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THYMOQUINONE: THE EVALUATION OF ITS CYTOTOXIC POTENTIAL, EFFECTS ON P53 STATUS AND THE CELL CYCLE IN VARIOUS CANCER CELL LINES

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ABSTRACT OF THESIS

THYMOQUINONE: THE EVALUATION OF ITS CYTOTOXIC POTENTIAL, EFFECTS ON P53 STATUS AND THE CELL CYCLE IN VARIOUS CANCER CELL LINES

Cancer is a group of diseases that are the second leading cause of human mortality in the United States. Discovering new therapies is vital to conquer cancer. Thymoquinone (TQ) is found in the plant *Nigella sativa.* TQ was found to be cytotoxic to the human ovarian cancer cell lines PA-1, CAOV-3 and SKOV-3, which have varying p53 status. PA-1 cells were the most sensitive, indicating that TQ was effective against cells having wild-type (WT) p53. Western blots indicated an increase in p53 in cell lines having WT p53. TQ when given concurrently with cisplatin resulted in antagonism for PA-1, A172 and H460 cell lines. Sequential exposure to TQ followed by cisplatin resulted in synergy or additive effects in these cell lines. Sequential exposure to cisplatin followed by TQ resulted in additive or moderate antagonism in these cell lines. Concurrent exposure to TQ and paclitaxel showed synergy in PA-1 and H460 cells. Sequential exposure to TQ followed by paclitaxel resulted in synergism or antagonism in A172, PA-1, and H460 cells. Paclitaxel followed by TQ resulted in antagonism or synergism in these cells. These results demonstrate that TQ has a potential as an anti-neoplastic agent and may affect p53 levels.

Key words: Thymoquinone, p53 protein, cancer, synergism, antagonistic.

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THESIS

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The Graduate School
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THYMOQUINONE: THE EVALUATION OF ITS CYTOTOXIC POTENTIAL, EFFECTS ON P53 STATUS AND THE CELL CYCLE IN VARIOUS CANCER CELL LINES

THESIS

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2003

For James Howard Anderson

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Chapter One

Introduction

In normal tissues, homeostasis is maintained because of tightly controlled balances between cell proliferation and cell death. A disruption in this balance can lead to uncontrollable cell growth, which is frequently characterized by the loss of ability to undergo apoptosis, and cellular growth spreads without restraint. This type of cell behavior characterizes a group of diseases commonly referred to as cancer (American Cancer Society).

Cancer is a genetic disease that can result from both internal factors such as hormones, inherited mutations, or immune conditions or from external factors such as diet, tobacco, radiation, chemical exposure, or infectious organisms to name a few. Typically chemical carcinogenesis requires six or seven mutagenic events to occur over twenty to forty years (Cross, et al. 1991). This process is generally described to occur in four distinct steps: initiation, promotion, malignant conversion, and tumor progression. The initial step, initiation, results from irreversible DNA damage. The selective clonal expansion of initiated cells comprises tumor promotion. The third step, malignant conversion is the stage at which preneoplastic cells are transformed into cells that express the malignant phenotype. Lastly tumor progression is the stage in which malignant cells are expressed and they tend to acquire more aggressive characteristics.

Cancers can arise almost anywhere in the body. They are initially characterized as carcinomas, sarcomas, lymphomas, and leukemias. Carcinomas, the most common type of cancer originates from the epithelial cells that cover internal and external body surfaces. Breast cancer is the most frequent type of carcinoma diagnosed in the United States. The second types of cancer, sarcomas, arise from cells found supporting the tissues of the body such as fat, muscle, cartilage, and bone cells. Cancers, which originate in the lymph nodes and tissues of the body's immune system are called lymphomas. The fourth type of cancer, known as leukemia, is a cancer of the immature

blood cells, which grow in the bone marrow and accumulate in large numbers in the blood stream (National Cancer Institute).

Today, cancer is the second leading cause of the death in the United States (following heart disease) causing one out of every two deaths in males and one in three deaths in females. On a worldwide scale, cancer causes seven million deaths per year (World Health Organization). While there are methods for treating cancer that have been shown to improve the quality of life of cancer patients, including, chemotherapy, surgery, radiation, immunotherapy, and hormones, the improvement in mortality rates has been only slight. It is estimated that by the year 2020 there will be twenty million new cancer patients per year (American Cancer Society). This data suggests that cancer prevalence and incidence is on the rise. New modalities of treatments must be discovered which have either the ability to prevent or to destroy existing cancers.

A drug discovery effort to identify a compound, which will provide preventative and/or therapeutic treatment, is essential. Over the last millennia herbal medicines have been used for the successful prevention and treatment of numerous diseases. Recently, herbal medication has also been successfully demonstrated to have anti-neoplastic potential and is the source of many chemotherapeutic agents (paclitaxel, vincristine, etc). *Nigella sativa* is a herb that shows encouraging chemopreventive as well as chemotherapeutic properties.

Chapter Two

Nigella Sativa

Historical Overview

Black Seed, Black Cumin, Kalunji, Nutmeg Flower, Kalajira, and Roman Coriander are all commonly used names for the herbaceous plant, *Nigella sativa L.* This plant is widely distributed, belonging to the botanical family *Ranunculaceae.* The genus *Nigella* is native to the Mediterranean region and Western Asia, the name being derived from the Latin word *niger*, meaning black, in reference to the color of the seeds (United States Department of Agriculture).

N*igella sativa* has an erect 30 cm branched stem. The leaves consist of 2-3 cm segments, linear to oblong-lanceolate in shape. The flowers are 3.5-4.5 cm in diameter, and are white tinged blue, without involucres (Fig 2.1). The fruits are inflated follicles, 3-7 in number, fused to the base of the outspread styles, forming a capsule containing the seeds (Hukley 1992). A more extensive description of *Nigella sativa* can be found in Tutin *et al*. (1964), Rechinger (1964), and Davis *et al*. (1965).

Figure 2.1: Nigella Sativa flower and seeds

N*igella sativa* has an extensive history going back thousands of years. It was referred to by the prophet Mohammed of Islam as having healing powers for every illness except death (Khan, 1976). According to Birdwood, Black Seed is the "Black Cumin" mentioned in the Bible, the "Melanthion" of Hippocrates and Dioscorides, and is the Gith of Pliny (Atta-ur-Rahman *et al*. 1985a). On the Indian subcontinent, Black Seed has been used for hundreds of years as a natural remedy for many health conditions and diseases.

Nigella sativa was first botanically described and characterized by Linnaeus in 1753 (Abou-Basha *et al*. 1995). The seed has been used as a spice in cooking, and as a preservative for cheese products (El-Sayed et al. 1994). Raw seeds, seed oil, or seed extract have been used alone or in combination with other incrediants, as a traditional medicine in the treatment of various health conditions, such as eczema, cough, headache, diabetes, asthma, infection, and hypertension.

In recent years, the seeds of *Nigella sativa* have been subjected to a growing number of phytochemical and pharmacological investigations. The chemical composition of the seed has been determined using a variety of analytical and spectroscopic techniques (Houghton *et al*. 1995, Aboul-Enein and Abou-Basha, 1995, Abou-Basha *et al*. 1995). Also, pharmacological activities of seed extracts have been documented, including activities against human and animal diseases, and against pests (Kumar, 1989; Schweig, 1999; Khan, 1999).

Nigella sativa's potential role in cancer therapy

In vitro studies

Salomi et al. (1992) studied the antitumor activity of a methanolic extract of Nigella sativa seed against different types of cancer cells *in vitro*, and demonstrated a 50% cytotoxicity of this extract against Ehrlich Ascites Carcinoma, Dalton's Lymphoma Ascites, and Sarcoma-180 cells at concentrations of 1.5, 3, 1.5 µg/ml, respectively, with little toxicity against lymphocytes. Cell growth of KB (human epidermal carcinoma of the mouth) cells was also inhibited (approximately 60%) by the active principle of *Nigella sativa* at a concentration of 0.5 µg/ml, while K-562 (human erythroleukemic) cells

resumed growth at near control values on days two and three. Tritiated thymidine incorporation studies indicated that the action of the active principle was possibly at the DNA level.

In vivo studies

Salomi *et al*. (1991) found that topical application of a methanolic extract of *Nigella sativa* inhibited two-stage initiation/promotion of skin carcinogenesis in mice. The same group also demonstrated that an injection (intraperitoneal) of the methanolic extract of *Nigella sativa* reduced the number of soft tissue sarcomas formed in the mice induced by an initiator (33% treated versus 100% controls).

Salomi *et al*. (1992) also studied the antitumor activity of a methanolic extract of the *Nigella sativa* seed against Ehrlich Ascites Carcinoma *in vivo* and demonstrated that the development of malignancy was completely inhibited at a dose of 2 mg/mouse per day for 10 days.

Abdel-Salam et al. (1992) also treated mice having Ehrlich Ascites Carcinoma with a single intraperitoneal injection of *Nigella sativa* extract (160 mg/kg body weight). It was found that the lifespan was significantly increased with 37.5% of the treated rats living one month and 25.0% surviving two months. All rats, which did not receive *Nigella sativa*, died by day fourteen. It was also determined that no detectable side effects resulted at the administered dose; such as increases in the levels of blood glucose, cholesterol aspartate aminotransferase (GOT), and serum alanine aminotransferase (GPT), total proteins, albumin; as well as, liver RNA and DNA.

In a study performed by Mabrouk *et al.* (2002) the protective effect of *Nigella sativa* seeds on oxidative stress and carcinogenesis induced by methylnitrosourea (MNU) was demonstrated *in vivo*. Rats were injected with a single dose of MNU (50 mg/kg, i.v.) and then treated with 0.2 grams of *Nigella sativa* grains orally. The authors found that the oral administration of *Nigella sativa* grains reduced MNU-induced oxidative stress and tumorogenesis by 80%, as compared to the control group. It also lowered serum

Malondealdehyde (MDA) and nitric oxide (NO) levels further demonstrating that *Nigella sativa* has a protective effect against MNU-induced oxidative stress and carcinogenesis.

Pharmacology

Hailat *et al*. (1995) determined the effect of the volatile oil from *Nigella sativa* seeds on Jurkart T cell leukemia polypeptides and found that the volatile oil changed protein expression. They concluded that this effect could reflect a role in its biological activity, perhaps, through post-translational modification.

In a later study, Medenica *et al*. (1997) suggested that a *Nigella sativa* seed extract may have strong antitumor activity. The investigators found that the seed extract inhibits cancer and endothelial cell progression through the cell cycle, decreases the production of the angiogenic protein-fibroblastic growth factor (FGF), and inhibits endothelial cell growth factor.. Seed extract was shown to suppress FGF-2, which is commonly present in aggressive breast cancers.

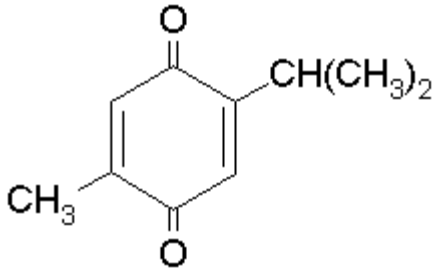
Tumor growth angiogenesis is dependent on neo-vascularization a crucial determinant of the metastatic potential of the tumor. Formed vessels in tumors are hyperpermeable to plasma protein due to gaps in the endothelial lining. These vascular abnormalities can facilitate entry of the tumor cells into the circulation. The authors also studied breast cancer, prostate cancer and melanoma cells for acidic fibroblast growth factor, which were suppressed by the extract. An attempt was made to recapitulate early angiogenic evidence *in vitro* by developing a model of endothelial growth migration and extracellular matrix interaction. The *in vitro* assay revealed that stimulated endothelial cells could produce degradative proteinase and invade the extracellular matrix similar to tumor cells. Moreover, this model system indicated that a fine-tuned balance between proteinase and proteinase inhibitor regulates vascular morphogenesis and invasion. Migrating endothelial cells produce Type 4 collagenase (a member of the matrix metalloproteinase family) and serine proteinase. The authors further demonstrated that specific inhibitors of Type 4 collagenase, general metalloproteinase inhibitors and serine proteinase inhibitors blocked endothelial cell invasion of the extracellular matrix. These

inhibitors blocked tumor cell invasion in the same assay. The extract was compared with these factors and shown to have the same action. The endothelial cells in culture reverted to a non-angiogenic state when the angiogenic stimulus was neutralized by the extract. The authors concluded that the activity of the seed extract blocked the tumor growth and dissemination in metastasis and has remarkable promise for clinical use.

Unfortunately, only a few studies have related certain biological activities to specific chemical entities in blackseed.

One particular chemical entity found in *Nigella sativa* is thymoquinone. Mahfouz and El-Dakhakhny (1960a) reported the identification of "a polymer of the active principle" and named it nigellone: This component was separated from the essential oil of the seed. Three years later, El-Dakhakhny (1963) isolated a crystalline substance from the essential oil, which he identified as thymoquinone (Fig 2.2). Ghosesh et al., (1999) demonstrated that this component is the major constituent of *Nigella sativa*. Since its discovery it has been demonstrated to exhibit anti-microbial, antioxidant, anti-histaminergic, anti-inflammatory, anti-diabetic, analgesic, anti-pyretic, and anti-neoplastic activity. It is the anti-neoplastic activity, which has recently gained a significant amount of attention.

FIGURE 2.2: Chemical structure of Thymoquinone



Thymoquinone's Potential Role in Cancer Therapy

In vitro toxicity

Worthen *et al*. (1997, 1999) investigated the *in vitro* anti-tumor activity of purified components of *Nigella sativa* seeds. Thymoquinone was assayed *in vitro* for its cytotoxicity against several human tumor cell lines. Thymoquinone was cytotoxic for all the cell lines tested [IC_{50}'s 78-393 μM]. Both the parental cell lines and their corresponding MDR (multi-drug resistant) variants (which were over 10 times more resistant to the standard antineoplastic agents doxorubicin and etoposide, as compared to their respective parental controls) were equally sensitive to thymoquinone. The inclusion of the competitive MDR modulator quinine in the assay reversed MDR DX-5 cell resistance to doxorubicin and etoposide by 6 to 16 times, but had no effect on the cytotoxicity of thymoquinone. Quinine also reduced MDR DX-5 cell accumulation of the P-glycoprotein substrate ^{3}H-paclitaxel in a dose-dependent manner. However, thymoquinone did not significantly alter cellular accumulation of ^{3}H-paclitaxel. The inclusion of 0.5% (w/v) of the radical scavenger DMSO in the assay reduced the cytotoxicity of doxorubicin by as much as 39%, but did not affect that of thymoquinone. These studies suggest that thymoquinone is cytotoxic for several types of human tumor cells, may not be a P-glycoprotein substrate, and that radical generation may not be critical to its cytotoxic activity. The investigators concluded that thymoquinone may serve as a useful anticancer drug and as a lead compound for the development of novel agents designed for the treatment of MDR tumors resistant to standard antineoplastics.

Chemoprotection

Badary *et al.* (1999) examined the effect of thymoquinone on Ifosfamide-induced Fanconi (IFO) syndrome and its antitumor activity in rats and mice. Ifosfamide was injected (50 mg/kg/day, i.p.) for five days to induce Fanconi syndrome, which is characterized by renal wasting of glucose, electrolytes, and organic acids. It also resulted in elevated levels of serum creatinine and urea. Thymoquinone was administered in drinking water (0.5 mg/kg/day) for five days before and during Ifosfamide treatment. This administration significantly reduced the elevated serum urea and creatinine, prevented the decrease in serum phosphate and albumin, reduced the

fractional and total excretion of Na^+, K^+, PO_{4-}^{3}, glucose, and organic acids as compared to ifosfamide only treated rats. The creatinine clearance rate was also significantly increased. As in the 1997 study by Badary *et al*., the authors found that IFO and thymoquinone when given to EAC bearing mice resulted in decreased mortality and less body weight loss as compared to IFO treated mice.

Tumor initiation and prevention

Badary *et al* (1999) further characterized the affect of thymoquinone on benzo-(a)-pyrene-induced (BP) forestomach tumors in mice. BP was administered orally (1mg), twice weekly for four weeks. Thymoquinone (100mg/ml) was administered in drinking water one week before, during, and after BP treatment until the end of the experiment. The authors found that thymoquinone inhibited BP-induced forestomach tumor incidence and multiplicity by 70% and 67%, respectively. Thymoquinone alone showed a significant induction of the enzyme activities of hepatic glutathione-s-transferase and DT-diaphorase. This data indicates that thymoquinone acts as a powerful chemoprotective agent against BP-induced forestomach tumors in mice. The authors speculate that the possible modes of action of thymoquinone may be through its anti-inflammatory and antioxidant activities.

Badary *et al* (2000) also examined the affects of thymoquinone on 20-methylcholanthrene-induced fibrosarcoma (MC) tumorogenesis. MC was injected (200 µg/kg, subcutaneous). Administration of thymoquinone inhibited the tumor incidence and tumor burden by 43% and 34%, respectively compared to the group receiving MC alone. Again, thymoquinone showed a significant induction of the enzyme activities of hepatic GST and DT-diaphorase. These last three studies by Badary *et al* have shown that thymoquinone has promise as a chemoprotective agent.

Pharmacology

Most recently Shoeib *et al* (2002) demonstrated the *in vitro* inhibition of growth and induction of apoptosis in cancer cell lines by thymoquinone. Thymoquinone cytotoxicity was investigated using canine osteosarcoma (COS31), its cisplatin-resistant variant

(COS31/rCDDP), human breast adenocarcinoma (MCF-7), and human ovarian adenocarcinoma (MDCK) cell lines. All cell lines were sensitive to thymoquinone. Mechanistic studies showed that 25μM of thymoquinone induced apoptosis of COS31 cells six hours after treatment and decreased the number of cells in S-phase while increasing the number of cells in G1 phase, indicating cell cycle arrest at G(1). The authors believe that thymoquinone exerts its effects by inducing apoptosis and cell cycle arrest.

From the above discussion it is apparent that the curative and preventative properties of *Nigella sativa* are significant. To advance the knowledge of this plant and its constituent, thymoquinone, led to the studies discussed in this thesis. However, in order for meaningful data to be collected it is essential to have basic knowledge of the cell cycle and proteins affected by cancer. This knowledge will allow for appropriate experiments to be designed and end points to be monitored.

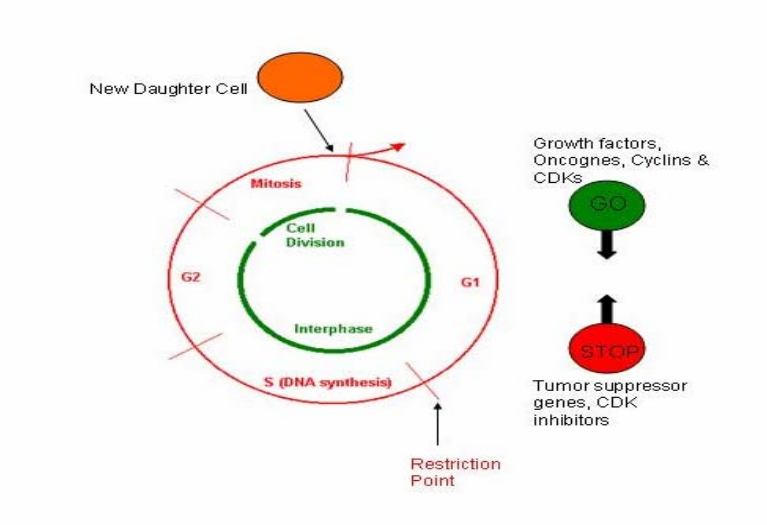
Chapter Three

Cell Cycle Control and Protein Expression in Cancer

Normal Cell Cycle and Protein Expression

In order for healthy cells to maintain a balance between cell production and cell death, intracellular and extracellular signals must be integrated in a set order. The cell cycle can be broken down into four phases. It is during these phases that a mammalian cell will replicate the entire genome and divide into genetically identical daughter cells. During the first phase, G(1) (gap 1), biochemical changes take place within the cell which prepare it for entry into the next phase. This next phase is called S phase (synthesis), at this time new DNA is synthesized and the cell creates an entire copy of its genetic material. The third phase, G(2) (gap 2), is a preparatory phase leading to entry into the last phase, M phase (mitosis). During M phase DNA condensed into chromosomes. These chromosomes are segregated into two daughter cells where each receive a full copy of the genetic material. A fifth phase does exist called G(0). Cells at G(1) can enter into G(0) if environmental growth factors are not right for the cell to proceed to S phase. Entry into S phase is irreversible (Rosenberg, 2001).

Figure 3.1: Cartoon illustration of the normal cell cycle



In the cell cycle, there are two interacting components: (a) the “mechanical” component, which refers to DNA replication, mitosis and cytokinesis; and (b) the “regulatory” components which refer to events in G(1) that control entry into the S phase and those in G(2) that regulate entry into mitosis . The proper progression of the cell cycle events depends primarily upon accurate chromosome replication and segregation, which is achieved by assuring that each step, is completed before the next one begins. Damage to these mechanisms, which results in failure to control cell cycle events, can cause alterations and mutations that may cause cell death or cancer. (Pusztai, 1996).

In order to avoid irreversible mistakes, initiation of any event in the cell cycle is dependent upon completion of earlier events. For this ordered dependency to be enforced the cell has developed control mechanism called checkpoints. These cell cycle checkpoints are regulated by components of the cells cycle machinery. In cases where nuclear DNA has been damaged normal cells cease to progress through the cell cycle at one of two points: either prior to entry into S-phase or prior to entry into mitosis. Arrest at these phases will limit propagation of genetic mutations to daughter cells by allotting time for DNA repair or initiation of cell death, however loss of a checkpoint can lead to a number of abnormalities including cancer (Rosenberg, 2001.)

Cell-cycle progression is governed by key regulatory proteins, which are controlled by posttranslational modification. Posttranslational modifications function to alter the stability and increase the protolytic degradation of these proteins (Blagosklonny, 2001).

A detailed discussion of the cell cycle transitions and the proteins that control them is presented below.

G(1)-S transition in cells and cell cycle checkpoints

In order for cells to complete the G(1) to S phase transition extracellular growth factors must be present for the cells to pass the first restriction point. This restriction point is defined as a point in late G(1) at which growth factors are no longer required. If

adequate growth factors are not present the cell will enter G(0) (Pardee, 1974). During the transition past this initial restriction point a series of biochemical events occur. First the protein complex cyclin D/cdk 4 is activated. This activated complex will hyperphosphorylate the protein RB. Upon hyperphosphorylation, RB will disengage from its partner E2F-1. The physical interaction of RB with E2F-1 blocks gene activation by E2F-1, a necessary step for cell cycle progression. Thus, upon hyperphosphorylation of RB, the E2F-1 gene is released and can now mediate gene activation and progression to the S phase (Nevins, 1998).

DNA damage at G(1) will result in cell cycle arrest due to the tumor suppressor gene, p53. Following DNA damage p53 levels and activity are increased. An increase in p53 can lead to an increase in the level of p21 (WAF1/CIP1) which serves to inhibit the activity of the cyclin/cdk complexes. Inhibition of cyclin/cdk will prevent hyperphosphorylation of RB thus preventing E2F-1 release which has previously been shown to drive the G(1) - S transition. p53 also functions as a cell cycle checkpoint protein by driving apoptosis following DNA damage (Levine, 1997).

S-phase

During S-phase a complete copy of the genetic material must be generated. For this to occur, DNA synthesis must begin at a defined location in the genome or an origin of replication (ORC). For DNA synthesis to be initiated and progress multiple proteins must bind to the DNA to aid in this process. These proteins are called the origin of replication complex (ORC). At different times, this phase of the cell cycle, two ORCs can be observed, a pre-ORC and a post-ORC. The pre-ORC binds to and prepares the DNA for replication at the end of G (1) but does not allow for replication to begin. Upon entry into S phase the post-ORC complex forms and allows the cell to proceed with DNA synthesis. Regulation of the pre-ORC and post-ORC is likely responsible for the mechanism by which cells cannot re-replicate DNA during the S phase (Rosenberg, 2001).

While little is known about the DNA-damage induced checkpoint controls during this phase of the cell cycle, a recently identified gene, ATM, a cytoplasmic protein kinase, is known to phosphorylate certain proteins such as p53 which are involved in DNA damage repair (Banin *et al.*1998).

M Phase

Following the S phase the cell enters G(2) and prepares for mitosis. During mitosis the duplicated genome is segregated into two daughter cells. It is crucial that M phase be successfully completed because errors in this process can lead to alterations in the genetic material such as loss of a chromosome (Hartwell, and Weinert, 1989). The M phase is also irreversible.

There are several stages within M-phase. During the first stage cells enter mitosis; this is controlled to prevent the segregation of chromosomes that have not finished DNA synthesis. During the second stage structural changes occur that prepare the sister chromatids to separate. The next stage at which the sister chromatids separate is an important checkpoint for the cell because this process is not easily reversed. During the last stage the chromosomes decondense, reform their nuclear envelope, and the cells undergo cytokinesis to complete the cell cycle (Rosenberg, 2001).

There are three levels of regulation during mitosis. Activation of the protein complex cyclin B/cdk1 is the first crucial regulatory mechanism, which governs whether cells enter mitosis. Cyclin B levels increase during late S and G(2) phase. The newly synthesized cyclin B will bind to dephosphorylated cdk1 (Blow, J., 1989). This is then activated by phosphorylation from a cdk-activating complex and localizes to the nucleus. The second level of regulation involves a family of kinases known as Polo-Like Kinases (PLKs). These are localized to spindle pole bodies, kinetichores and the spindle mid-zone at different stages in mitosis, thus playing a role in spindle apparatus assembly, paring of the sister chromatids and separation. PLKs are also involved in activation of the APC/cyclosome. The APC cyclosome serves as the third regulatory mechanism. The APC cyclosome causes destruction of certain proteins by ubiquination.

Thus the APC cyclosome controls the entry into, transition through, and exit from mitosis (Rosenberg, 2001).

During mitosis a checkpoint exists to prevent the metaphase to anaphase transition until sister chromatid pairs are aligned and attached at the spindle apparatus. This checkpoint is also thought to be controlled by the p53 protein.

Cancer Development

The molecular mechanisms that control cellular proliferation include both positive and negative regulators. Many regulatory proteins involved in the stimulation of proliferation, such as growth factors, growth factor receptors or proteins that participate in signal transduction, have been identified.

Cancer develops when the delicate balance between cell formation and cell death is disrupted. Dysregulation of cell cycle control is a phenomenon common to all forms of cancer. This can occur in one of two ways: lack of a cellular death program in response to stress, or lack of appropriate control responses to signals which normally cause the cells to halt progression through the cell cycle. Following discoveries made in the 1970s and 1980s, it has become clear that tumor cells results from too many cell cycle accelerators (oncogenes) and too few cell cycle decelerators (tumor suppressor genes e.g. p53) (Cross, 1991). This model has since been revised to recognize the roles of cell death controls. It now appears that tumor formation also results from decreased pro-apoptotic signals (p53), and increased anti-apoptotic signals (BCL-2) (Jacobson et al. 1993).

There are many known oncogenes such as c-SIS, an abnormally activated growth factor, and RAS, an intercellular signaling molecule. While these oncogenes do exist in some types of cancer it appears that the most influential factor leading to cancer is the loss of the tumor suppressor gene p53 (Rosenberg, 2001). p53 directly influences cell cycle machinery by, as previously mentioned, inducing p21 (WAF1/CIP1) to inhibit activation of cdk. Cdk will not be able to phosphorylate RB and thus E2F-1 will not be

able to mediate gene activation. If p53 function is lost both, inappropriate progressions through the cell cycle after DNA damage and survival of cells, which might have been destined to die, will occur. It is not surprising that p53 is the most commonly mutated gene and that an estimated 50% of human malignant tumors have mutated p53 (Soussi, 2000).

In conclusion, the cell cycle consists of a complex set of sequential, well coordinated specific events that result in cell division, and thus is central to proliferative diseases such as cancer. The genes which regulate the cell cycle, its checkpoints, and the factors which control it, are essential for the initiation, promotion, and progression of cancer.

Chapter Four

p53: the Core of DNA damage Pathways

The p53 tumor suppressor protein is a transcription factor with “sensor” functions integrating signals from the external and internal environment. The p53 tumor suppressor protein is a 53kDa nuclear phosphoprotein that can function as a sequence specific transcriptional activator and nonspecific transcriptional repressor. The p53 protein is also involved in cellular differentiation, DNA repair, senescence, and angiogenesis. Wild type p53 and intact signaling pathways minimize the formation of cancer, which is consistent with a high tumor incidence observed in p53-deficient mice (Choi, *et al.,* 1999). It is estimated that approximately one half of human cancer cells contain a mutation of p53. The increased predisposition to tumor development in the absence of p53 is due to the accumulation of genetic alterations in cells and the failure to eliminate these defective cells (Soussi, 2000). Thus, p53 is a major DNA damage checkpoint gene and is clearly required for a complete DNA damage response.

Wild type p53 is a labile protein with a short half life (Linzer *et al*., 1979). Accumulation and activation of this protein can be triggered by a variety of stress signals including DNA damage, hypoxia, nuclear deprivation, viral infection, heat shock, and mitogenic or oncogenic activation. The specific activity of p53 is further enhanced by posttranslational modifications and by a variety of positive and negative regulators (Bennett, 1998). Activated p53 elicits cellular responses that ultimately lead to growth arrest and/or apoptosis.

Regulation of p53

The p53 protein is subject to tight regulation at multiple levels. This is achieved by a variety of positive and negative regulators often creating feedback loops. Three major levels of regulation are recognized; protein stability, protein activity, and sub-cellular distribution (Blagosklonny, 2001). These are described in detail below.

Mdm-2 negative regulatory feedback loop

The major negative regulator of p53 is the Mdm-2 proto-oncogene. Mdm-2 is a transcription factor induced by p53. Thus, p53 causes its own destruction through a negative feedback loop (Momand, 1997). This feedback loop leads to variations in the expression of both proteins following DNA damage. Over expression of Mdm-2 can result in inactivation of p53 without further mutation and similarly loss of Mdm-2 is sufficient to induce p53 mediated apoptosis. Therefore, Mdm-2 is critical in keeping p53 in check and hence is referred to as "the master regulator "(Momand et al. 2000).

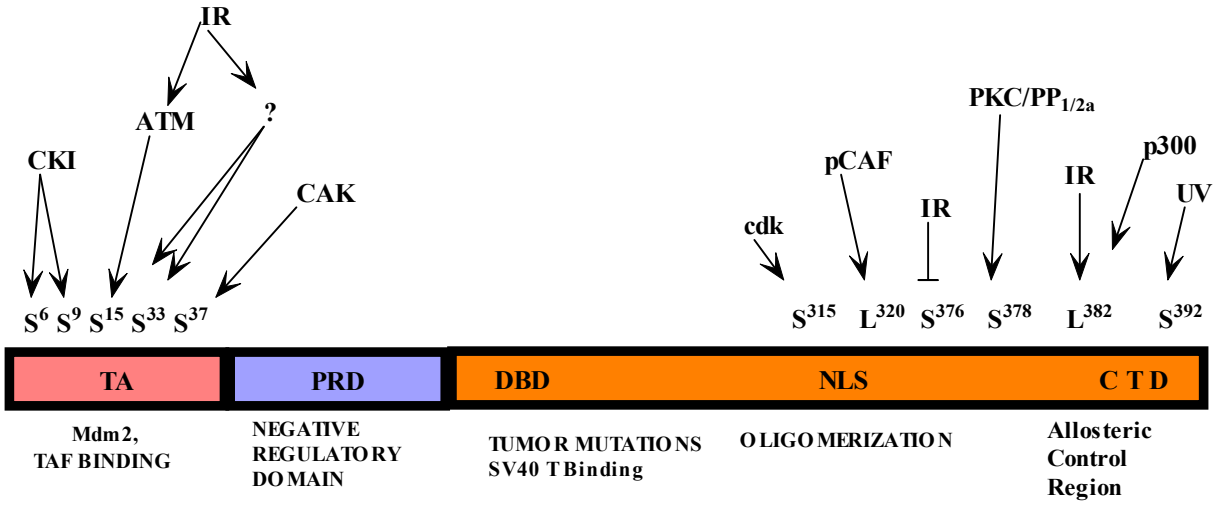
Mdm-2 and p53 proteasomal degradation

Haupt *et al*. (1997) demonstrated that Mdm-2 promotes p53 degradation through the ubiquitin proteasome system. Mdm-2 promotes p53 for degradation by acting as an E3 ubiquitin ligase. The exact mechanism of proteasomal degradation is not completely understood. It is possible that additional proteins may be involved in ubiquination of p53 by Mdm-2 *in vivo*. A possible candidate is Janus Kinase (JNK) which binds p53 and has been implicated in the ubiquination of p53 in unstressed cells (Fuchs et al.1998).

Mdm-2 inhibits p53 activity

Mdm-2 binds the trans-activation domain of p53 in a region important for the interaction of p53 with the components of the transcription machinery, such as TATA-binding protein (TBP) and associated factors (TAFs) and with its transcriptional coactivator p300. These observations prompted the construction of a model, called a "masking model," which hypothesizes that the binding of Mdm-2 to p53 conceals its transactivation domain thus inactivating it (Oliner et al. 1993). In spite of all of the studies done on Mdm-2 and p53 it is difficult to distinguish between the effects of Mdm-2 on p53 stability from its effect on p53 activity. A schematic diagram illustrating the various domains present in p53 including the Mdm-2 binding domain is shown in fig. 4.1.

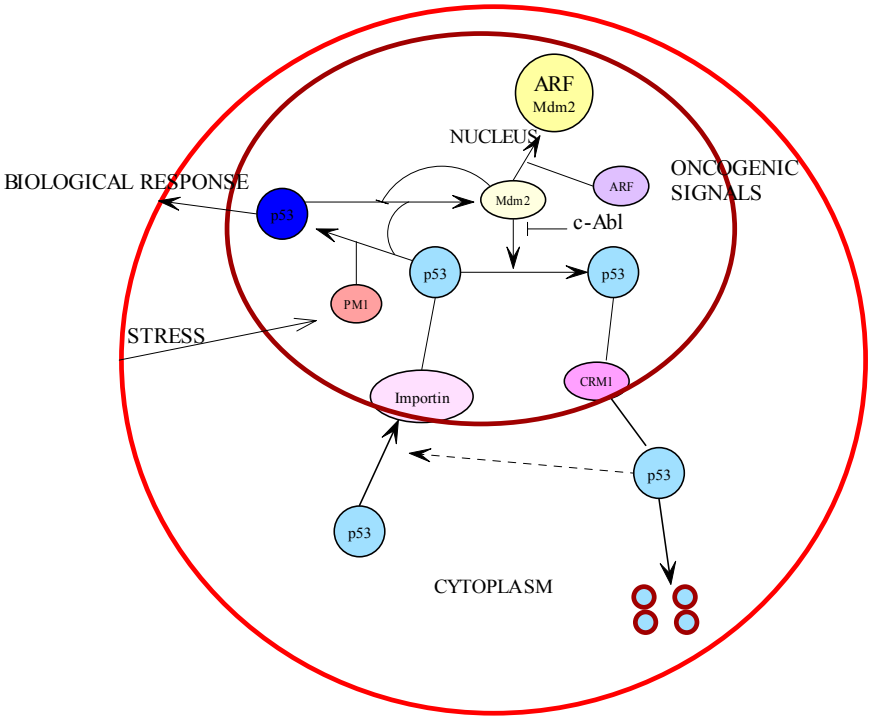
Figure 4.1 Illustration of the various domains present on the p53 gene



Involvement of interacting proteins

The majority of the positive regulators of p53 can be classified into two groups: (a) proteins that activate and stabilize p53 by neutralizing the inhibitory effects of Mdm-2 and (b) proteins that bind the C-terminus of p53 and activate it by protecting p53 from the inhibitory effects of this region, this is illustrated in fig. 4.1. Thus, p53 is regulated by a variety of proteins and a variety of stimuli (Blagosklonny, 2001). Figure 4.2 is a composite illustration showing a few of the regulatory interactions of p53 and its subcellular distribution.

Figure 4.2: A possible model for p53 regulation



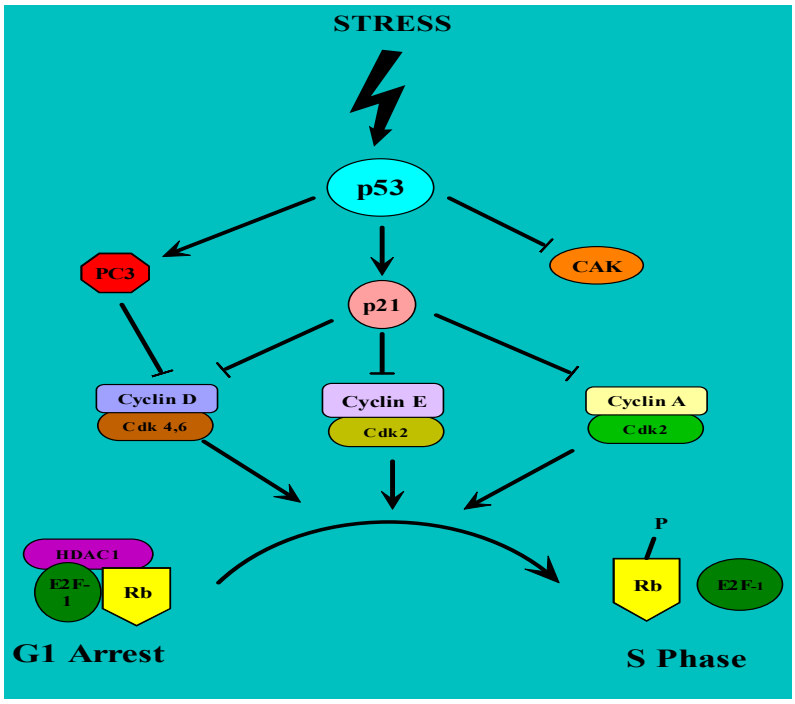
p53-mediated growth regulation

Once activated p53 triggers either growth arrest or apoptosis. p53 is crucial for the induction of growth arrest by numerous stress signals.

<u>p53 mediated-cell cycle arrest in G(1) phase</u>

The p53 target gene which is the key factor in G(1) arrest is p21 (the WAF1/Cip1). p21 inhibits different complexes of cyclin/cdk which sequentially phosphorylate the Rb protein, and as a result reduce the S-phase promoting E2F-1 transcription factor. p53 also promotes G(1) arrest by directly inhibiting the activity of cdk-activating kinase (CAK) which activates cyclin A/cdk2 by phosphorylation. Binding of p53 to CAK results in an increase in G(1) arrest. In addition p53 may activate Rb via PC3 (TIS21, BTG2), a newly identified p53 target gene. The PC3 gene product promotes accumulation of hyperphosphorylated Rb by reducing cyclin D1 protein levels and thereby inhibiting CDK4 activity (Brehm, et al. 1998) Figure 4.3 summarizes these pathways.

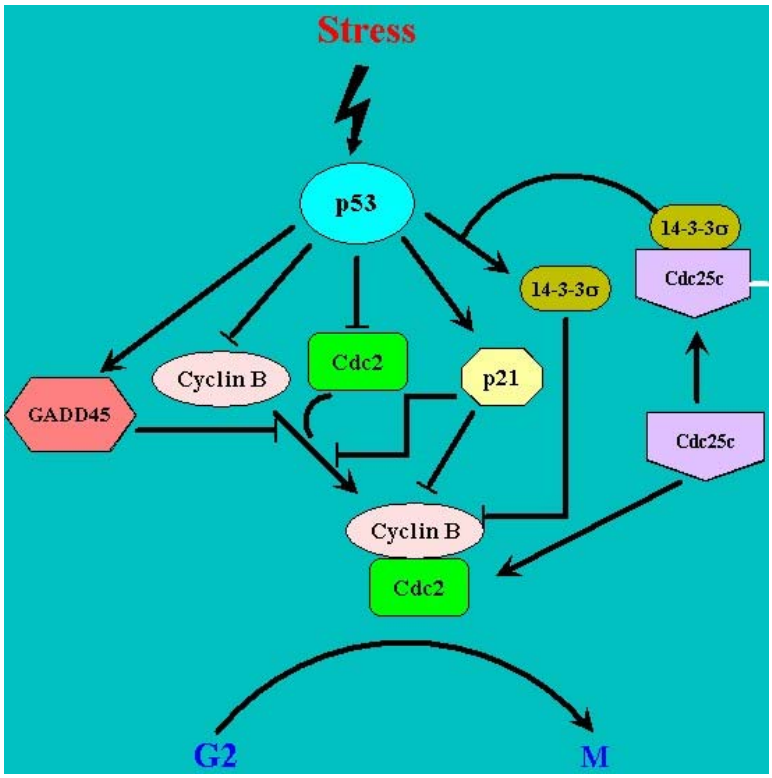
Figure 4.3: Role of p53 in G1 arrest



p53 mediated G(2) arrest

Activation of p53 can promote and maintain G(2) arrest. This depends on functional Rb protein and is mediated by several target genes. The p53 protein and its target genes effectively inhibit the cyclin B1/Cdc2 activity, which is essential for cells to enter mitosis. p21 inhibits the activity of the cyclin B1/Cdc 2 complex. The other important protein activated by p53 is GADD45. This protein binds Cdc2 and disrupts its ability to complex cyclin B1 and mediated G(2) arrest. The other protein activated by p53 is 14-3-3-σ. The 14-3-3-σ protein sequesters and inhibits the phosphorylated form of Cdc25c (Hermeking et al. 1997.) 14-3-3-σ also sequesters Cdc2 in the cytoplasm and prevents it from translocating into the nucleus in the late G(2) phase. The effects of the above proteins are further compounded by the transcriptional repression of Cdc2 and cyclin B1 by p53. Figure 4.4 summarizes these pathways.

Figure 4.4: Role of p53 in G2 arrest



p53 and Mitotic Spindle Checkpoint

Loss of p53 function leads to genomic instability, abnormal centrosome duplication, and formation of aneuploid and polypoid cells. This suggests that p53 plays a role in the control of centrosome duplication and normal chromosomal segregation. The exact mechanism is not known but may involve a variety of proteins. A cooperative role may exist between p53 and its interacting protein BRCA-1 in regulating chromosomal segregation. Both the proteins associate with centrosomes in mitosis and bind to gamma tubulin; however the importance of the interactions are unknown (Meek, 2000).

p53 mediated apoptosis

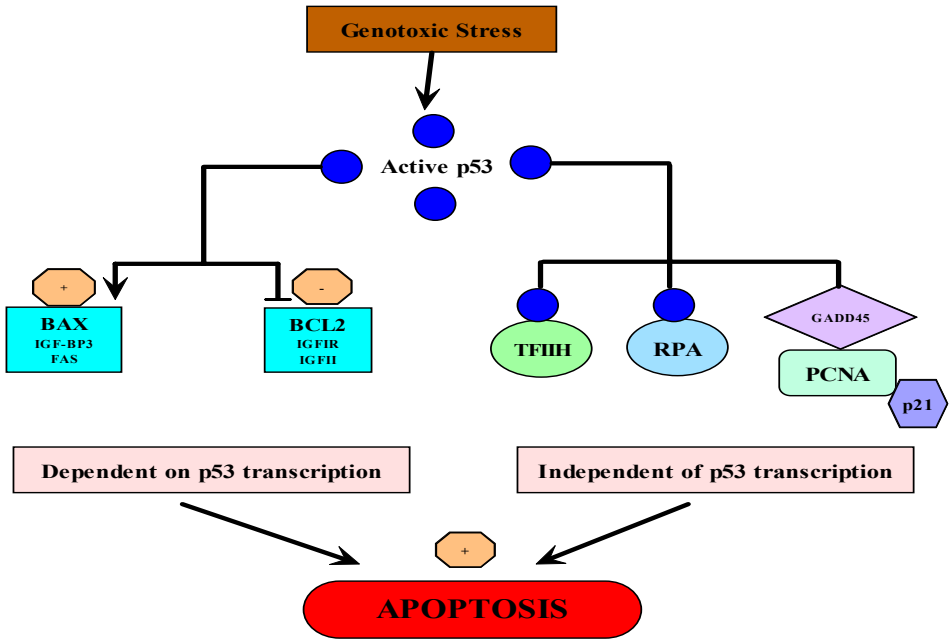
p53's role in modulating the apoptotic response likely plays an important role in p53's tumor suppressor activity. p53 is known to trigger apoptosis following the appropriate signals. The mechanistic pathways connecting p53 to the apoptotic death machinery

have remained illusive. No single mediator of p53's apoptotic activity has emerged. In fact, a variety of experimental systems have suggested that p53 may fundamentally utilize at least two apoptotic pathways. The two pathways leading to apoptosis are the death receptor and the mitochondrial pathways.

p53 may mediate apoptosis in a transcription dependent or transcriptional independent pathway. The transcription dependent pathway of apoptosis suggests that p53 activates death promoting genes such as BAX, CD95/Fas/Apo1, the PIG genes involved in redox regulation, and TNF-receptor family of genes containing death domains (Miyashita *et al.,* 1995 and Zhan *et al*., 1994). In spite of the growing list of p53 target genes involved in apoptosis the precise mechanism by which activation of these genes lead to apoptosis is not clear.

Information has emerged from studies that suggest, p53 may trigger apoptosis without the apparent need for transcriptional activity (Chao *et al*., 2000, and Attardi *et al*., 1996). The original data supporting this possibility came from the observation that p53's apoptotic activity was resistant to actinomycin D and cyclohexamide treatment. p53 has a proline rich region which contains several SH3 binding motifs (PXXP) and displays SH3 binding activity. This proline rich domain is important for p53's apoptotic function (Walker *et al*., 1996). A mutation in this region does not affect the ability p53 to arrest the cell cycle but prevents it from triggering apoptosis. This region perhaps serves as a regulatory domain for p53 mediated transcription independent apoptosis.
The possible pathways are illustrated in Figure 4.5.

Figure 4.5: Transcription dependent and independent pathways of apoptosis by p53



It appears that p53 is a major determinant in cancer development, and thus a drug which could target this protein and promote its up-regulation or block its down regulation would be of great value in cancer treatment.

Chapter Five

Hypothesis and Aim of Study

With the promising results from the *Nigella sativa* and thymoquinone studies and the knowledge of how cancer cells differ from normal cells, as well as, insight into the role of p53 in the cancer development process, it was possible to develop a hypothesis, ask some novel questions, and devise a set of experiments to answer these. The goal of the following research was to further monitor and evaluate thymoquinone's cytotoxic effects in common human tumors, determine if these effects are related to p53 status, determine the effect on the cell cycle, and evaluate the mechanism of cell death (apoptotic versus necrotic).

Hypothesis: Thymoquinone functions as a cytotoxic agent through the upregulation of the p53 protein.

Specific Aim 1: To assess cytotoxicity of thymoquinone in common human tumor cell lines with varying p53 expression levels.

The first step in the investigation was to determine the cytotoxicity of thymoquinone in three human ovarian cancer cell-lines (PA-1, SKOV-3 and CAOV-3). These cell lines were chosen because of their varying p53 status. With these experiments it was possible to assess if varying p53 expression played a role in thymoquinone's ability to kill these cells.

Specific Aim 2: To determine the sensitivity of common tumor cell lines with wild-type p53 to thymoquinone, and determine the effect of thymoquinone on p53 expression in these cell lines.

If p53 does play a role in thymoquinone's ability to manifest as an anti-neoplastic agent then the next step is to determine if cell-lines with wild-type p53 are consistently susceptible to the anti-neoplastic activity of thymoquinone. The cell-lines chosen were PA-1, A172, H460, and A549. All of these cell lines posses a wild-type p53 protein.

Experiments would be performed to determine changes in p53 expression in thymoquinone treated compared to untreated cells.

Specific Aim 3: To determine if thymoquinone works in synergy with cell cycle non-specific or cell cycle specific agents.

The next set of experiments was to determine the effect of thymoquinone on the cell cycle, when administered in combination with other chemotherapeutic agents. If thymoquinone's cytotoxic function is p53 dependent than this could play a role in determining the drug regime for cancer therapy. The drugs chosen for these combination studies were the cell-cycle specific agent, paclitaxel, and the non cell-cycle specific agent, cisplatin. The cell-lines used for these studies were PA-1, A172, and H-460.

Specific Aim 4: To determine the effect of thymoquinone on the cell cycle.

To further elucidate the effect of thymoquinone on the cell cycle, flow-cytometry analyses were conducted. The aim of this experiment was two-fold; to assess if thymoquinone affected the cell lines at a particular phase in the cell cycle as would be predicted by p53 upregulation, and to analyze if thymoquinone caused its cytotoxic effect via necrosis or apoptosis.

Chapter Six

Materials and Methods

Media and Chemicals

The established human ovarian cancer cell lines PA-1, SKOV-3, and CAOV-3, the human glioblastoma cell line A172, and the non-small cell lung cancer cell lines A549 and H460 were used in these experiments. All cells were obtained from American Type Cell Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (Gibco BRL, Gaithersburg, MD, USA). The following supplements were added to the media 10% fetal bovine serum (Gibco), 4mM glutamine (Gibco), sodium pyruvate (Gibco), and penicillin/streptomycin solution (100ug/ml) (Gibco). The PA-1 cells further required a supplement of non-essential amino acids (Gibco). The cells were maintained at 37°C in humidified 5% CO_2 atmosphere for a minimum of 72 hours before each experiment.

Additional Materials included: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), which was purchased from Promega (Madison, WI, USA), and ApoAlert Annexin V-FITC Apoptosis Kit (BD Biosciences Palo Alto, CA, USA). Phenazine methosulfate (PMS), ribonuclease A, and propidium Iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary monoclonal p53 antibody was obtained from Oncogene (San Diego, CA, USA). The secondary antibody, goat anti-mouse, was obtained from Chemicon International, Inc. (Temecula, CA, USA). Paclitaxel was purchased from Abbott Laboratories (Chicago, Il, USA) and cisplatin was purchased from GensiaSicor Pharmaceuticals (Irvine, CA, USA). Lastly, thymoquinone was purchased from Acros Organic (New Jersey, USA).

Preparation of Thymoquinone

Thymoquinone was reconstituted in methanol at a concentration of 10mg/ml. This stock was stored at 4°C in 15ml centrifuge tubes wrapped in aluminum foil to avoid dimer

formation. This stock was diluted as needed in DMEM to produce the concentrations to be tested.

Cell Proliferation Experiments

The proceeding colorimetric experiments were analyzed by using KC4 General Data Reduction Package Software and the EL_x808 Ultra Microplate Reader (BioTek instruments, Inc. Winooski, VT, USA). KC4 software is used to quantify data generated from the MTS colorimetric assay. The MTS assay calculates the % of live cells using the following equation:(sample - blank)/(control - blank). The blank well contains only the MTS solution and is subtracted out in order to remove background absorbance. The control wells contain untreated cells, while the sample wells represent the treat cells. To monitor the effects of concurrent or sequential drug exposure the data obtained from the KC4 analysis was further subjected to analysis by Calcusyn (Biosoft ®) software to predict additive, synergistic, or antagonistic effects.

<u>24-hour exposure experiments:</u>

PA-1, and SKOV-3 cells were plated in 96 well plates at a concentration of 1 X 10^3 cells/well and CAOV-3 cells were plated in 96 well plates at 5 X 10^3 cells per well. The cells were allowed to adhere overnight before addition of thymoquinone. Thymoquinone stock was diluted in media by one-fourth for seven concentrations starting at 28μM for PA-1 cells, 320μM for SKOV-3 cells, and 800μM for CAOV-3 cells. The cells were exposed to the thymoquinone for 24 hours. Following this exposure the thymoquinone was removed and 100μl of media was reapplied to the cells. Cell viability was assessed using the MTS assay.

<u>48-hour exposure experiments:</u>

PA-1, A172, H460 cells were plated in 96 well plates at concentrations of 3 X 10^3 cells per well, and A549 cell were plated at 1 X 10^3 cells per well. The cells were allowed to adhere overnight before addition of thymoquinone. Thymoquinone stock was diluted in DMEM (serial dilutions 1:4) to obtain the final concentrations of 28 - 0.0017μM for PA-1 cells, 448 -1.75μM for A172 and H460 cells, and 256 - 0.0625μM and for A549 cells. Each cell line was exposed to seven concentrations within the designated concentration

range. These cells were exposed to the thymoquinone for 48 hours. Following this exposure the thymoquinone was removed and 100µl of media was reapplied to the cells. Cell viability was assessed using the MTS assay.

48-hour exposure followed by 24 hours of recovery in media:

PA-1, A172, H460 cells were plated in 96 well plates at concentrations of 3 X 10^3, 3 X 10^3 and 2 X 10^3 cells per well respectively. The cells were allowed to adhere overnight before addition of thymoquinone. Thymoquinone stock was diluted in DMEM (serial dilutions 1:4) to obtain the final concentrations of 512 – 0.0078µM for PA-1 and 1024 – 0.015µM for A172 and H460 cells. Each cell line was exposed to seven concentrations within the designated concentration range. These cells were exposed to thymoquinone for 48 hours. Following this exposure the thymoquinone was removed and 100µl of media was reapplied for 24 hours to the cells. Cell viability was then assessed using the MTS assay.

120-hour exposure experiments:

PA-1, A172, H460, and A549 cells were plated in 96 well plates at concentrations of 7.5 X 10^2, 5 X 10^2, 1 X 10^3, and 5 X10^2 cells per well, respectively. The cells were allowed to adhere overnight before addition of thymoquinone. Thymoquinone stock was diluted in DMEM (serial dilutions 1:4) to obtain the final concentrations of 128-0.03125µM for PA-1, A172, and A549, and 256- 0.0625µM for H460 cells. Each cell line was exposed to seven concentrations within the designated concentration range. After 48 hours of exposure the thymoquinone was removed and reapplied in fresh media. Following another 48 hours the thymoquinone was again removed and reapplied in fresh media. After 24 hours cell viability was monitored using the MTS assay.

P53 quantification by Western Blot Analysis

Protein extraction

PA-1, A549, A172, and H460 cells were plated into 100cm Petri dishes at concentrations of 1.5 X10^6 for PA-1, 3X10^6 for A549, and 2 X 10^6 for both A172 and H460 cells per dish. Thymoquinone was administered for 48 hours at a concentration

that resulted in 50% cell death. After 48 hours of exposure protein extracts were prepared by washing the cells with PBS and counting them. They were then pelleted and suspended in Laemelli Lysis Buffer. The lysis buffer was boiled for five minutes at 95°C. The samples were then stored at 4°C for a maximum of six months. A total of four thymoquinone treated samples were prepared for H460, A549, and A172, and six treated samples were prepared for the PA-1 cells. Control samples were also prepared by treating A549 cells with 25nM Paclitaxel which is known to upregulate p53 levels (Giannakakou, P., 2001).

Gel loading, transfer, and development

The protein extracts were loaded onto a 12% polyacrylamide gel and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad apparatus. The running time was approximately one hour. The gel was transferred to a nitrocellulose membrane for 45 minutes using a Bio-Rad gel transfer apparatus. Following the transfer, the nitrocellulose membrane was immersed in blocking buffer (5% powdered milk in tris buffer) for one hour. The membrane was then subjected to an immunoblotting procedure, which involved adding primary monoclonal antibodies to p53 and β-actin (concentration of p53 100:1, and actin 5000:1) and incubating for two hours at room temperature or overnight at 4°C with slow constant shaking. This was followed by three 10-minute washes with tris-buffer prior to a one hour incubation with the secondary antibody (goat anti-mouse IgG horse radish peroxidase conjugated). The membrane was washed again as above before subjecting the membrane to the development procedure. The membrane was developed using the chemilumensent ECL system. The membranes were exposed to x-ray film for varying times periods (0.5 to 4 minutes). Equal protein loading was verified by actin probing. Quantification was performed by acquiring an image of the film with a CCD camera (Syngene, Cambridge, UK), which allowed densitometry with computer software. (GeneSnap and GeneTool software (Syngene, Cambridge, UK)). The results are normalized to the paclitaxel treated A549 positive control before comparisons are made.

Combination Drug Therapy Studies (Synergy/Antagonism)

Concurrent exposure to thymoquinone and cisplatin

PA-1, A172, H460 cells were plated in 96 well plates at concentrations of 3 X 10^3 cells per well for the PA-1 and A172 and 2 X 10^3 cells per well for the H460. The cells were allowed to adhere overnight before addition of thymoquinone and cisplatin. Each 96 well plate was divided into regions: region one was thymoquinone and cisplatin, region two was thymoquinone alone, and region three was cisplatin alone. Thymoquinone and cisplatin were added to the 96 well plates in ratios of 4:1, 1:1, and 1:4 on separate plates respectively. These ratios were chosen based on the monotherapy studies which indicated that thymoquinone was effective at concentrations similar to cisplatin. For PA-1 cells at a 4:1 ratio (thymoquinone: cisplatin) the concentrations were 28 – 0.0068μM: 7- 0.0017μM, at a 1:1 ratio the concentrations were 7-0.0017μM: 7-0.0017μM, and at a 1:4 ratio the concentrations were 7-0.0017μM: 28-0.0068μM. For A172 and H460 at a ratio of at 4:1 (thymoquinone: cisplatin) the concentrations were 448 – 7μM: 112-1.75μM, at a 1:1 ratio the concentrations were 112-1.75μM: 112-1.75μM, and at a 1:4 ratio the concentrations were 112-1.75μM: 448- 0.1094μM. Each cell line was exposed to seven concentrations within the designated concentration range and these concentrations were diluted by one-fourth starting from the top concentration. After twenty four hours of exposure to thymoquinone, cisplatin or both, the 96 well plates were washed and the thymoquinone solutions were again re-added to regions one and two at the same concentrations and media was re-added to region three. The following day the plates were washed and 100μl of media added and cell viability was assessed using the MTS assay.

This data was quantified via the MTS assay and then analyzed using the Calcusyn (Biosoft ®) software program, which utilizes the T.C. Chou method of determining synergy and antagonism. The combination index was determined at a 25%, 50%, and 75% toxicity level for each cell line and at each drug ratio (1:1, 1:4, and 4:1 thymoquinone to cisplatin).

Sequential exposure to thymoquinone and cisplatin

PA-1, A172, H460 cells were plated in 96 well plates at concentrations of 1 X 10^3 cells per well for PA-1 and A172 and 5 X 10^3 cells per well for H460 cells. The cells were allowed to adhere overnight before addition of thymoquinone and cisplatin. Each of the 96 well plates were divided into regions: region one was thymoquinone and cisplatin, region two was thymoquinone alone, and region three was cisplatin alone. Thymoquinone or cisplatin were added to the 96 well plates at a ratio of 4:1, respectively. For PA-1 cells the concentrations used were 28-0.0068μM: 7-0.0017μM, for A172 the concentrations used were 448-7μM: 112-1.75μM, and for H460 the concentrations used were 1792-0.4375μM: 448 - 0.1094. Each cell line was exposed to seven concentrations within the designated concentration range and these concentrations were diluted by one fourth starting from the top concentration. The protocol was as follows: on day one, cells were allowed to adhere, on day two, thymoquinone was added to regions one and two and media added to region three, or cisplatin was added to regions one and three and media was added to region two. On day three, the solution was removed from all regions and either thymoquinone was added to regions one and two with media added to region three or cisplatin was added to regions one and three and media was added to region two. On day four, the solution was removed from all regions and media was added at 100μl per well. The MTS assay was performed to assess cell viability.

This data was quantified via the MTS assay and then analyzed using the Calcusyn (Biosoft ®) software program. The combination index was determined at 25%, 50%, and 75% cell toxicity levels for each cell line at the drug ratio of 4:1 (thymoquinone to cisplatin).

Concurrent exposure to thymoquinone and paclitaxel

PA-1, A172, and H460 cells were plated in 96 well plates at concentrations of 3 X 10^3 cells per well. Each 96 well plate was divided into three regions: region one contained thymoquinone and paclitaxel, region two contained thymoquinone alone, and region three contained paclitaxel alone. Thymoquinone and paclitaxel were added to the 96

well plates in ratios of 100:1, 400:1, and 1600:1 on separate plates respectively. For PA-1 cells at a 100:1 ratio (thymoquinone: paclitaxel) the concentrations were 32 - 0.00781μM: 0.32 - 0.0000781μM, at a 400:1 ratio the concentrations were 128μM - 0.03125: 0.32- 0.0000781μM, and at a 1600:1 ratio the concentrations were 512 - 0.125μM: 0.32- 0.0000781μM. For A172 and H460 at a ratio of at 100:1 (thymoquinone: paclitaxel) the concentrations were 64 - 0.0156μM: 0.64 - 0.000156μM, at a 400:1 ratio the concentrations were 256 - 0.0625μM: 0.64 - 0.000156μM, and at a 1600:1 ratio the concentrations were 1024 –0.25μM: 0.64 - 0.000156μM. Each cell line was exposed to seven concentrations within the designated concentration range and hese concentrations were diluted by one-fourth stating from the top concentration. The protocol was as follows: on day 1 cells were allowed to adhere, on day two thymoquinone and paclitaxel were added to region one, thymoquinone was added to region two and paclitaxel was added to region three, the cells were allowed to incubate until day four, on day four the solution was removed from all regions and media was reapplied at 100μL per well. The MTS assay was performed to monitor cell viability after incubation for twenty-four hours.

This data was quantified via the MTS assay and then analyzed using the Calcusyn (Biosoft ®) software program. The combination index was determined at a 25%, 50%, and 75% toxicity level for each cell line and at the above drug ratios of 100:1, 400:1, and 1600:1 (thymoquinone to paclitaxel).

Sequential exposure to thymoquinone and paclitaxel

PA-1, A172, and H460 cells were plated in 96 well plates at concentrations of 1X 10^3 cells per well. Each of the 96 well plates were divided into three regions: region one contained thymoquinone and paclitaxel, region two contained thymoquinone alone, and region three contained paclitaxel alone. Thymoquinone and paclitaxel were added to the 96 well plates at a ratio of 1600:1. For PA-1 cells the concentrations used were 128-0.031:0.08-0.0000195μM, for A172 and H460 cells the concentrations used were 512-0.125:0.32-0.000078μM. These concentrations were diluted by one-fourth with seven concentrations applied within each range noted above. The protocol was as follows: on

day one cells were allowed to adhere, on day two thymoquinone was added to regions one and two and media added to region three, or paclitaxel was added to regions one and three and media was added to region two. These plates were incubated for forty-eight hours. On day four the solutions were removed from all regions and either thymoquinone was added to regions one and two with media added to region three or paclitaxel was added to regions one and three and media was added to region two. These plates were again incubated for forty-eight hours. The MTS assay was performed to monitor cell viability.

This data was quantified via the MTS assay and then analyzed using the Calcusyn (Biosoft ®) software program. The combination index was determined at 25%, 50%, and 75% cell toxicity levels for each cell line at a drug ratio of 1600:1 (thymoquinone to paclitaxel).

Analysis of additive, synergistic, or antagonistic effects

The median-effect equation and combination index analysis was used to calculate the interaction between treatment modalities. This analysis determines if the effect is antagonistic, additive or synergistic. The analysis is termed median effect analysis and used extensively in biological systems. For further review see Chou TC (1976) and Chou TC et al., (1984). The mathematical equation of the median-effect equation is: $f_a/f_u + (D/D_m)^m$, where f_a is the fraction affected by dose, f_u is the fraction unaffected by the dose, D is the drug dosage, D_m is the dosage of the drug required to inhibit the growth of 50% of the cells and is determined from the x-intercept, and m is the coefficient signifying the sigmoidicity of the dose-effect curve. The logarithmic form of the median-effect equation was used to linearize the dose-response curves, and can be plotted to generate the median-effect plot. Log (D) plotted vs. log (f_a/f_u), the x-intercept obtained is log (D_m), from which the IC_{50} can be evaluated and the slope is the m value. The m and the D_m values obtained thereafter can be used to calculate the effective dose for any effect level (Bence, A. *et al*., 2003).

The combination index (CI) is used to determine synergism or antagonism. The equation is based on the multiple-drug effect equation. A CI value of one indicates that the effect of one drug is additive to the second, a CI value of greater than one indicates antagonism between the two drugs, and a CI value of less than one indicates synergism between the drugs,. The equation used to calculate CI is as follows: $(D)_1/(D_x)_1 + (D)_2/(D_x)_2 + \alpha\ (D)_1(D)_2/(D_x)_1(D_x)_2$, where $(D_x)_1$ and $(D_x)_2$ are the doses for x% inhibition by drug 1 and drug 2 alone (Chou et al. 1984). These values are obtained from the median-effect equation. $(D)_1$ and $(D)_2$ are the doses in combination that inhibit cell growth by x%. A more detailed description of degrees of synergism and antagonism is listed in the table 6.1 adapted from Chou and Hayball (1996).

Table 6.1: Combination Index Values (CI)

Range of Combination Index Values	Description of Effect
< 0.1	Very strong synergism
0.10 – 0.30	Strong synergism
0.30 – 0.70	Synergism
0.70 – 0.85	Moderate synergism
0.85 – 0.90	Slight synergism
0.90 – 1.10	Nearly additive
1.10 – 1.45	Moderate antagonism
1.45 – 3.30	Antagonism
3.30 – 10	Strong antagonism
> 10	Very strong antagonism

The entire analysis was performed with the automated computer software Calcusyn (Biosoft®, Ferguson, MO USA).

Cell Cycle Analysis using Flow Cytometry

A172 and PA-1 cells were plated in 10ml of media in 100cm dishes at concentrations of 2 X 10^6 and 1 X 10^6 cells/dish, respectively. The cells were allowed to adhere overnight before addition of thymoquinone (21μM, and 8μM, respectively). These concentrations were determined to result in 50% cellular death. At 48 hours post drug treatment the cells were harvested, pelleted, washed with cold PBS, and resuspended in 0.5ml of PBS. 5ml of 70% ethanol was added drop wise while vortexing. The cells were left in fixative at 4°C for at least 15 minutes. The cells were then centrifuged and resuspended in a propidium iodide/RNAse A solution at a final concentration of 1 X 10^6 cells/ml. The cells were incubated for thirty minutes at 37°C before analysis by flow cytometry. Propidium Iodide staining allowed for the percentage of cells in G(1), S, and G(2)/M phase of the cell cycle to be determined.

Determination of Apoptosis or Necrosis using Flow Cytometry

A172 and PA-1 cells were plated in 10ml of media in 100cm dishes at concentration of 2 X 10^6 and 1 X 10^6 cells/dish, respectively. The cells were allowed to adhere overnight before addition of thymoquinone (21μM, and 8μM, respectively). At 48 hours post drug treatment the cells were harvested, pelleted (with initial media solution added), re-pelleted, and resuspended in media. A cell count was then taken and approximately 1 X 10^6 cells were added to polystyrene tubes and pelleted. The media was removed and replaced with 1ml of binding buffer. Cells were pelleted, and then suspended in 200μl of binding buffer, except tube 5, which was resuspended in 200μl of 70% ethanol. Five control tubes of cells, which were not exposed to thymoquinone, were required. The experiment was carried out according to the table below:

Table 6.2 Experimental Setup for Flow Cytometry

Tube #	PA-1 untreated cells	A172 untreated cells
1	Nothing Added	Nothing Added
2	Added 5µl Annexin	Added 5µl Annexin
3	Added 10µl PI	Added 10µl PI
4	Added 5µl annexin and 10µl PI	Added 5µl annexin and 10µl PI
5	Added 5µl annexin and 10µl PI in 200µl ethanol	Added 5µl annexin and 10µl PI in 200µl ethanol
6	Added 5µl annexin and 10µl PI	Added 5µl annexin and 10µl PI

All of the tubes were then incubated for five to fifteen minutes in the dark, at room temperature before addition of 800µl binding buffer. The tubes were analyzed by flow cytometry and the percentage of apoptotic and necrotic cells was determined.

Cytostatic or Cytotoxic Determination

PA-1 and A172 cells were plated in 96 well plates (two plates per cell line: one plate as a control and the other plate to be treated with thymoquinone) at a concentration of 3 $X10^3$ cells per well. After 24 hours thymoquinone was administered to one 96 well plate from each cell line in concentrations ranging from 128-0.03125µM. The plates were incubated for 48 hours at 37°C. To the other 96 well plate from each cell line the MTS assay was performed. The time at which the MTS assay was started was noted (and called the time zero reading) and readings were collected every half hour for up to four hours. These plates were discarded. To the plates with thymoquinone, after the 48 hour incubation period the plates were washed and 100µl of media was added. The MTS assay was started at the exact time of the day as the plates without thymoquinone and readings were collected every thirty minutes until a value of 1.00 had been reached in the control wells. Using the absorbance measurements collected at time zero (Tz) (all control wells), and from the thymoquinone treated cells (Ti) after matching MTS

development time, the cytotoxicity can be assessed. More specifically an LD50 can be calculated with the following equation [(Ti-Tz)/Tz] x 100 = -50. More details about antineoplastic drug screening can be found at (National Cancer Institute) web site under the *In Vitro* Cell Line Screening Project (IVCLSP).

Statistical Analysis

All statistical analysis were conducted using the Microsoft Excel program. Statistical differences were analyzed using two-sample student t-test. Significance was determined at $p \leq 0.05$.

Chapter Seven

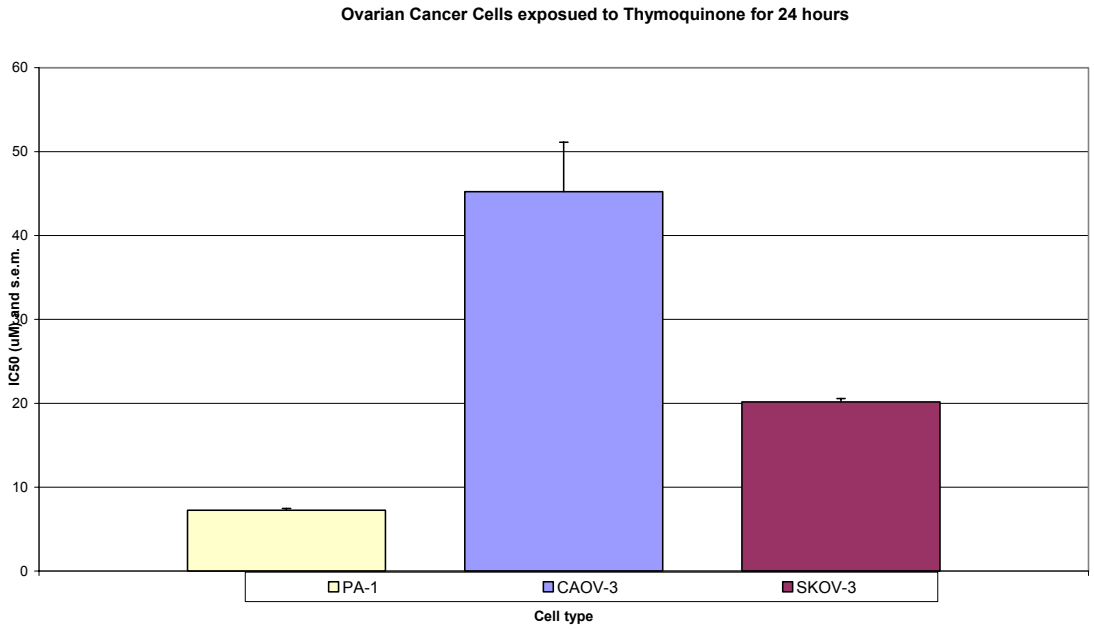
Results

Cell Proliferation and Viability Assays

24-hour exposure experiments

Initial cell viability assays were undertaken to see if thymoquinone exhibited its cytotoxic effects in a p53-dependent manner. The human ovarian cancer cells, PA-1 with wild type p53, CAOV-3 with mutated p53, and SKOV-3 with null p53, were used to elucidate the effects of thymoquinone. For PA-1 cells treated with thymoquinone, the IC_{50} was determined to be 7.26μM ± 0.21. For CAOV-3 cells, the IC_{50} was 45.22μM ± 5.88. The IC_{50} for SKOV-3 cells was found to be 20.16μM ± 0.41. All experiments were done in quadruplicate per plate and each experiment was repeated at least three times. All values are reported as the standard error of the mean. The data shows that the PA-1 cells are most susceptible to thymoquinone, which suggests that p53 maybe important for activity. The data is shown in figure 7.1.

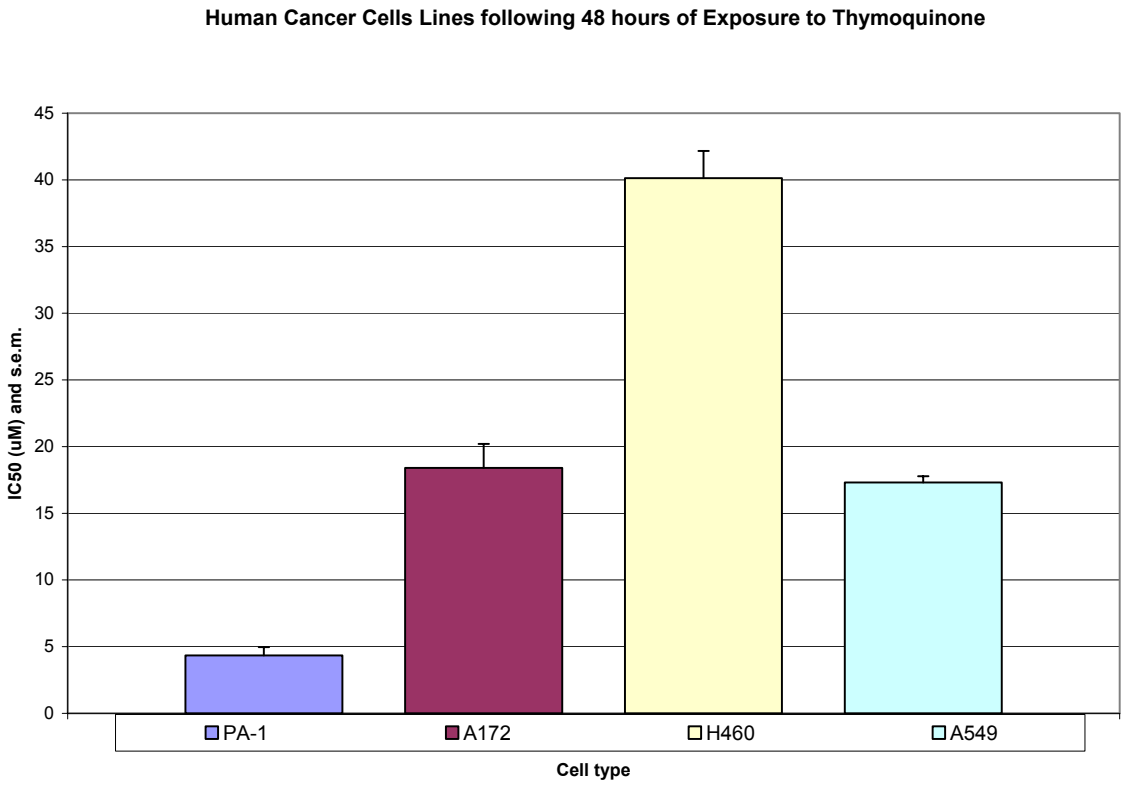
Figure 7.1: Effect of thymoquinone on PA-1, CAOV-3, and SKOV-3 cell lines after 24 hours of exposure.



48-hour exposure experiments

The cell lines PA-1, A172, A549 and H460 cells all posses wild type p53. PA-1 cells treated with thymoquinone had an IC_{50} of 4.35μM ± 0.63. A172 cells exposed to thymoquinone had an IC_{50} was of 18.41μM ± 1.70, and H460 cells exposed to thymoquinone had an IC_{50} of 40.1μM ± 2.04. For A549 cells treated with thymoquinone over the range of 256 to 0.0625μM, the IC_{50} was found to be 17.31μM ± 4.70. All experiments were done in quadruplicate per plate and each experiment was repeated at least three times. All values are reported as the standard error of the mean. The data is demonstrated in figure 7.2. and suggests that wild type p53 does not clearly indicate sensitivity to thymoquinone.

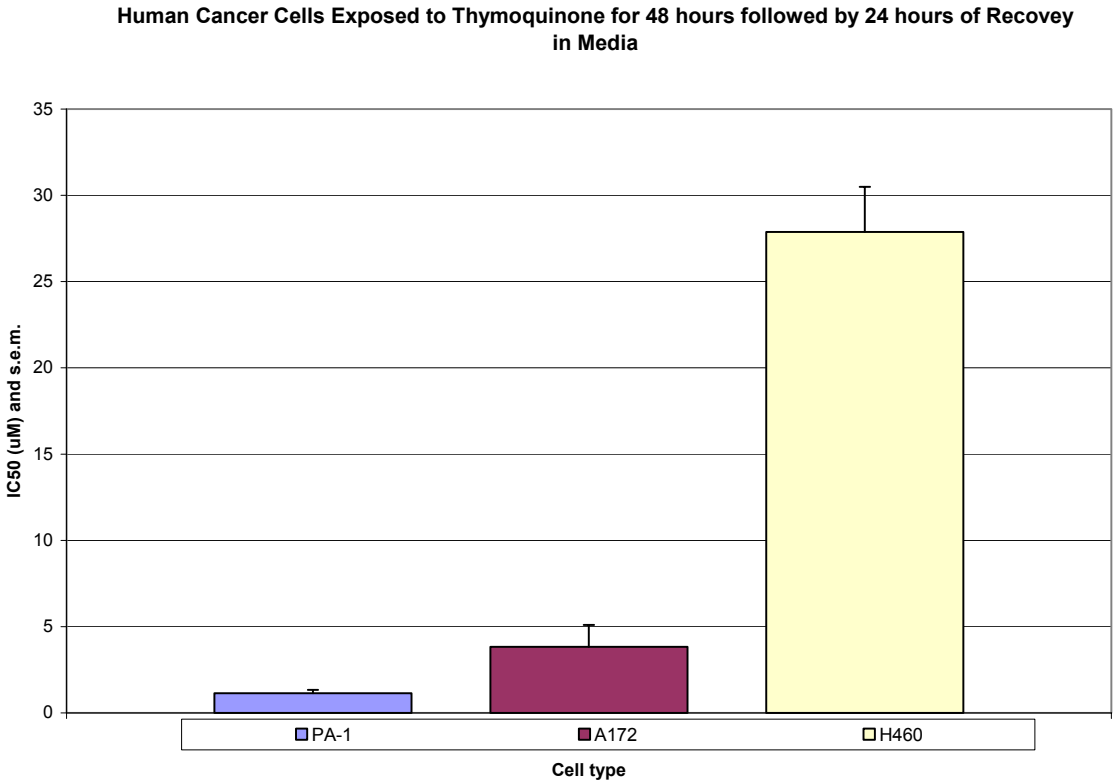
Figure 7.2: Effect of thymoquinone on PA-1, A172, H460, and A549 cell lines after 48 hours.



48-hour exposure followed by 24 hours of recovery in media

The cell line PA-1, A172, and H460 were subjected to thymoquinone for 48 hours followed by recovery in media for 24 hours. The IC_{50} for PA-1 cells was determined to be 1.14μM ± 0.19. A172 cells exposed to thymoquinone have an IC_{50} of 4.09μM ± 1.27. In H460 cells the IC_{50} was found to be 27.88μM ± 2.61. All experiments were done in quadruplicate per plate and each experiment was repeated at least three times. All values are reported as the standard error of the mean. The data is demonstrated in figure 7.3. These data suggest that growth inhibition/cytotoxicity is not rapidly reversible after drug removal.

Figure 7.3: Effect of thymoquinone on PA-1, A172, and H460 cell lines after 48 hours followed by 24 hour recovery in media.

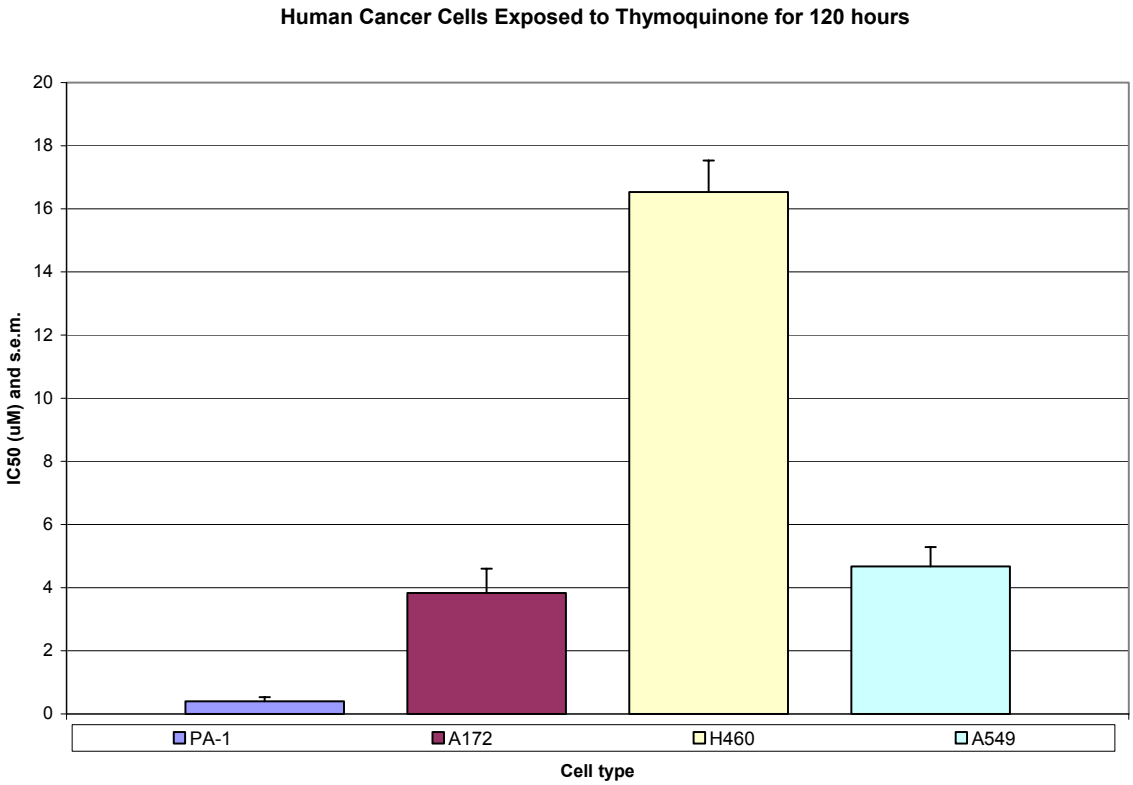


120-hour exposure experiments:

PA-1, A172, H460, and A549 cells were exposed to thymoquinone for 120 hours. The IC_{50}s for PA-1, A172, and A549 were determined to be: 0.39μM ± 0.13, 3.83μM ± 0.17,

and 4.67μM ± 0.61, respectively. H460 cells exposed to thymoquinone had an IC_{50} of 16.53μM ± 1.00. All treatments were done in quadruplicate per plate and each experiment was repeated at least three times. All values are reported as the standard error of the mean. This data is demonstrated in figure 7.4.and suggest that prolonged exposure may optimize growth inhibition/cytoxic activity.

Figure 7.4: Effect of thymoquinone on PA-1, A172, H460, and A549 cell lines after 120 hours.



p53 quantification by Western Blotting

In order to study the ability of thymoquinone to mediate growth-signaling pathways in neoplastic cells, Western blots were performed to quantify changes in p53 levels between untreated cells and those treated with thymoquinone. p53 protein was the protein of choice to monitor because it is a known tumor suppressor protein and is mutated in 50% of human cancers. Many chemotherapeutic agents exert their effects

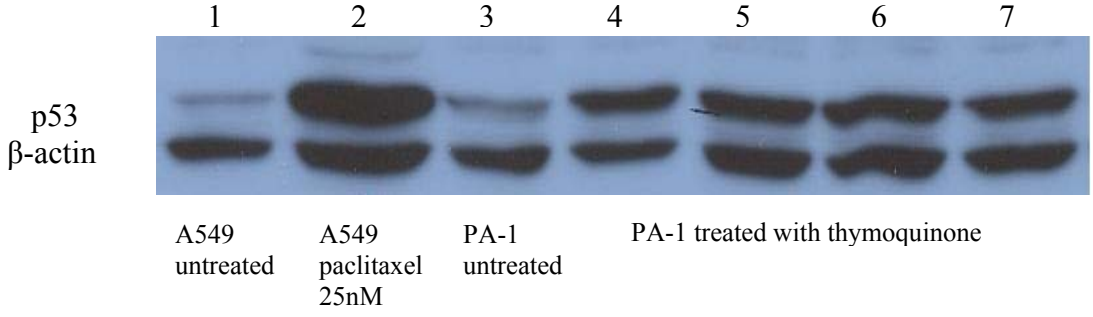
through upregulating p53. A549 cells treated with 25nM paclitaxel served as a positive control.

The cells used in this study were PA-1, A172, H460, and A549. p53 is reported as the percent of positive control prior to comparisons between untreated and thymoquinone treated cells. The results are as follows:

PA-1 cells

In PA-1 cells the average p53 level in untreated cells was 23.32% while the average p53 level in thymoquinone treated cells was 52.94%, relative to the A549 treated positive control. This is a 127% increase in the p53 levels in the thymoquinone treated cells versus the untreated cells. Using a two sample t-test these values were found to be significantly different ($p= 5.17 \times 10^{-7}$). A total of six thymoquinone treated protein samples were prepared and utilized, and each sample was subjected to Western blot analysis and repeated at least three times. All values are reported as the standard error of the mean. Figure 7.6 represents a sample Western blot analysis.

Figure 7.5: Western blot analysis demonstrating up regulation of p53 by thymoquinone in PA-1 cells. The lanes are as follows (1) untreated A549 cells, (2) A549 cells treated with 25nM paclitaxel, serving as the positive control, (3) untreated PA-1 cells, (4-7) treated PA-1 cells.



A172 cells

In A172 cells the average p53 level in untreated cells was 26.21% while the average p53 level for thymoquinone treated cells was 38.55%, relative to the A549 treated

positive control. This is a 47% increase in p53 levels in the thymoquinone treated cells versus the untreated cells. Using a two sample t-test these values were found to be significantly different (p= 0.04). A total of four thymoquinone treated protein samples were prepared and utilized, and each sample was subjected to Western blot analysis and repeated at least three times. All values are reported as the standard error of the mean. The data is illustrated if figure 7.6.

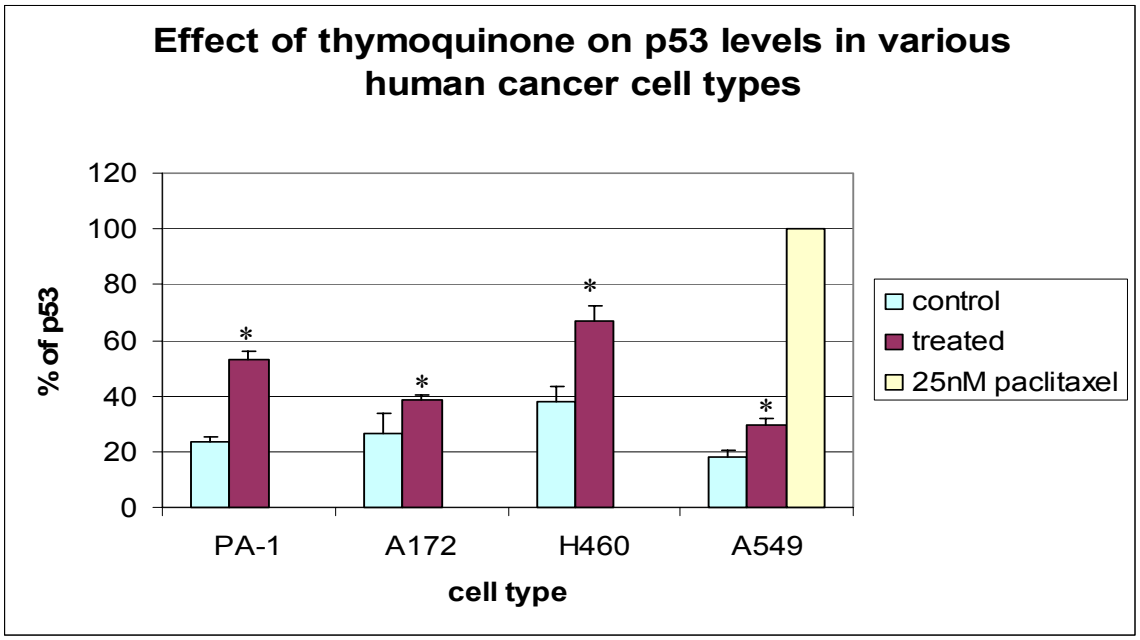
H460 cells

In H460 cells the average p53 level in untreated cells was 37.75% while the average p53 level in thymoquinone treated cells was 67.03%, relative to the A549 positive control. This is a 76% increase in the p53 levels in the treated cells versus the untreated cells. Using a two sample t-test these values were found to be significantly different (p= 0.008). A total of four thymoquinone treated protein samples were prepared and utilized, and each sample was subjected to Western blot analysis and repeated at least three times. All values are reported as the standard error of the mean. This data is illustrated in figure 7.6.

A549 cells

In A549 cells the average p53 level in untreated cells was 18.07% while the p53 level for thymoquinone treated cells was 29.49%, relative to the A549 positive control. This is a 61% increase in the p53 levels for the treated cells versus the untreated cells. Using a two sample t-test these values were found to be significantly different (p= 0.003). A total of four thymoquinone treated protein samples were prepared and utilized, and each sample was subjected to Western blot analysis and repeated at least three times. All values are reported as the standard error of the mean. This data is illustrated in figure 7.6.

Figure 7.6: The comparative levels of p53 protein in various cell lines, which have been treated with or without thymoquinone. The values are represented as a percent of the control. p≤ 0.05



* indicates statistical significance ($p < 0.05$)

Combination Drug Therapy Studies

Concurrent exposure to thymoquinone and cisplatin

PA-1, A172, and H460 cells were all exposed concurrently to thymoquinone and cisplatin.

PA-1 cells

Administration of thymoquinone and cisplatin in a 1:1 ratio resulted in moderate antagonism at the 25% toxicity level and antagonism at the 50% and 75% toxicity levels. At a 1:4 ratio the treatment resulted in a nearly additive effect at 25% toxicity, a moderate antagonistic effect at 50% and 75% toxicity. .At the 4:1 ratio the combination treatment resulted in antagonism at 25% toxicity, moderate antagonism at 50% toxicity, and was nearly additive at 75% toxicity. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.1.

Table 7.1: Effect of concurrent exposure of thymoquinone and cisplatin on PA-1 cells. The data represents an n≥3.

PA-1 cells with concurrent exposure to thymoquinone and cisplatin			
1:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.37	1.81	1.92
SEM	0.16	0.13	0.16
1:4 ratio			
% Toxicity*	25	50	75
Combination Index	1.02	1.14	1.29
SEM	0.07	0.05	0.06
4:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.95	1.27	1.01
SEM	0.27	0.08	0.07

* **The percent of non-viable cells.**

A172 cells

Administration of thymoquinone and cisplatin in a 1:1 ratio resulted in antagonism at the 25, 50, and 75% toxicity levels. At a 1:4 ratio the combination effect was also antagonistic at the 25, 50 and 75% toxicity levels. At a 4:1 ratio the combination drug effect was moderately antagonistic at 25%, 50%, and 75% toxicity. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.2.

Table 7.2: Effect of concurrent exposure of thymoquinone and cisplatin on A172 cells. The data represents an n≥3.

A172 cells with concurrent exposure to thymoquinone and cisplatin			
1:1 ratio			
% Toxicity*	25	50	75
Combination Index	2.54	1.87	1.69
SEM	0.05	0.09	0.12
1:4 ratio			
% Toxicity*	25	50	75
Combination Index	2.32	2.48	2.69
SEM	0.17	0.01	0.48
4:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.34	1.16	1.10
SEM	0.03	0.03	0.05

* **The percent of non-viable cells.**

H460 cells

Administration of thymoquinone and cisplatin in a 1:1 ratio resulted in moderate antagonism at 25% toxicity levels, and antagonism at the 50 and 75% toxicity levels. At a 1:4 ratio the effect of the drug combination was moderate antagonism at 25% and 50% toxicity levels and full antagonism at a 75% toxicity level. At the 4:1 ratio the combinatorial effect of the drug treatment resulted in antagonism at the 25% toxicity level, nearly additive at the 50% toxicity level, and moderate synergism at 75% toxicity. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table7.3.

Table 7.3: Effect of concurrent exposure of thymoquinone and cisplatin on H460 cells. The data represents an n≥3.

H460 cells with concurrent exposure to thymoquinone and cisplatin			
1:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.41	1.54	1.93
SEM	0.30	0.14	0.31
1:4 ratio			
% Toxicity*	25	50	75
Combination Index	1.29	1.38	1.67
SEM	0.27	0.20	0.05
4:1 ratio			
% Toxicity*	25	50	75
Combination Index	2.01	1.05	0.838
SEM	0.29	0.09	0.21

* **The percent of non-viable cells.**

Sequential Exposure to thymoquinone and cisplatin

PA-1, A172, and H460 cells were all exposed sequentially to thymoquinone and cisplatin.

PA-1 cells

Administration of thymoquinone followed by cisplatin resulted in an overall nearly additive effect at 25%, 50%, and 75% toxicity levels**.** Administration of cisplatin followed by thymoquinone resulted in moderate antagonism at 25% toxicity, a nearly additive effect at 50% toxicity, and moderate synergism at 75% toxicity. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.4 below .

Table 7.4: Effect of thymoquinone and cisplatin added sequentially at a ratio of 4:1 on PA-1 cells. The data represents an n≥3.

PA-1 cells treated with thymoquinone and cisplatin sequentially at a 4:1 ratio (thymoquinone to cisplatin)			
Thymoquinone first			
% Toxicity*	25	50	75
Combination Index	0.95	0.92	0.94
SEM	0.05	0.06	0.08
Cisplatin first			
% Toxicity*	25	50	75
Combination Index	1.30	0.99	0.84
SEM	0.16	0.07	0.07

* **The percent of non-viable cells.**

A172 cells

Administration of thymoquinone followed by cisplatin resulted in an overall moderate synergistic effect 25%, 50%, and 75% toxicity levels**.** Administration of cisplatin followed by thymoquinone resulted in moderate antagonism at 25%, 50%, and 75% toxicity levels. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.5.

Table 7.5: Effect of thymoquinone and cisplatin added sequentially at a ratio of 4:1 on A172 cells. The data represents an n≥3.

A172 cells treated with thymoquinone and cisplatin sequentially at a 4:1 ratio (thymoquinone to cisplatin)			
Thymoquinone first			
% Toxicity*	25	50	75
Combination Index	0.83	0.75	0.70
SEM	0.06	0.04	0.06
Cisplatin first			
% Toxicity*	25	50	75
Combination Index	1.32	1.18	1.13
SEM	0.01	0.05	0.09

* **The percent of non-viable cells.**

H460 cells

Administration of thymoquinone followed by cisplatin resulted in moderate synergism at 25%, slight synergism at 50%, and a nearly additive effect 75% toxicity levels. Administration of cisplatin followed by thymoquinone resulted in moderate antagonism at 25% toxicity, and a nearly additive effect 50%, and 75% toxicity levels. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.6.

Table 7.6: Effect of thymoquinone and cisplatin added sequentially at a ratio of 4:1 on H460 cells. The data represents an n≥3.

H460 cells treated with thymoquinone and cisplatin sequentially at a 4:1 ratio (thymoquinone to cisplatin)			
Thymoquinone first			
% Toxicity*	25	50	75
Combination Index	0.84	0.88	0.93
SEM	0.01	0.04	0.06
Cisplatin first			
% Toxicity*	25	50	75
Combination Index	1.19	0.97	0.95
SEM	0.08	0.06	0.11

* **The percent of non-viable cells.**

Concurrent exposure to thymoquinone and paclitaxel

PA-1, A172, and H460 cells were all exposed concurrently to thymoquinone and paclitaxel.

PA-1 cells

Administration of thymoquinone and paclitaxel in a 100:1 ratio resulted in moderate antagonism at the 25% and 50% toxicity, and antagonism at 75% toxicity. At a 400:1 ratio the drug combination was moderately antagonistic at 25%, 50%, and 75% toxicity levels. At a 1600:1 ratio the drug combination was synergistic at the 25%, 50%, and 75% toxicity levels. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.7.

Table 7.7: Effect of the concurrent exposure of thymoquinone and paclitaxel on PA-1 cells. The data represents an n≥3.

PA-1 cells with concurrent exposure to thymoquinone and paclitaxel			
100:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.20	1.34	1.48
SEM	0.10	0.04	0.05
400:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.34	1.28	1.22
SEM	0.12	0.05	0.05
1600:1 ratio			
% Toxicity*	25	50	75
Combination Index	0.58	0.57	0.617
SEM	0.12	0.14	0.16

* **The percent of non-viable cells.**

A172 cells

Administration of thymoquinone and paclitaxel in a 100:1 ratio resulted in moderate antagonism at 25 and 50% toxicity levels and antagonism at the 75% level. At a 400:1 ratio the drug effect was antagonistic at 25% toxicity, moderately antagonistic at 50% toxicity, and nearly additive at 75% toxicity. At a 1600:1 ratio the combinatorial drug effect was nearly additive at 25%, 50%, and 75% toxicity levels. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.8.

Table 7.8: Effect of the concurrent exposure of thymoquinone and paclitaxel on A172 cells. The data represents an n≥3.

A172 cells with concurrent exposure to thymoquinone and paclitaxel			
100:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.11	1.29	1.56
SEM	0.25	0.08	0.14
400:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.77	1.28	1.04
SEM	0.20	0.11	0.18
1600:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.01	1.04	1.02
SEM	0.05	0.12	0.18

* **The percent of non-viable cells.**

H460 cells

Administration of thymoquinone and paclitaxel in a 100:1 ratio resulted in moderate synergism at 25% toxicity, slight synergism at 50% toxicity, and moderate antagonism at 75% toxicity. At a 400:1 ratio moderate antagonism was the result at the 25% toxicity level, moderate synergism at the 50% toxicity level, and synergism at the 75% toxicity level. At a 1600:1 ratio the combination drug effect was moderate antagonism at 25% toxicity, moderately synergistic at 50% toxicity, and synergistic at 75% toxicity. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.9.

Table 7.9: Effect of the concurrent exposure of thymoquinone and paclitaxel on H460 cells. The data represents an n≥3.

H460 cells with concurrent exposure to thymoquinone and paclitaxel			
100:1 ratio			
% Toxicity*	25	50	75
Combination Index	0.74	0.88	1.43
SEM	0.10	0.10	0.45
400:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.32	0.71	0.54
SEM	0.29	0.09	0.03
1600:1 ratio			
% Toxicity*	25	50	75
Combination Index	1.28	0.728	0.48
SEM	0.09	0.05	0.07

* **The percent of non-viable cells.**

Sequential exposure to Thymoquinone and Paclitaxel

PA-1, A172, and H460 cells were all exposed sequentially to thymoquinone and paclitaxel.

PA-1 cells

Administration of thymoquinone followed by paclitaxel resulted in an overall slight synergism at 25%, 50%, and 75% toxicity levels**.** Administration of paclitaxel followed by thymoquinone resulted in antagonism at 25%, 50%, and 75% toxicity levels. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.10.

Table 7.10: Effect of thymoquinone and paclitaxel added sequentially at a ratio of 1600:1 on PA-1 cells. The data represents an n≥3.

PA-1 cells treated with thymoquinone and paclitaxel sequentially at a 1600:1 ratio (thymoquinone to paclitaxel)			
Thymoquinone first			
% Toxicity*	25	50	75
Combination Index	0.89	0.85	0.86
SEM	0.02	0.08	0.17
Paclitaxel first			
% Toxicity*	25	50	75
Combination Index	1.87	1.67	1.53
SEM	0.24	0.22	0.20

* **The percent of non-viable cells.**

A172 cells

Administration of thymoquinone followed by paclitaxel resulted in a synergism effect at 25%, 50%, and 75% toxicity levels**.** Administration of paclitaxel followed by thymoquinone resulted in a nearly additive effect at 25% toxicity, a moderately synergistic effect at 50% and 75% toxicity levels. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.11.

Table 7.11: Effect of thymoquinone and paclitaxel added sequentially at a ratio of 1600:1 on A172 cells. The data represents an n≥3.

A172 cells treated with thymoquinone and paclitaxel sequentially at a 1600:1 ratio (thymoquinone to paclitaxel)			
Thymoquinone first			
% Toxicity*	25	50	75
Combination Index	0.66	0.64	0.66
SEM	0.07	0.07	0.08
Paclitaxel first			
% Toxicity*	25	50	75
Combination Index	1.04	0.80	0.77
SEM	0.22	0.18	0.13

* **The percent of non-viable cells.**

H460 cells

Administration of thymoquinone followed by paclitaxel resulted in an antagonistic effect at 25%, 50%, and 75% toxicity levels**.** Administration of paclitaxel followed by thymoquinone resulted in an overall slight synergistic effect at 25%, 50%, and 75% toxicity levels. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. This data is summarized in table 7.12.

Table 7.12: Effect of thymoquinone and paclitaxel added sequentially at a ratio of 1600:1 on H460 cells. The data represents an n≥3.

H460 cells treated with thymoquinone and paclitaxel sequentially at a 1600:1 ratio (thymoquinone to paclitaxel)			
Thymoquinone first			
% Toxicity*	25	50	75
Combination Index	1.68	1.60	1.63
SEM	0.36	0.20	0.07
Paclitaxel first			
% Toxicity*	25	50	75
Combination Index	0.87	0.89	0.87
SEM	0.13	0.08	0.15

* **The percent of non-viable cells.**

Effect of Thymoquinone on Cell Cycle Distribution

PA-1 and A172 cells were treated with thymoquinone at a dose which caused 50% cell death (7uM and 21μM, respectively). These cells, as well as, untreated PA-1 and A172 cells were analyzed by flow cytometry to monitor cell cycle distribution. Thymoquinone treatment of PA-1 cells and A172 cells with the above mentioned dose did not cause a significant accumulation of cells in the G(0)-G(1), S, or G(2)-M phases of the cell cycle after 48 hours of thymoquinone treatment. The results are summarized in figure 7.7 and figure 7.8.

Figure 7.7: The cell cycle distribution of PA-1 cells, either with or without thymoquinone. All values are represented as the percent of cells in different phases of the cell cycle.

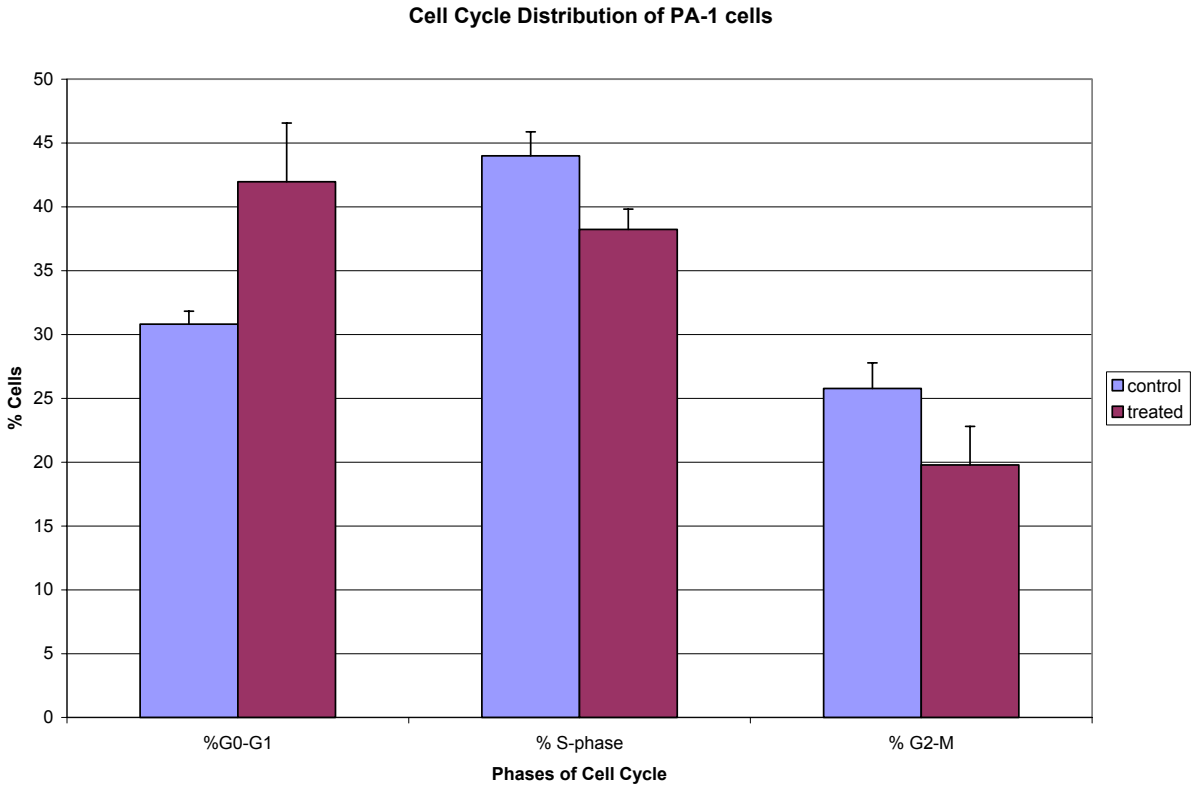
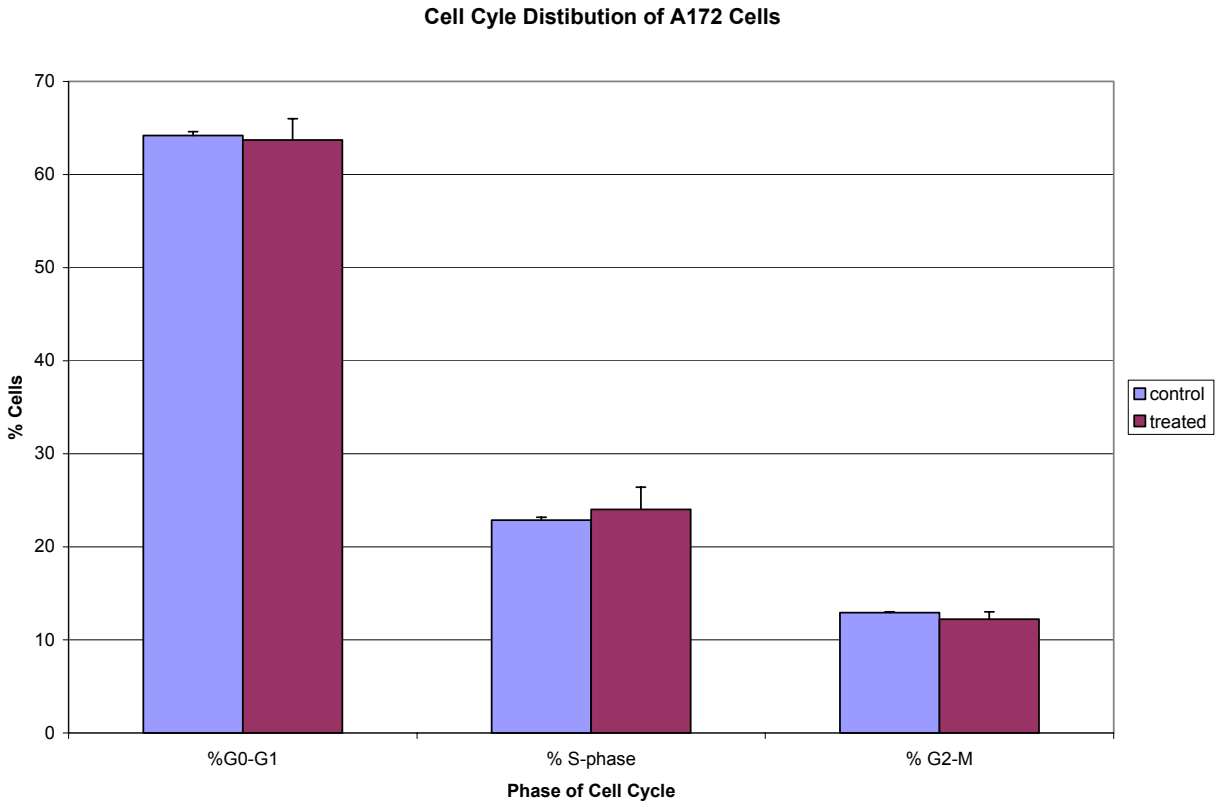


Figure 7.8: The cell cycle distribution of A172 cells, either with or without thymoquinone. All values are represented as the percent of cells in different phases of the cell cycle.



Thymoquinone mediated apoptosis and necrosis

PA-1 and A172 cells were treated with thymoquinone at a dose which caused 50% cell death at 7μM and 21μM respectively. These cells as well as untreated PA-1 and A172 cells were treated with annexin and/or propidium iodide to distinguish between apoptotic and necrotic cellular death. These cells were analyzed by flow cytometry. The ratio of apoptotic to necrotic cells was determined to monitor if an increase in apoptosis occurred following thymoquinone treatment. Thymoquinone treatment of PA-1 cells and A172 cells at the above mentioned doses did not cause a significant change in the number of apoptotic to necrotic cells. All values were determined with an n≥ 3 and all values are reported as the standard error of the mean. The data is summarized in figures 7.9 and 7.10

Figure 7.9: Ratio of apoptotic/necrotic PA-1 cells with or without thymoquinone.

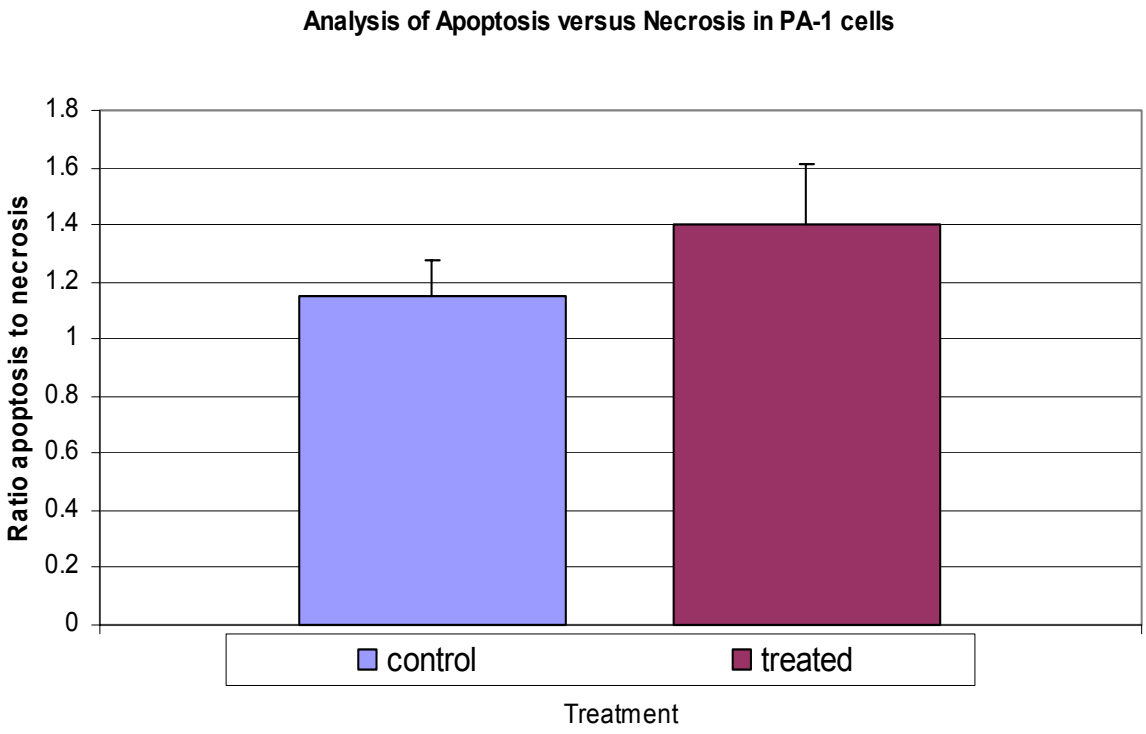
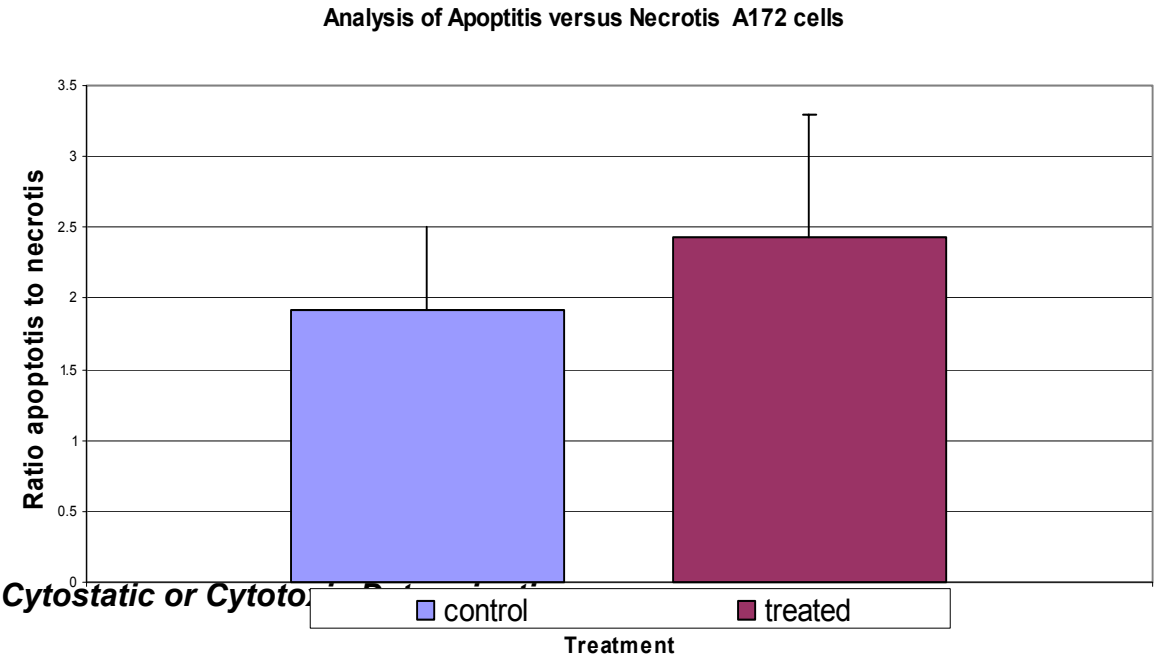


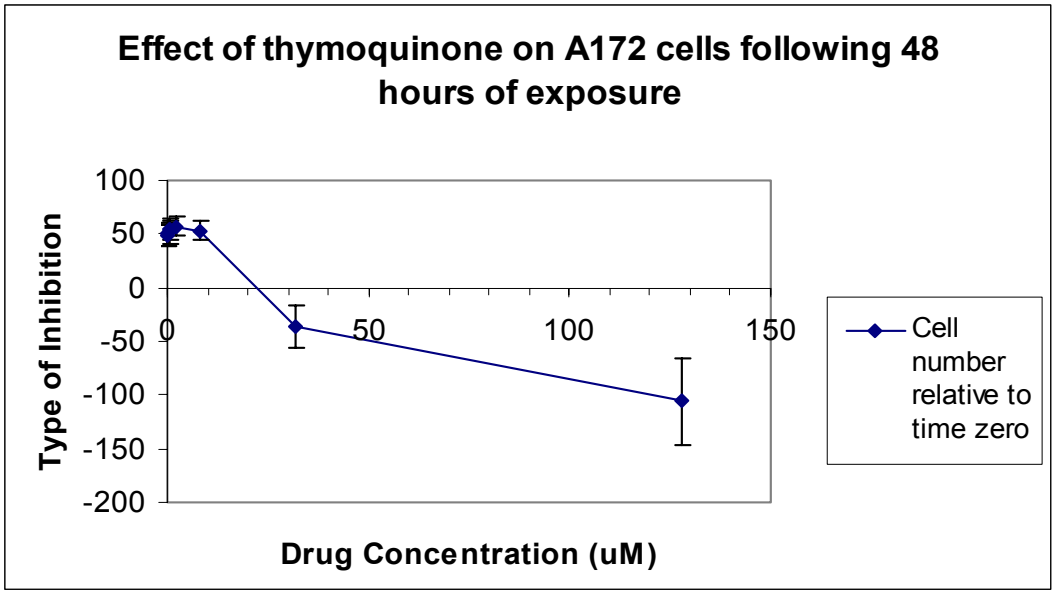
Figure 7.10: Ratio of apoptotic/necrotic A172 cells with or without thymoquinone.



Cytostatic or Cytoto

PA-1 cells and A172 cells were treated with thymoquinone over a concentration range of 128-0.3125 µM for 48 hours to monitor if thymoquinone functioned as a cytotoxic or cytostatic agent. The experiments were performed and analyzed using methods set forth by the *In Vitro* Cell Line Screening Project (IVCLSP). The control cells (cells exposed to media for 48 hours) are displayed when x=0. Values of y larger than the control indicate increased cellular growth following drug treatment. Values of y less than the control but greater than zero indicated cellular growth inhibition (cytostatic effect). When the y-axis equals zero, total growth inhibition (TGI) has occurred, this is when the number of cells prior to thymoquinone treatment equals the number of cells after 48 hours of drug treatment indicating that thymoquinone has completely inhibited cells from growing. Values of y less than zero indicate cellular death (cytotoxic effect). Based on this methodology it was determined that thymoquinone acts as a cytotoxic agents because the relative number of cells at the end of thymoquinone treatment was less than the number of cells at time zero (start of thymoquinone treatment) (National Cancer Institute). The graph for A172 cells (n = 3) is shown in figure 7.11.

Figure 7.11: The type of inhibition produced by thymoquinone in A172 cells



Chapter Eight

Discussion

Queries

The research conducted herein focused on thymoquinone, the active constituent of the plant *Nigella sativa*. The following queries were raised. Is thymoquinone cytotoxic through a mechanism which up regulates p53? This query was raised because it had previously been reported that thymoquinone causes G(1) arrest (Shoeib et al. 2002). G(1) arrest is known to be initiated by up regulation of the tumor suppressor gene p53. If thymoquinone initiated its function through this pathway then its cytotoxic properties should be greatly reduced in cells which have mutated or null p53 status. The next question was: could the up regulation of the p53 protein be demonstrated following thymoquinone treatment? At this point in time p53 up regulation had been speculated but not proven.

Another query was: can thymoquinone alter the cytotoxicity of other known chemotherapeutic agents and is its effect, antagonistic, additive, or synergistic, does this effect change if the drugs are given sequentially instead of concurrently, and does this effect vary if thymoquinone is administered with a cell cycle specific agent (paclitaxel) or with a non-cell cycle specific agent (cisplatin)? This study was important to undertake because in some parts of the world Nigella sativa is consumed in high levels by the general population. If thymoquinone is found to antagonize the effects of chemotherapy it would be of importance for physicians in these regions of the world to be able to make their patients aware of this possible drug-drug interaction. However, if synergistic this may be a new treatment option for physicians

Does thymoquinone treatment result in necrosis or apoptosis? This endpoint was evaluated to further elucidate thymoquinone mechanism of action as a chemotherapeutic agent. In addition it would be useful to know if thymoquinone is a true cytotoxic or cytostatic agent. This would allow clinicians in the near future to decide on the dosing regimen of thymoquinone, if it proves to be of clinical importance.

Summary of Research

The initial study evaluating thymoquinone's effects on p53 status indicated that the PA-1 cells (wild type p53) were the most sensitive. Based on this, studies were conducted in cell types which only express wild-type p53. From these studies it was found that thymoquinone was cytotoxic to all cell lines in the micro-molar range. These studies were repeated at various time points (48 to 120 hours) and it appeared that the degree of cytotoxicity was directly related to the length of exposure. These cells were then analyzed by Western blot analysis. All p53 wild type cell lines tested had a significant increase ($p \leq 0.05$) in p53 levels after treatment with thymoquinone as compared to their untreated counterparts.

The drug combination studies with concurrent exposure to thymoquinone and cisplatin in the cell types tested (PA-1, A172, and H460) revealed some degree of antagonism. These results suggest that it may not be beneficial to combine these drugs concurrently. The ratio that caused the least antagonism was the 4:1 ratio. Hence this ratio was used to assess the efficacy of the sequential combination therapy.

When thymoquinone was administered first followed by cisplatin, a nearly additive effect was observed in PA-1 cells. The same effect was seen when cisplatin was administered first. Sequential administration of these two drugs seemed to have a more promising effect than when given concurrently in PA-1 cells. When thymoquinone was administered first followed by cisplatin a moderate synergistic effect was observed in A172 cells. Interestingly, a moderate antagonistic effect was seen when cisplatin was administered first in A172 cells. Sequential administration of thymoquinone first, followed by cisplatin may have the most beneficial effect of the possible combination therapies tested. When thymoquinone was administered first followed by cisplatin an overall slight synergistic effect was observed in H460 cells. A nearly additive effect occurred when cisplatin was administered followed by thymoquinone. Sequential administration of thymoquinone first, followed by cisplatin may have the most beneficial effect of the possible combination therapies tested in the H460 cell line. These sets of

experiments suggest that sequential administration of thymoquinone with cisplatin seemed more efficacious than concurrent administration. However, the degree of efficacy and order of administration is cell line dependent.

Thymoquinone was also monitored when given concurrently and sequentially with paclitaxel. The following cell lines were used; PA-1, A172 and H460. For PA-1 and A172 cells overall antagonism was seen at the lower drug ratios while the high drug ratio there was an additive effect for the A172 cells and synergy in the PA-1 cells. For H460 cells some degree of synergism was seen in all drug ratios. The ratio that resulted in maximum synergy was the 1600:1 ratio. Hence this ratio was used to assess the efficacy of the sequential combination therapy.

When thymoquinone was administered first followed by paclitaxel a slight synergistic effect was observed in PA-1 cells. Interestingly, antagonism was seen when paclitaxel was administered first. Sequential administration of thymoquinone followed by paclitaxel seemed to have a most promising effect of the possible combination therapies. When thymoquinone was administered first followed by paclitaxel a synergistic effect was observed in A172 cells. A nearly additive to moderate synergistic effect was observed when paclitaxel was administered first. Sequential administration of thymoquinone first followed by paclitaxel may have a most beneficial effect of the possible combination therapies tested for A172 cells. When thymoquinone was administered first followed by paclitaxel an overall antagonistic effect was observed in H460 cells. Surprisingly, a slight synergistic effect occurred when paclitaxel was administered followed by thymoquinone. Sequential administration of paclitaxel first followed by thymoquinone may have the most beneficial effect of the possible combination therapies tested for H460 cells. These sets of experiments suggest that sequential administration of thymoquinone with paclitaxel seemed more efficacious than concurrent administration.

PA-1 and A172 cells appeared to be the most sensitive to thymoquinone treatment, for this reason the remainder of the studies focus only on these two cell lines. For both cell lines, no significant difference was observed in cell cycle distribution between the

untreated control cells and the thymoquinone treated cells. These cells were also monitored to determine the effect of thymoquinone on apoptotic versus necrotic death. No significant difference was observed in the apoptotic:necrotic ratio between the untreated control cells and the thymoquinone treated cells in these cell lines. Lastly, PA-1 and A172 cells were used to determine if thymoquinone exerts its effects either by being a cytotoxic or a cytostatic agent. It was found that thymoquinone functioned as a cytotoxic agent in both these cell lines.

Interpretations of data

Thymoquinone was most effective in the ovarian cancer cell type with wild-type p53. This result supports previous findings by Shoeib *et al*. 2002, who demonstrated that thymoquinone causes *in vitro* inhibition of growth and induction of apoptosis in cancer cells, with cell cycle arrest occurring at G(1). G(1) arrest is known to be initiated by up regulation of the tumor suppressor gene p53. If thymoquinone initiated its function through this pathway then its cytotoxic properties should be greatly enhanced in cells which have wild-type p53 status.

Thymoquinone was cytotoxic to all cell lines tested in the micro molar range. This result supports the previous findings by Worthen *et al*. 1998 which examined thymoquinone's cytotoxic properties in various human cancers compared to their MDR-variants. This study found that thymoquinone was cytotoxic in both the cell lines tested and their MDR-variants in the micro-molar range.

The increase in p53 levels of the thymoquinone treated cancer cells supports the hypothesis by Shoeib *et al*. 2002., that thymoquinone exerts its effect through a p53 mediated pathway.

Combination drug therapy studies were undertaken to determine how thymoquinone behaves with other cytotoxic agents. Since thymoquinone is believed to work by halting cellular progression at G(1), it was hypothesized that its administration with a cell cycle non-specific agent like cisplatin would result in an additive to synergistic effect because

the two drug would work through different pathways. The concurrent thymoquinone and cisplatin studies however indicated that this combination was antagonistic in all tested cell lines. The exact mechanism by which this effect occurs is not known but it is possible that cisplatin and thymoquinone react with each other to decrease the cytotoxic capacity of each drug alone. When the two agents were given sequentially with thymoquinone first the PA-1 cells exhibited a nearly additive effect and the A172 and H460 cells showed some degree of synergism. When the cells were exposed to cisplatin first, PA-1 and H460 cell lines exhibited a nearly additive effect whereas the effect on A172 cells was antagonistic. These results are in agreement with previous studies done *in vivo* by Badary et al. 1997, which showed that thymoquinone enhanced the activity of cisplatin in mice when given both prior and following cisplatin treatment. The discrepancy in the A172 cell line is not clear, further studies need to be carried out in this particular cell line.

It was hypothesized that administration of thymoquinone with paclitaxel would show antagonism because paclitaxel exerts its effect at M-phase. If thymoquinone arrests cells at G(1) it would prevent the cells from entering the M-phase, thus enabling paclitaxel from exerting its effect. This was demonstrated in the PA-1 and A172 cells at a low drug-drug ratio; however at a 1600:1 ratio in these cells and in all ratios of treatment in H460 cells, synergy was seen. A possible explanation might be that thymoquinone is a cell cycle non-specific antineoplastic agent, which is consistent with our cell cycle studies. However, combinations of cell cycle and non-cell cycle agents are commonly synergistic regardless of sequence. When the two agents were given sequentially with thymoquinone first, the PA-1 and A172 cell lines showed synergy, whereas the effect on H460 cells was antagonism. When paclitaxel was administered first A172 and H460 cells showed moderate to slight synergism while PA-1 cells showed antagonism. While this data does not follow a trend and cannot lead to predictions in other cell lines, it is possible to explain why antagonism or synergy could occur. Antagonism may result from thymoquinone or paclitaxel halting progression of the cell cycle so that the other drug cannot exert its activity at the necessary phase. Synergy could be explained if one drug lines up the cells in one phase so that all of the cells in

the population progress at the same time to the next phase, thus allowing the second drug to kill all of those cells at once. Further experimentation is necessary to characterize the exact effects of the thymoquinone-paclitaxel drug interaction.

A significant difference was not seen in the cell cycle distribution analysis of thymoquinone treated cells compared to the untreated cells (PA-1 and A172). Cell cycle analysis was performed by Shoeib et al. 2002 using thymoquinone in cancer cells. These authors found that thymoquinone arrested all cell types tested at the G(1) phase. The apparent discrepancies in the results seen in this study may be due to the methodology used. Shoeib et al. analyzed the cells at six hours post-treatment, and these cells were treated with extremely high doses of thymoquinone. The study conducted herein used micro molar concentrations and the cell cycle analysis was performed 48 hours post-treatment. While the results obtained by Shoeib *et al*. 2002 are possibly valid, the methodology may not have been the best. It is possible that the cellular distribution seen at six hours in their study may be the result of cellular shock and not due to thymoquinone; cells need some time to recovery from cellular shock before an appropriate analysis can be undertaken. In addition the drug concentrations which they chose to expose the cells to were also very high and not likely achievable in human subjects.

A significant difference was not seen in the apoptotic to necrotic ratio. While these results are not conclusive, possible explanations may be that the drug concentration added was either too high which caused 100% cell death which would not allow for the apoptosis analysis or that the concentration added was too low to have an effect on the cell viability and apoptosis. It was very difficult to find a median concentration due to a threshold effect. The last study set of experiments performed demonstrated a trend which, indicates that thymoquinone functions as a cytotoxic agent. This may be explained by the increase in p53 which is known to lead to apoptosis.

The studies reported in this thesis support the hypothesis that thymoquinone is indeed a cytotoxic agent, which upregulates p53. However, the results gathered in this thesis

suggest that this may not be its only mechanism of cytotoxicity. Combination drug therapy with thymoquinone seems to be an attractive option, but conflicting results with different drugs and the variability between cell lines seem to suggest that this may not be a viable alternative. More studies need to be performed with thymoquinone to assess the true chemotherapeutic potential of this amazing phytochemical agent.

Bibliography

1. Abdel-Salam, I., Abdel-Wahab, S., l-Aaser, A., and El-Merzabani, M. (1992) Biochemical and cytotoxic effects of *Nigella sativa L*. *The Egyptian Journal of Biochemistry* 12(2): 348-355.
2. Abou-Basha, L., Rashed, M., and Aboul-Enein, H. (1995) TLC assay of thymoquinone in black seed oil (*Nigella sativa Linn*.) and identification of dithymoquinone and thymol. *Journal of Liquid Chromatography* 18(1): 105-115.
3. Aboul-Enein, H., and Abou-Basha, L. (1995) Simple HPLC method for the determination of thymoquinone in black seed oil (*Nigella sativa Linn.*) *Journal of Liquid Chromatography* 18(5): 895-902.
4. Attardi L., Lowe S., Brugarolas J, and Jacks T. (1996) Transcriptional activation by p53, but not induction of the p21 gene, is essential for oncogene-mediated apoptosis. *EMBO J.* 15(14): 3693-701.
5. Atta-ur-Rahman, Malik, S., Cun-heng, H., and Clardy, J. (1985 a) Isolation and structure determination of Nigellicine, a novel alkaloid from the seeds of *Nigella sativa*. *Tetrahedron Letters* 26(23): 2759-2762.
6. Badary, O. (1999) Thymoquinone attenuates ifosfamide-induced fanconi syndrome in rats and enhances its antitumor Activity in Mice. *Journal of Ethnopharmacology* 67: 135-142.
7. Badary, O., Al-Shabanah, O., Nagi, M. Al-Bekairi, A. and Elmazar, M. (1998) Inhibition of benzo (a) pyrene-induced forestomach carcinogenisis in mice by thymoquinone. *European Journal of Cancer Prevention* 8: 435-440.
8. Badary, O. and El-Din, A. (2001) Inhibitory effects of thymoquinone against 20-Methylcholanthrene-induced fibrosarcoma tumorogenesis. *Cancer Detection and Prevention* 25(4): 362-368.
9. Banin, S., Moyal, L., and Shieh, S. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 281: 1674-1677.
10. Bannach, P., Kanduc, D., Papa, S., and Tager, J. (eds.) (1998) *Cell Growth and Oncogenesis.* Molecular and Cell Biology Updates, Boston, USA.

11. Bence, A., Adams. V, and Crooks, P. (2003) L-Canavanine as a radiosensitization agent for human pancreatic cancer cells. *Molecular and Cellular Biochemistry* 244:73-43.

12. Bennett, M., MacDonald, K., and Chan, S. (1998) Cell surface trafficking of FAS: a rapid mechanism of p53-mediated apoptosis. *Science* 282: 290-293.

13. Blagosklonny, M. (ed.) (2001) *Cell Cycle Checkpoints and Cancer.* Landes Bioscience, Texas, USA.

14. Blow, J. (1989) DNA replication and its control. *Current Opin. Cell Biol*. 1:263-267.

15. Brehm, A., Miska, E., and McCance, D. (1998) Retinoblstoma protein recruits histone deacteylase to repress transcription. *Nature* 391:597-601.

16. Chao C., Saito S., Kang J., Anderson C., Appella E., and Xu Y. (2000) p53 transcriptional activity is essential for p53-dependent apoptosis following DNA damage. *EMBO J.* 19(18):4967-75.

17. Choi J., and Donehower L. (1999) p53 in embryonic development: maintaining a fine balance. *Cell Mol Life Sci.* 55(1): 38-47.

18. Chou, T. (1976) Derivation and properties of Michaelis-Menten type and Hill type equations for reference ligands. *J Theor Biol*. 59(2):253-76.

19. Chou, T.C., Hayball, M.P. (1996) Calcusyn Manual, Biosoft, U.K.

20. Chou, T. and Talalay, (1984) P. Quantitative Analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* 22:27-55.

21. Cross M, and Dexter M. (1991) Growth factors in development, transformation, and tumorogenesis. *Cell* 64(2):271-80.

22. Davis, P., Cullen, J., and Coode, M. (1965) *Flora of Turkey*, vol. 1 Univ. Press, Edinburgh.

23. Dhar, M. L., Dhar, M. M., Dhawan, B., Mehrotra, B., and Ray, C. (1968) Screening of Indian plants for biological activity: Part 1. *Indian Journal of Experimental Biology* 6: 232-247.

24. El-Dakhakhny, M. (1963) Studies on the chemical constitution of Egyptian *Nigella sativa L.* seeds II. The Essential Oil. *Planta Medica* 11: 465-470.

25. El-Sayed, M., El-Banna, H., and Fathy, F. (1994) The use of *Nigella sativa* oil as a natural preservative agent in processed cheese spread. *Egyptian Journal of Food Science* 22(3): 381-396.

26. Fuchs S., Adler V., Buschmann T., Wu X., and Ronai Z. (1998) Mdm2 association with p53 targets its ubiquitination. *Oncogene* 17(19): 2543-7.

27. Ghosheh, O., Houdi, A., and Crooks, P. (1999) High Performance Liquid Chromatographic Analysis of the Pharmacologically Active Quinones and Related Compounds in the Oil of the Black Seed (*Nigella sativa L*.). *J. Biomedical and Pharmaceutical Analysis* 19: 757-762.

28. Giannakakou, P. Robey, R., Fojo, T., and Blagosklonny, M. (2001) Low concentrations of induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of -induced cytotoxicity. Oncogene 20:3806-3813

29. Hahn W. (2002) Immortalization and transformation of human cells. *Mol Cells*. 13(3):351-61.

30. Hailat, N., Bataineh, Z., Lafi, S., Raweily, E., Aqel, M., Al-Katib, M., and Hanash, S. (1995) Effect of *Nigella sativa* volatile oil on Jurkat T cell Leukemia Polypeptides. *International Journal of Pharmacognosy* 33(1): 16-20.

31. Harley, C. (2002) Telomerase is not an oncogenes. *Oncogene* 21(4):494-502.

32. Hartwell, L. and Weinert, T. (1989) Checkpoints: controls that insure the order of cell cycle events. *Science* 246: 629-634.

33. Haupt Y., Maya R., Kazaz A., and Oren M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature* 387(6630): 296-9.

34. Hermeking H., Lengauer C., Polyak K., He T., Zhang L., Thiagalingam S., Kinzler K., and Vogelstein B. (1997)14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell.* 1(1): 3-11.

35. Houghton, P., Zarka, R., Heras, B., and Hoult, J. (1995) Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosaniod generation in leukocytes and membrane lipid peroxidation. *Planta Medica* 61: 33-36.

36. Hukley, A. (1992) *The New Royal Horticultural Society, Dictionary of Gardening,* Vol. 3. Macmillan Press, London. p. 321-323.

37. Jacobson, M., Burnej, J., King, M., Miyashita, T., Reed, J., and Raff, M. (1993) BCL-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature* 361: 365-369.

38. Khan, M. (1976) A translation of *Al-Bukhari.* (Collection of Authentic Prophet saying), Division 71 (the book of Medicine), chapter 7. Publish. Hilal Yayinlari. Ankara, Turkey 2nd ed.

39. Khan, M. (1999) Chemical composition and medicinal properties of *Nigella sativa* Linn. *Inflammopharmacology 7*(1): 15-35.

40. Kumar, B. and Thakur, S. (1989) Effect of certain non-edible seed oil on the growth of *Dysdercus similis* (F.). Journal of Animal Morphology and Physiology 36(2): 209-217.

41. Levine, A. (1997) p53, the cellular gatekeeper for growth and division. Cell 88: 323-331.

42. Linzer, D., and Levine, A. (1979) Characterization of a 54kD cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* 17:43-52.

43. Mabrouk, G., Moselhy, S., Zohny, S. Ali, E., Helal, T., Amin, A., and Khalifa, A. (2002) Inhibition of Methylnitrosourea (MNU) induced oxidative stress and carcinogenisis by orally administered bee Honey and *Nigella* grains in Sprague Dawley rats. *J. Exp. Clinical Cancer Research* 21: 341-346.

44. Mahfouz, M., and El-Dakhakhny, M. (1960a) The isolation of a crystalline active principle from *Nigella sativa L*. seeds*. Journal of Pharmaceutical Sciences of United Arab Republic* 1: 9-19.

45. Medenica, R., Janssens, J., Tarasenko A., Lazovic, G., Corbitt,W., Powell, D., Jocic, D., and Mujovic, V. (1997) Anti-angiogenic activity of *Nigella sativa* plant extract in cancer therapy. *Proceeding of the American Association for Cancer Research Annual Meeting* 38: A1377.

46. Meek DW. (2000)The role of p53 in the response to mitotic spindle damage. *Pathol Biol (Paris).* 48(3): 246-54.

47. Merlin, J. (1994) Concepts of Synergism and Antagonism. Anticancer Research 14: 2315-2320.

48. Miyashita T., and Reed J. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 80(2): 293-9.

49. Momand J. and Zambetti G. (1997) Mdm-2: "big brother" of p53. *J Cell Biochem* 64(3): 343-52.

50. Momand J., Wu H., and Dasgupta G. (2000) MDM2--master regulator of the p53 tumor suppressor protein. *Gene* 242(1-2): 15-29.

51. Nair, S. , Salomi, M. , Panikkar, B, and Panikkar, K. (1991) Modulatory effects of Crocus sativas and *Nigella sativa* extracts on cisplatin induced toxicity in mice. *Journal of Ethnopharmacology* 31(1): 75-83.

52. Nevins, J. (1998) Towards an understanding of the functional complexity of the E2F and retinoblastoma families. *Cell Growth Diff.* 9:585-593.

53. Oliner J., Pietenpol J., Thiagalingam S., Gyuris J., Kinzler K., and Vogelstein B. (1993) Oncoprotein MDM2 conceals the activation domain of tumor suppressor p53. *Nature* 362(6423): 857-60.

54. Pardee, A. (1974) A restriction point control of normal animal cell proliferation. *Proc Natl Acad Sci USA* 71:12861290.

55. Pusztai, L., Lewis, C., and Yap, E. (eds.) (1996) *Cell Proliferation in Cancer.* Oxford Press. Oxford.

56. Rechinger, K. (ed.) (1964) *Flora of lowland Iraq.* Cramer, Weinheim

57. Rosenberg, S. (2001) *Cancer Principles and Practice of Oncology, 6th edition.* Lippincott, Williams, and Wilkins, Philadelphia.

58. Salomi, N., Nair, S., and Panikkar, K. (1991) Inhibitory effects of *Nigella sativa* and Saffron (*Crocus sativas*) on chemical carcinogenesis in mice. *Nutrition and Cancer* 16(1): 67-72.

59. Salomi, N., Nair, S., Jayawardhanan, K., Vorghese, C., and Panikkar, K. (1992) Antitumor principles from *Nigella sativa* seeds. *Cancer Letters* 63(1): 41-46.

60. Schweig, T. (1999*) Nigella sativa* seeds of current interest again. *Pharm Ztg* 144 (33):2582-2587

61. Shoieb, A., Elgayyan, M., Dudrich, P., Bell, J., and Tithof, P. (2002) *In vitro* inhibition of growth and induction of apoptosis in cancer cell lines by thymoquinone. *International Journal of Oncology* 22:107-113.

62. Soussi, T. (2000) The p53 tumor-supressor gene: from molecular biology to clinical investigation. *Ann. N.Y. Acad. Sci.* 1910:221-237.

63. Tutin, T. , Heywood, V. , Burges, N. , Valentine, D. , Walters, S. , and Webb, D. (eds) (1964) *Flora europaea* vol. 1 Univ. Press, Cambridge

64. Walker K. and Levine AJ. (1996) Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc Natl Acad Sci U S A*. 93(26):15335-40.

65. Worthen, D., Ghosheh, O., and Crooks, P. (1997) An *in vitro* evaluation of the antineoplastic activity of some crude and pure constituents of black seed, *Nigella sativa. Pharmaceutical Research (Supplement*) 14(11): S-386 (2527).

66. Worthen, D., Ghosheh, O., and Crooks, P. (1998) The *in vitro* Anti-tumor Activity of some crude and purified components of black seed, *Nigella sativa L.. Anticancer Research* 18: 1527-1532.

67. Zhan Q., Fan S., Bae I., Guillouf C., Liebermann D., O'Connor P., and Fornace A. Jr. (1994) Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. *Oncogene* (12): 3743-51.

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