resistance. Obtaining an
understanding of how 4NQO signaling pathways function may in turn lead to the understanding of how other drugs, such as the commonly used chemotherapeutic drug cisplatin, operate. This new understanding could lead to new advances to improve the effectiveness of chemotherapeutic drugs. These studies could be used to improve the therapy or could possibly lead to the development of a new cancer diagnosis technique.
Proteomics

Proteomics is defined as the global study of gene expression at the protein level. It can give a complete description of living cells in terms of all of their functional components, or proteins, brought about by the direct analysis of those components rather than the genes that encode them. The entire protein complement expressed by a cell or tissue type is termed the proteome [92-95].

The genomics era brought about factory-style automated DNA sequencing which resulted in sequencing of the genome of many organisms, including the human genome which was finished in 2003 [95]. These genomic sequencing projects sequenced many genes that did not have known functions. Microarray hybridization could assess RNA expression and, thus gene expression. These assays were limiting as they could not analyze the end product, the protein. Thus, the proteomic field has emerged in order to identify specific proteins and correlate those proteins with the gene from which it originated [95].

Sequencing of the genome lead to the realization that while the genomes are large and very complex they are finite. However, each gene can produce different mRNAs and thus proteins. Different transcripts can be generated by alternative splicing, alternative
promoter or polyadenylation site usage and special processing strategies such as RNA editing. Different proteins can be generated by the alternative use of start and stop codons, and these proteins can be further modified post-translationally by processes such as glycosylation or phosphorylation. Over 400 types of post-translational modifications to proteins have been documented [93]. The human genome contains about 30,000 genes, but it is estimated that over a million proteins make up the human proteome after post-translational modification are taken into account, adding increased complexity to human biology [93, 95].

There are many aspects of proteomics technology: two-dimensional gel electrophoresis, multidimensional liquid chromatography, mass spectrometry, sequence analysis, structural analysis, methods for studying protein interactions, modifications, protein chips, localization and function [93, 95]. We have used: two-dimensional gel electrophoresis (2DGE) and matrix assisted laser deionization time of flight mass spectrometry (MALDI-TOF-MS) for proteomic studies aimed at identifying breast cancer markers.

The challenges of proteomics

There are certain significant challenges facing the proteomics field. Only a fraction of genes in a cell type is switched on at any given time. If a cell expresses between 5,000 and 10,000 genes, there will be approximately 15,000 to 30,000 distinct cellular proteins expected as a result of mRNA splicing and post-translational
modifications. In addition, the dynamic range of protein abundance in cells could be as high as eight orders of magnitude and even higher for proteins in biological fluids [93, 94]. Thus, methods to handle this level of complexity are needed.

Sample preparation varies not only from organism to organism but also between different tissue types and types of samples. A serum sample must have a different preparation method as compared to an organ such as the kidney. Working with new samples may prove to become an issue taking time and using what could be valuable sample to determine the best method of preparation. Other issues for sample preparation include: extraction of membrane proteins, functional inactivation, protein degradation, and sample contamination [93].

There are also challenges with the separation and identification of proteins in proteomics. There are no methods to amplify proteins. Thus proteins with lower abundances will be difficult to detect [93, 94], and there is no current way to detect and isolate only membrane proteins. There are many different types of protein staining, which each differently stain different sets of proteins. Reproducibility and separation of proteins with extreme PI values may also be problematic [93].

The bioinformatics databases pose other possible issues with proteomics. Many sources of bioinformatics contain diverse data types and sources making it hard and cumbersome to use. Sometimes identification of proteins in an experiment may lead to a
sequence not in the database [93]. This issue will arise when working with an organism in which the genome is not completely sequenced.

Principles of two-dimensional gel electrophoresis (2DGE)

Two-dimensional gel electrophoresis is used to resolve large numbers of proteins. Many studies today use 2DGE to display larger numbers of proteins and to monitor proteins involved in signaling pathways and their perturbation by disease and drug action [94].

The 2DGE is a two-step process. The protein samples are first separated by charge and then by mass. The first step of 2DGE is isoelectric focusing (IEF), in which the proteins are separated by their net charge. Electrophoresis is carried out on a pH gradient, allowing each protein to migrate to its isoelectric point, i.e., the point at which its pI value is equivalent to the surrounding pH and its net charge is zero. When electric current is applied the charge density of each protein decreases as it moves along the pH gradient towards its isoelectric point. Once the isoelectric point has been reached its protein charge density is zero and it no longer can move in the electric field. The pI value is determined by the number and type of acidic and basic amino acid residues they contain [93, 96].

The second step of 2DGE is carried out by standard sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates the proteins by their
molecular mass. The detergent sodium dodecylsulfate (SDS) binds to the polypeptide backbones of proteins giving them a negative charge proportional to their mass. The stoichiometric binding ensures separation on the basis of mass alone. The protein-SDS complexes have essentially the same charge density, and the relative differences in mass between proteins are maintained in the protein-SDS complexes [93, 96].

After 2DGE, the gels are stained and analyzed either manually or by use of a computer based program. Proteins that are of interest are excised from the gel and identified by using mass spectrometry.

A mass spectrometer is an instrument that measures the mass/charge ratio (m/z) of ions in a vacuum, which can determine the molecular composition of a given sample. In proteomics the samples are composed of peptides derived from a protein sample which has been digested by trypsin or other proteases. Two types of analysis can be made: the analysis of intact peptide ions, and the analysis of fragmented ions [93].

In a mass spectrometer the sample is ionized by converting the sample to gas phase ions in a vacuum. Using an electric field the ions are then accelerated toward the analyzer, which separates them according to their m/z ratios on their way to the detector. The function of the detector is to record the impact of individual ions [93, 96]. The results are then compared to a theoretical computer generated database used to identify the gene that encoded the peptide fragments.
MALDI-TOF MS is a type of mass spectrometry commonly used for proteomics. MALDI-TOF MS is known for its highly sensitive analysis of high-molecular-weight compounds. MALDI-TOF MS uses a solid nonvolatile matrix in which the sample is suspended or dissolved. A pulsed laser beam is directed at the matrix/sample causing the matrix and sample to vaporize. Part of the matrix will remain on the sample creating a charge in which an electrical pulse shoots the charged ions towards the mass analyzer [97].

Once the samples are vaporized, time-of-flight mass analysis is used to separate the ions according to their $m/z$ ratio. The mixture of ions all have the same charge and are accelerated toward the detector with the same amount of energy, but different masses. The heavy ions will take longer to travel to the detector than the lighter ones. The $m/z$ ratio is determined from the time it takes an ion to reach the detector from the matrix [97]. The $m/z$ is compared to a theoretical computer database which identifies the proteins.

We have used proteomic analysis to identify breast cancer biomarkers in nipple aspirate fluid (NAF). The NAF was chosen because it is a promising candidate for a non-invasive way to identify markers of breast cancer. Current methods of breast cancer detection such as mammography and breast examinations miss up to 40% of breast cancers, and are very ineffective in young women. Furthermore, current methods of detection use invasive needles or surgical biopsy to confirm a malignant disease. Thus, a non-invasive detection of breast cancer for women would prove to be beneficial [98].
Previous breast cancer studies have analyzed gene expression attempting to find genetic markers that will facilitate early prognosis of breast cancer. Studies have been conducted to examine global gene expression or protein profiles in cancer vs. normal samples [99, 100]. These studies found up-regulation of certain genes mainly at the initiation of breast cancer [98]. Other studies analyzed gene expression of normal versus cancer tissues, established cell lines, and samples of before and after treatment with chemotherapeutic agents [101, 102]. These studies lead to gene expressions that could be classified into subgroups that may become useful for early prognosis of breast cancer [98].

Previous breast cancer studies have used proteomics to compare normal and cancerous breast cells [103]. These studies mapped a proteome of breast cancer cells which led to being able to distinguish normal, benign and cancerous breast tissues based on their protein profile [104], and identifying proteins that showed differing protein expression based on disease stage.

Many of the proteins found to be expressed differently in breast cancer differ from those found in the gene expression studies suggesting that some differences in normal vs. cancer samples may be due to post-translational modification such as glycosylation or truncation [105]. Thus, it was logical to examine the NAF proteome for biomarkers associated with breast cancer.
The NAF is a good candidate to examine genetic biomarkers for breast cancer. NAF analysis is a low cost, noninvasive procedure making it easy to obtain samples from women, and would be suitable to conduct a screen as an early prognosis with patients in the clinic. NAF is composed of a relatively small number of proteins, making it easier to analyze than the complex protein composition of tumor cells. The NAF proteins are secreted, thus it provides a sample that represent its final processed form [98]. Other studies indicate that NAF could contain diagnostic or prognostic markers associated with breast cancer [106-108].

In this study part of the proteome of the NAF was defined. Using NAF samples from healthy women we performed 2DGE and excised as many protein spots from the gel as possible. Using MALDI-TOF-MS we identified 41 different proteins, in which 25 were previously known to be secreted proteins. Furthermore, we also compared NAF samples from women that had pathologically confirmed ductal carcinoma in situ (DCIS) (10) and normal/healthy samples (10) with 2DGE and identified three up-regulated proteins in the cancerous samples. These included: gross cystic disease fluid protein (GCDFP)-15, apolipoprotein D (apoD), and alpha1-acid glycoprotein (AAG) [98], which had previously been linked to breast cancer [109-111]. Using enzyme-linked immunosorbent assay (ELISA), 53 normal NAF and 52 DCIS NAF samples, were examined using antibodies for the GCDFP-15, apoD, and AAG. It was found that AAG and GCDFP-15 correlate with disease presence and stage [98].
This study strongly supports the idea that NAF can be used in the diagnosis and prognosis of breast cancer. Furthermore, with future studies leading to the identification of known biomarkers associated with breast cancer and the use of antibodies to screen patient samples, this noninvasive breast cancer prognosis technique could be developed and used in a clinical setting. In turn early detection will lead to enhancing the patients chances of survival, and a decline in breast cancer deaths in women.
REFERENCES


