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The Use of DNA Microarrays to Compare the Gene Expression Profiles of Paclitaxel and Acetic Acid 2,2'-thiobis, bis [[(2-hydroxyphynyl) methylene]hydrazid] in A549 Cells: Part 1

Jennifer Diane Ellzey

THE USE OF DNA MICROARRAYS TO COMPARE THE GENE EXPRESSION PROFILES OF PACLITAXEL AND ACETIC ACID 2,2'-THIOBIS-, BIS [(METHYLENE SUBSTITUTED)]HYDRAZIDE] IN A549 CELLS: PART I

by
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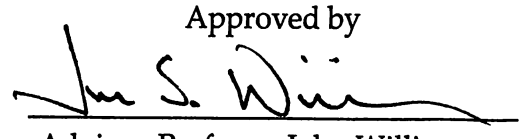
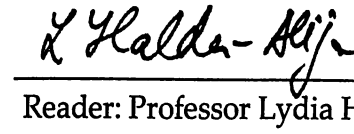
Approved by



Advisor: Professor John Williamson



Reader: Professor Lydia Halda-Alija

Reader: Dean Barbara Wells

To my parents, whose constant support of my pursuits has always made me feel that anything is possible, to my teachers, whose mentorship has meant the world to me, to my friends, whose kind encouragement and just plain craziness has kept me sane, and to my sovereign Lord, without whose divine guidance and unwavering comfort I would have given up on this thing a long time ago.

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I would like to thank Dr. Donald Sittman at University of Mississippi Medical Center and the members of his research group for allowing me to visit and observe their lab and for generously giving me their protocols. Thanks also to Nick Hammond for his cell culture expertise and endless patience.

ABSTRACT

JENNIFER DIANE ELLZEY: The Use of DNA Microarrays to Compare the Gene Expression Profiles of Paclitaxel and Acetic Acid 2,2'-thiobis-, bis [(methylene substituted)hydrazide] in A549 Cells: Part I
(Under the direction of Dr. John Williamson)

There are several classes of anticancer drugs, including the antimicrotubule drugs, which halt mitosis by interfering with the activity of microtubules, the cylindrical protein polymers that play a role in segregating chromosomes during mitosis. While most antimicrotubule drugs act by destabilizing the microtubule polymer and reducing microtubule assembly, paclitaxel, a drug first isolated from the bark of *Taxus brevefolia*, halts mitosis by stabilizing microtubules, thus interfering with the dynamics of polymerization and depolymerization that are crucial to their function. In light of the mounting resistance to paclitaxel, it is more important than ever to discover new drugs with a similar mechanism of action. Our research group has discovered a novel compound, acetic acid 2,2'-thiobis-, bis [(methylene substituted)hydrazide], that has the same effect on tubulin as paclitaxel. The purpose of this study was to use DNA microarrays to compare the gene expression profile of paclitaxel and our lab's new compound in A549 non-small cell lung cancer cells. DNA microarrays are small glass slides or silicon chips whose ordered arrays of thousands of DNA sequences hybridize with fluorescently labeled messenger RNA probes. A laser excites the fluorescent labels, and the relative gene expression of each gene is measured as the fluorescent intensity of

each spot. However, microarray work is very complex and requires a considerable amount and variety of equipment and materials. Since our lab did not have the necessary materials or any background knowledge, extensive preparation of the lab was necessary to initiate a project of this magnitude. The study was divided into Part I, the preparatory steps, and Part II, the experimental steps. I was responsible for Part I and thus reviewed literature, learned techniques, adapted cell culture and RNA extraction protocols, purchased materials, set up the laboratory, and designed a basic scheme for the experiment. These steps required extensive research and took two-and-a-half years to complete. As the preparatory work took longer than expected, we are just now nearing the start of the experimental phase of the project. We intend to conduct the study as follows. A549 cells will be cultured and treated with varying concentrations of paclitaxel and acetic acid 2,2'-thiobis-, bis [(methylene substituted)hydrazide], RNA will be extracted, labeled, and hybridized to microarrays, the microarrays will be scanned, and the gene expression profiles induced by each drug will be measured. We hope that our study will help advance the use of microarrays as a tool for drug screening that will accelerate the pace of anticancer drug discovery and development.

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CHAPTER 1: INTRODUCTION

Cancer Chemotherapy

While cancer chemotherapy in the form of herbal treatments has been used since ancient times, only recently has cancer research been a priority in the scientific community (Baguley, 2002). It was only after the rapid development of antibiotics during the so-called "antibiotic era" drastically reduced infections disease deaths that cancer became a more prominent killer than bacterial infections. This change along with the first reports of practical human cancer chemotherapeutic agents—those of Karnofsky and others on nitrogen mustards against lymphoma in 1948, Farber and others on the folic acid analog aminopterin against leukemia in 1948, and Burchenal and others on 6-mercaptopurine against leukemias in 1953—brought cancer to the forefront of biomedical research (reviewed by Baguley, 2002; Suffness and Wall, 1995). At that time, the main cancer drug discovery and development program was the Sloan-Kettering Institute for Cancer Research, which evaluated 75% of the compounds submitted as possible anticancer agents in the United States. However, this and the few other cancer research institutions in existence were not sufficient to evaluate the anticancer activity of the plethora of compounds that various researchers wanted to have tested. In response to pressure from researchers around the country, Congress instructed the National Cancer Institute (NCI) to establish a national cancer drug-screening program in 1955. In

1956, the NCI fulfilled the request of Congress by establishing the Cancer Chemotherapy National Service Center, whose purposes were to support drug discovery and cancer treatment research and to test both synthetic compounds and natural products, particularly plant extracts, for anticancer activity (Suffness and Wall, 1995).

Though cancer research has not been a high priority for very long, the diligent efforts of thousands of scientists have resulted in the discovery of several classes of anticancer drugs, which include the DNA-reactive drugs, such as the DNA platination-inducing cisplatin; inhibitors of DNA replication, including the thymine analog 5-fluorouracil and the purine analog 6-mercaptopurine; and topoisomerase inhibitors such as camptothecin and etoposide; among many others (Baguley, 2002). The compounds that are the subject of this study belong to the mitotic poisons, or drugs that halt mitosis by interfering with the activity of microtubules, whose function in the cell is discussed below.

Microtubules and Antimicrotubule Drugs

Microtubules are long, hollow cylindrically-shaped structures composed mainly of tubulin, a heterodimeric protein consisting of two polypeptide subunits, α and β (Alberts et al., 2002). Connected intimately to the tubulin subunits are microtubule-associated proteins (MAPs), proteins that enhance microtubule assembly (Manfredi and Horwitz, 1984). Microtubules are a major component of spindle fibers and thus play a crucial role in segregating chromosomes during mitosis (Ringel, 1995). They also are a key component of the cytoskeleton, participating in a variety of cellular functions,

including morphogenesis, motility, intracellular organelle transport, and anchorage of surface receptors to the plasma membrane (Abal, Andreu, and Barasoain, 2003; Manfredi and Horwitz, 1984).

Assembly of microtubules generally emanates from a single microtubule-organizing center (MTOC), which in animal cells consists of a single pair of centrioles (Manfredi and Horwitz, 1984). In addition to this so-called primary initiation site, there are two other classes of microtubule initiation sites: secondary sites, which are complex perinuclear sites other than the centriole from which emanate multiple microtubules, and tertiary sites, which produce single microtubules (Spiegelman, Lopata, and Kirschner, 1979). There are three phases of microtubule assembly: nucleation, elongation, and steady-state. During the nucleation phase, a small aggregate of tubulin called a nucleus is formed. As no microtubules are observed during this phase, it is often called the lag phase (Alberts et al., 2002). During the elongation or growth phase, tubulin monomers add at a rapid rate to both ends of the nucleated filaments. Polymerization at the microtubule ends occurs at different rates; the fast-growing end is called the plus end, while the slow-growing end is called the minus end (Dumontet and Sikic, 1999). During elongation, tubulin subunits assemble in a head-to-tail fashion to form long protofilaments, and 13 of these protofilaments assemble laterally to form the cylindrical microtubule (Orr et al., 2003). This process is GTP-dependent; as each tubulin subunit is added to the growing polymer, a GTP molecule bound to an exchangeable site on β-tubulin is hydrolyzed to a tightly bound GDP (Weisenberg, 1980). Elongation continues until the addition of tubulin subunits is equal to the rate of

subunit dissociation. At this point, microtubule assembly and disassembly has reached equilibrium, and the reaction is said to be in the steady-state or equilibrium phase (Alberts et al., 2002). However, there is still a net growing at the plus end of the microtubule and a net shortening at the minus end; this phenomenon, called treadmilling, is vital to many cellular processes involving microtubule dynamics, most notably the polar movement of chromosomes during metaphase (Dumontet and Sikic, 1999). The concentration of tubulin dimers at equilibrium, or the critical concentration, corresponds to the stability of the polymer, with a lower critical concentration denoting a more stable polymer (Ringel, 1995).

It is this process of tubulin assembly to form microtubules that is affected by antimicrotubule drugs. Most antimicrotubule drugs act by destabilizing the microtubule polymer and reducing microtubule assembly, thus inhibiting formation of the mitotic spindle. Drugs in this class include the vinca alkaloids, colchicine, podophyllotoxin, and nocodazole (Ringel, 1995). On the other hand, Taxol® (paclitaxel, Bristol-Myers Squibb), one of the compounds considered in this study, binds to the microtubule polymer and promotes microtubule assembly (Schiff, Fant, and Horwitz, 1979), thus interfering with the microtubule dynamics of polymerization and depolymerization that are crucial to their function.

Paclitaxel

History

Paclitaxel (see Figure 1.1 for structure) is a relatively new chemotherapeutic agent whose discovery would not have been possible without the collaboration formed in 1960 between the United States Department of Agriculture (USDA) and the NCI, by which USDA botanists collected diverse and unique plant samples for evaluation by NCI researchers. In 1962, Arthur Barclay of the USDA collection team collected samples of the Pacific yew, *Taxus brevifolia*, from the Gifford Pinchot National Forest in Washington State (Suffness and Wall, 1995). In April 1964, extracts from *T. brevifolia* were found to be cytotoxic against KB cancer cells by researchers in Bethesda, Maryland, a finding confirmed two months later at the University of Miami. Afterward, various extracts from *T. brevifolia* bark were found to be active *in vivo* against the L1210 leukemia, the P-1534 leukemia, the Walker 256 carcinosarcoma, and the P388 leukemia (Suffness and Wall, 1995).

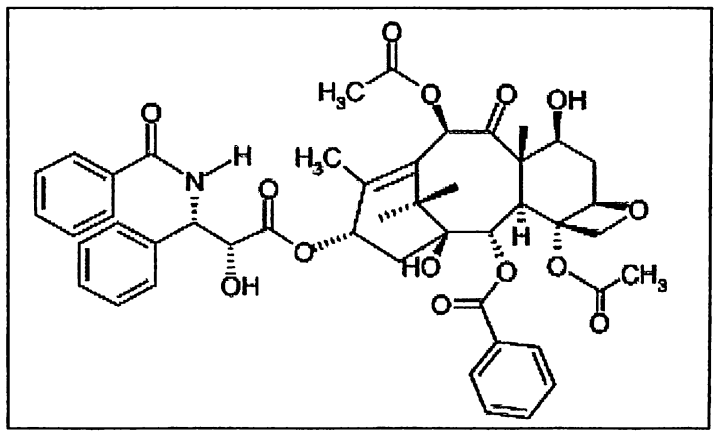

Figure 1.1. Structure of paclitaxel.

Paclitaxel in its pure form was first isolated from the bark of *T. brevefolia* by Wani and others (1971). However, the *in vivo* results with L1210 leukemia, P388 leukemia, P1534 leukemia, and Walker 256 carcinosarcoma in mice were fairly unremarkable; though paclitaxel did show limited activity, it failed to reproducibly meet the criteria to be considered a candidate for drug development by the NCI. Luckily, the B16 melanoma was introduced as a model for solid tumors during the 1970's. In 1974, Taxol was tested against the B16 melanoma and showed an optimal percent increase in lifespan (ILS) of 126%, which far exceeded the ILS of 50% required by the NCI for consideration for drug development. After this data was shown to be reproducible in 1975 by an experiment giving an ILS of 86% in the B16 melanoma, the NCI finally approved Taxol as a candidate for development in 1977 (Suffness and Wall, 1995).

Due to its low aqueous solubility and the ecological concerns associated with its isolation from a threatened species, paclitaxel was difficult and expensive to develop as a drug, (Kingston, 1995). Despite these problems, development of paclitaxel continued at a rapid rate, largely due to interest in the compound's novel mechanism of action, the promotion of the assembly of tubulin into microtubules, which was discovered by Dr. Susan Horwitz in 1979 (Schiff, Fant, and Horwitz, 1979). Preclinical trials of the drug began in 1978, phase I clinical trials begin in 1983, and phase II trials began in 1985 (Kingston, 1995). In 1989, the first phase II results were published, showing a promising 30% response rate in advanced ovarian cancer (McGuire et al., 1989). Just two years later, Holmes and collaborators discovered that paclitaxel had outstanding activity in primary metastatic breast cancer as well, with a response rate of 56% in their phase II

study (1991). Since then, paclitaxel has also been shown to have activity against non-small cell lung cancer (Chang et al., 1993), small cell lung cancer (Ettinger et al, 1993; Jett et al., 1995), squamous cell carcinoma of the head and neck (Forastiere et al., 1998), urothelial cancer (Roth et al., 1994), germ cell tumors (Motzer et al., 1994), esophageal cancer (Kelsen et al., 1994), testicular cancer (Bokemeyer et al., 1996), HIV-associated Kaposi's sarcoma (Welles et al., 1996), and non-Hodgkins lymphoma (Younes et al., 1997). Paclitaxel is currently approved by the Food and Drug Administration for treatment of advanced ovarian carcinoma, metastatic breast cancer, non-small cell lung cancer in combination with cisplatin, and AIDS-related Kaposi's sarcoma (*Drug Facts and Comparisons*, 2003).

Isolation and Synthesis

There are various methods used to isolate or synthesize paclitaxel for mass production. Though indeed groundbreaking, Wani's original isolation from *T. brevefolia* bark has proven to be an impractical long-term means for large-scale paclitaxel production. Not only did it produce only a 0.02% yield (Wani et al., 1971), but isolation from *T. brevefolia* bark is further problematic because it requires the destruction of a small, slow-growing, ecologically threatened tree (Kingston, 1995). However, paclitaxel has also been successfully isolated from the needles of *T. brevifolia* and other *Taxus* species. As this method does not require the destruction of the tree, it is more ecologically sound; however, the isolation from needles is more complicated than the isolation from bark, due to the abundance of lipid material in the needles (Kingston,

1995). Furthermore, this method also produces a low yield—0.025% on average—which is comparable to that from bark (reviewed by Kingston, 1995).

Paclitaxel can also be produced in cell and tissue cultures of *Taxus* species. The first published article in this area was in 1989 by Christen, Bland, and Gibson (reviewed by Kingston, 1995), and several other successes have been reported since then with cultures of *T. baccata, T. media, T. wallichiana,* and *T. cuspidata,* among others (Cusidó et al., 2002; Navia-Osorio et al., 2002; Aoyagi, DiCosmo, and Tanaka, 2002). Plant culture as a means to produce paclitaxel is a technique still under investigation. In January of 2004, Bai and others published an article reporting their success in producing not only paclitaxel but also fourteen other biologically active taxoids with a culture of *T. cuspidata* (Bai et al., 2004).

Another viable method of taxol production is via endophytic fungi of *Taxus* species. The first published report in this area was in 1993 with *Taxomyces andreanae,* a fungus growing on *T. brevifolia* (Stierle, Strobel, and Stierle, 1993), with subsequent successes with endophytic fungi of the Himalayan yew, *T. wallachiana* (Strobel et al., 1996; Shrestha et al., 2001). Remarkably, fungi unconnected to *Taxus* have also been found to produce paclitaxel, including an endophytic fungus of the bald cypress, *Taxodium distichum* (Li et al., 1996), and an epiphytic fungus of *Maguireothamnus speciosus,* a rubiaceous plant widespread in southeastern Venezuela (Strobel et al., 1999).

In addition to isolation and biosynthetic production, the partial or total synthesis of paclitaxel in the laboratory has been explored as another means to produce the drug for commercial use. The total synthesis of paclitaxel was first published in 1994 by two

different research groups, Holton and others (reviewed by Kingston, 1995), and by Nicolaou and others (1994). Due to the cost and inefficiency of total synthesis as compared to other methods, however, this method is not a feasible method to produce paclitaxel on a large scale, and few attempts at total synthesis have been made since the original studies. Nevertheless, the original syntheses are invaluable to paclitaxel research because they have opened the door to the preparation of paclitaxel analogs and may thus accelerate the development of versions of the drug that are more effective or less toxic (Kingston, 1995).

While total synthesis is currently not a viable option for the mass production of paclitaxel, the semisynthesis of paclitaxel, or its synthesis from a simpler and more readily available related compound, is quite feasible. The most widely used starting compound for the semisynthesis of paclitaxel is 10-deacetylbaccatin III (10-DAB). Likely the most readily available taxoid (Kingston, 1995), 10-DAB has been isolated from the needles of *Taxus* species (reviewed by Kingston, 1995) and cultures of various *Taxus* species (Navia-Osorio et al., 2002; Cusidó et al., 2002; Bai et al., 2004), with far higher yields than paclitaxel. Furthermore, 10-DAB can be derived from a larger diversity of *Taxus* species than can Taxol (Croom, 1995). The semisynthesis of taxol from 10-DAB was first published in 1988 by Denis and colleagues (reviewed by Kingston, 1995) and remains the most efficient means of paclitaxel production, currently used by Bristol-Myers Squibb to produce all its paclitaxel (Kingston, 1995). Not only is the semisynthesis of paclitaxel a preferred method due to the high availability of 10-DAB, but it is also quite attractive to researchers because it makes possible the creation of

Taxol analogues unobtainable from natural sources, some of which could be more effective than paclitaxel itself (Croom, 1995).

In addition to isolating and synthesizing paclitaxel, a prodrug approach is also an appealing option due to the drug's low aqueous solubility. Many researchers have attempted to synthesize water-soluble paclitaxel prodrugs, or analogs that are converted into active paclitaxel upon administration in the body, and several of these compounds have shown greater activity than paclitaxel in various types of cancer (Kingston, 1995).

Structure and Activity

Paclitaxel is classified among the taxoids, a subset of the diterpenoids containing the taxane ring system, or tricyclo[9.3.1.0.^{3,8}]pentadecane (Kingston, 1995). The unique biological activity of paclitaxel and various analogs in comparison to the numerous other taxoids has been shown to be related to distinctive two functional groups: a C-13 *N*-benzophenylisoserine ester group and a C-4,5 oxetane ring. Studies indicate that the southern hemisphere of paclitaxel is crucial to its cytotoxicity, while the northern hemisphere is not closely associated with its biological activity (see Figure 1.2). Various modifications of the structure along the southern hemisphere, particularly opening of the oxetane ring and removal of the C-2 benzoyl group, result in a dramatic reduction in or total loss of cytotoxicity and tubulin-assembly activity, whereas structural changes along the northern hemisphere have little or no effect on cytotoxicity or tubulin assembly (Kingston, 1995).

Paclitaxel's unique structure confers upon the compound an equally unique mechanism of action. Paclitaxel binds to tubulin only in the microtubule polymer,

preferentially to the β subunit (Ringel, 1995). Based on a study on the conformation of docetaxel (Taxotere), a paclitaxel analog, it is likely that during the binding of paclitaxel and its analogs to microtubules, the southern hemisphere of the taxane ring system is first recognized, followed by interaction of the ester side chain with tubulin to form a stable drug-receptor complex (reviewed by Kingston, 1995).

The binding of paclitaxel to microtubules promotes tubulin assembly into microtubules, even under conditions under which microtubule polymerization is impossible, including the absence of microtubule-associated proteins and GTP (Schiff and Horwitz, 1981), a low temperature of 0°C (Thompson, Wilson, and Purich, 1981), and mild alkaline pH of ≥7.5 (Ringel and Horwitz, 1991). The drug also stabilizes microtubules against depolymerization, retarding the release of tubulin dimers from microtubule polymers in a concentration-dependent manner (Wilson et al., 1985). Microtubules polymerized in the presence of paclitaxel resist depolymerization by various treatments, including dilution, cold (4°C), calcium chloride, and antimitotic drugs such as colchicine (Schiff and Horwitz, 1981). The extreme stability afforded by paclitaxel is evident in that administration of the drug in concentrations of 5-10 μM can reduce the critical concentration of tubulin from 0.2 mg/mL to 0.015 mg/mL or less (Schiff and Horwitz, 1981; Orr et al., 2003). Consequently, administration of the drug shifts the equilibrium towards polymerization (Schiff, Fant, and Horwitz, 1979) and thus induces further microtubule assembly when added to a microtubule preparation at steady state (see Figure 1.3). Stabilization of microtubules leads to a decrease in free tubulin subunits in the cell, which in turn results in the up-regulation of tubulin

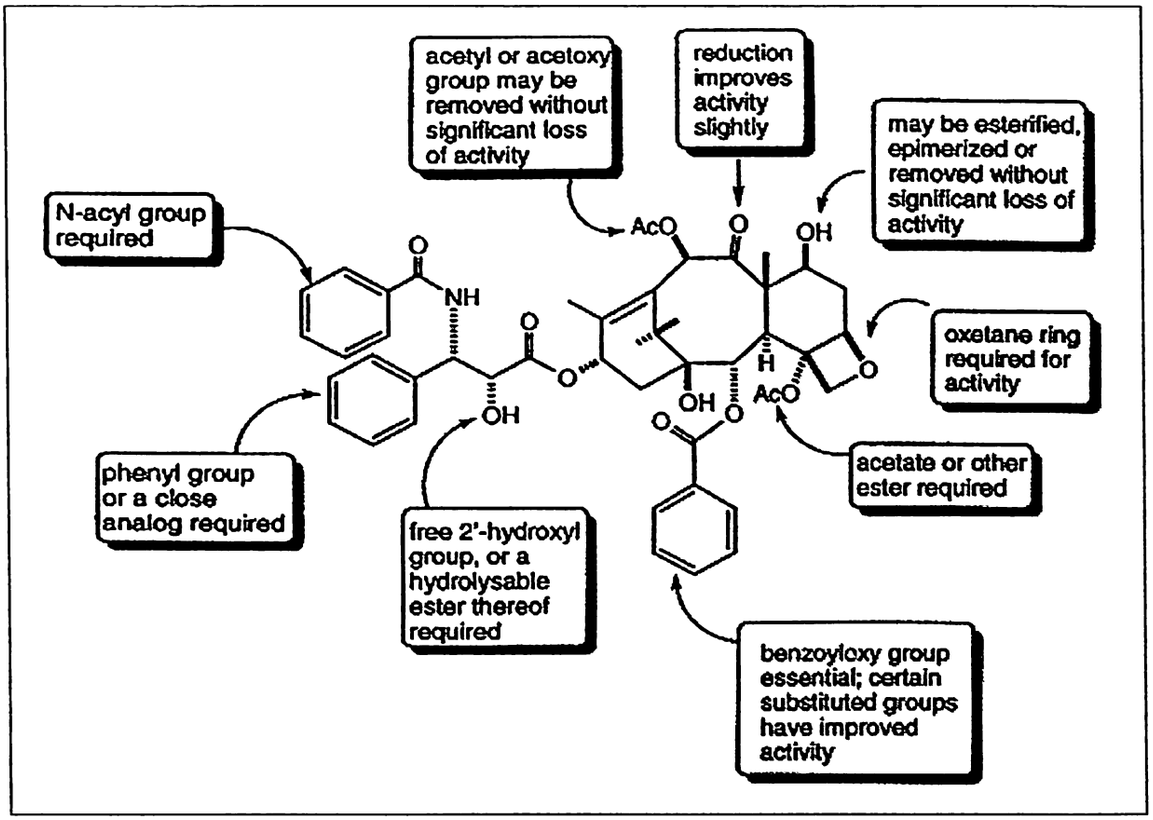

Figure 1.2. Summary of known structure-activity relationships of paclitaxel.
Note that many of the groups on the southern hemisphere are required for activity, whereas substitutions on the northern hemisphere generally do not result in a loss of activity.
Source: Kingston, D.G.I. 1995. History and chemistry. *In* Paclitaxel in Cancer Treatment. W.P. McGuire and E.K. Rowinsky, editors. Marcel Dekker, New York. 1-33.

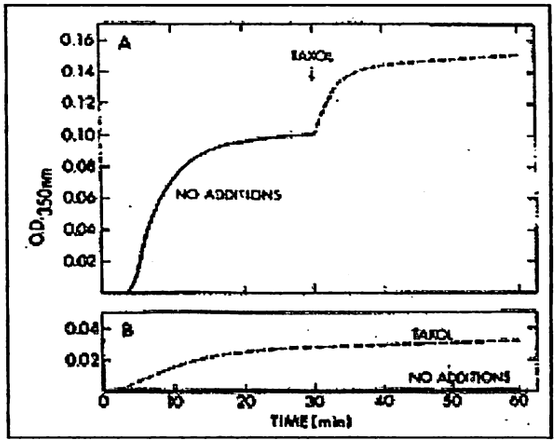

Figure 1.3. Addition of paclitaxel to microtubules at steady-state equilibrium results in increased microtubule assembly.
Optical density at 350 nm is proportional to microtubule polymer density.
A) Tubulin was assembled in the absence of paclitaxel. At 30 minutes, 5 μM paclitaxel was added.
B) Tubulin was assembled in the absence of paclitaxel. Paclitaxel was not added.
Source: Schiff, P.B., and S.B. Horwitz. 1981. Taxol assembles tubulin in the absence of exogenous guanosine 5'-triphosphate or microtubule-associated proteins. *Biochemistry*. 20:3247-3252.

synthesis, further promoting microtubule assembly via a concentration effect. It has been found that paclitaxel increases the rate and yield of microtubule polymerization maximally when the ratio of the concentration of the drug to the concentration of tubulin dimer is one (Kumar, 1981; Schiff and Horwitz, 1981).

The binding of paclitaxel to microtubules and resulting disturbance of the microtubule dynamics described above interferes with mitosis, causing cell death, or apoptosis. However, studies have shown that the mechanism of paclitaxel-induced cell death is concentration-dependent (see Figure 1.4). At low concentrations of paclitaxel, mitosis is not fully blocked; bipolar spindles are formed but do not segregate properly, leading to mitotic slippage, or the exit from mitosis without cell division, and subsequent apoptosis of the multinucleated state (Abal, Andreu, and Barasoain, 2003). High concentrations of paclitaxel, on the other hand, result in complete mitotic block at the metaphase-anaphase boundary resulting in apoptosis (Torres and Horwitz, 1998). Furthermore, the apoptotic mechanisms triggered by low and high concentrations of paclitaxel are different (Torres and Horwitz, 1998; Chen et al., 2003).

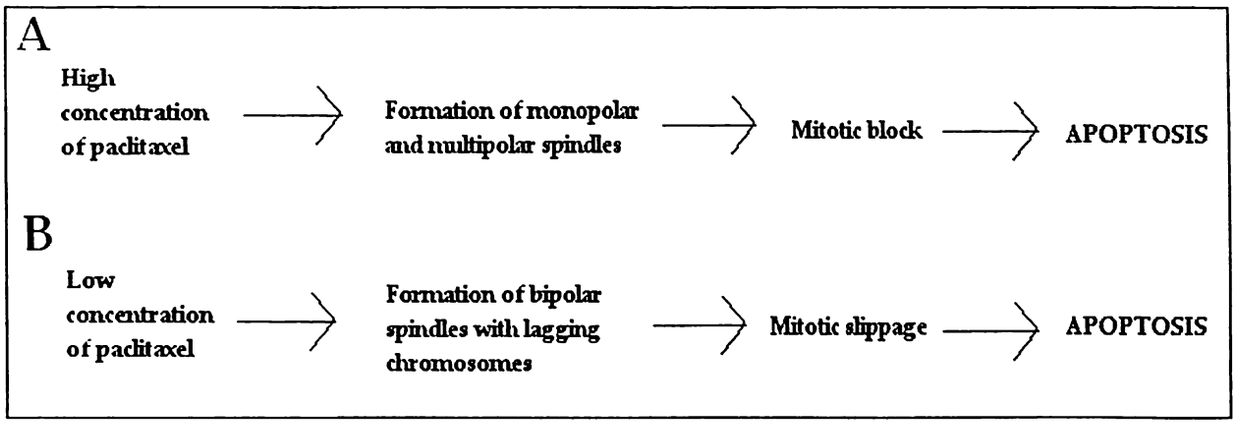

Figure 1.4. Concentration-dependent mechanism of action of paclitaxel.

A) At high concentrations, paclitaxel leads to the formation of multipolar and monopolar spindles, leading to mitotic block and apoptosis.

B) Low concentrations of paclitaxel are not sufficient to cause mitotic block but instead result in the formation of defective bipolar spindles leading to mitotic slippage into a multinucleated G1-like state followed by apoptosis.

Adapted from Abal, M., J.M. Andreu, and I. Barasoain. 2003. Taxanes: microtubule and centrosome targets, and cell cycle dependent mechanisms of action. *Current Cancer Drug Targets*. 3:193-203.

Paclitaxel Analogues and Taxane-Like Drugs

Though paclitaxel has often been touted as a wonder drug, there are ever-increasing problems with resistance to the drug. Various mechanisms are to blame, including overexpression of P-glycoprotein, an integral membrane protein that exports drugs from the cell to keep the intracellular concentration below cytotoxic levels (Horwitz et al., 1995); altered metabolism of the drug (Orr et al., 2003); decreased expression of *bcl-2*, a protein associated with paclitaxel-induced apoptosis (Rowinsky, 1997); expression of p53 tumor suppressor protein (Schmidt et al., 2003); and mutations in β-tubulin that interfere with paclitaxel binding (Orr et al., 2003).

In light of this mounting resistance, it is more important than ever to discover drugs that can take the place of paclitaxel, either paclitaxel analogs or drugs unrelated to paclitaxel that have a similar mechanism of action. Such drugs can not only replace

paclitaxel in the clinical setting but can also serve as lead compounds for the synthesis of still more anticancer drugs. Several analogs of paclitaxel have been synthesized with varying results with respect to cytotoxicity and tubulin activity. The most prominent of these analogs is Taxotere® (docetaxel, Aventis Pharmaceuticals), which differs from paclitaxel at the *N*-benzoyl group by a replacement of paclitaxel's phenyl group with a *tert*-butyl group (Kingston, 1995). Docetaxel has been found to be more active than J774.2 and P388 leukemia cells (Kingston, 1995) and about five times more active against paclitaxel-resistant cells (Ringel and Horwitz, 1991) and is currently approved for locally advanced or metastatic breast cancer after failure of prior chemotherapy and for locally advanced or metastatic non small-cell lung cancer after failure of platinum-based chemotherapy (*Drug Facts and Comparisons*, 2003).

A few paclitaxel-like drugs, including the epothilones and laulimalide, have been discovered as well. Epothilones, which are 16-member macrolides, were the first class of compounds discovered that exert the same mechanism of action as paclitaxel while having a different base structure. Epothilones A and B have the same microtubule binding site as paclitaxel and have been shown to be equally active; furthermore, they are much more effective against P-glycoprotein-expressing multidrug-resistant cells (Bollag et al., 1995). Laulimalide, an 18-membered macrocyclic lactone isolated from the marine sponge *Cacospongia mycofijiensis*, causes cell cycle arrest in G_2-M phase as does paclitaxel, has been shown to be more effective in stabilizing microtubules, and, like epothiliones, shows activity in paclitaxel-resistant cell lines (Mooberry et al., 1999). Unlike epothilones, however, laulimalide binds on a site on the microtubule distinct

from the taxoid binding site and shows toxicity against certain epothilone-resistant cell lines (Pryor et al., 2002).

A New Taxane-Like Drug: Acetic acid 2,2'-thiobis-, bis[(methylene substituted)hydrazide]

In the face of the ever-growing need for new anti-cancer drugs, the discovery by Dr. Williamson's research group of a novel paclitaxel-like microtubule-stabilizing agent is quite exciting. Like paclitaxel, the new compound, acetic acid 2,2'-thiobis-, bis[(methylene substituted) hydrazide], induces an increase in the rate of tubulin assembly and stabilizes microtubules to disassembly (Muri et al., 2002; see Figure 1.6 for structure). Our research group is interested in other similarities as well as differences between the cellular effects of paclitaxel and our novel compound. An exciting and fairly new way to accomplish this is with microarray technology, which allows the analysis of the expression of thousands of genes at once.

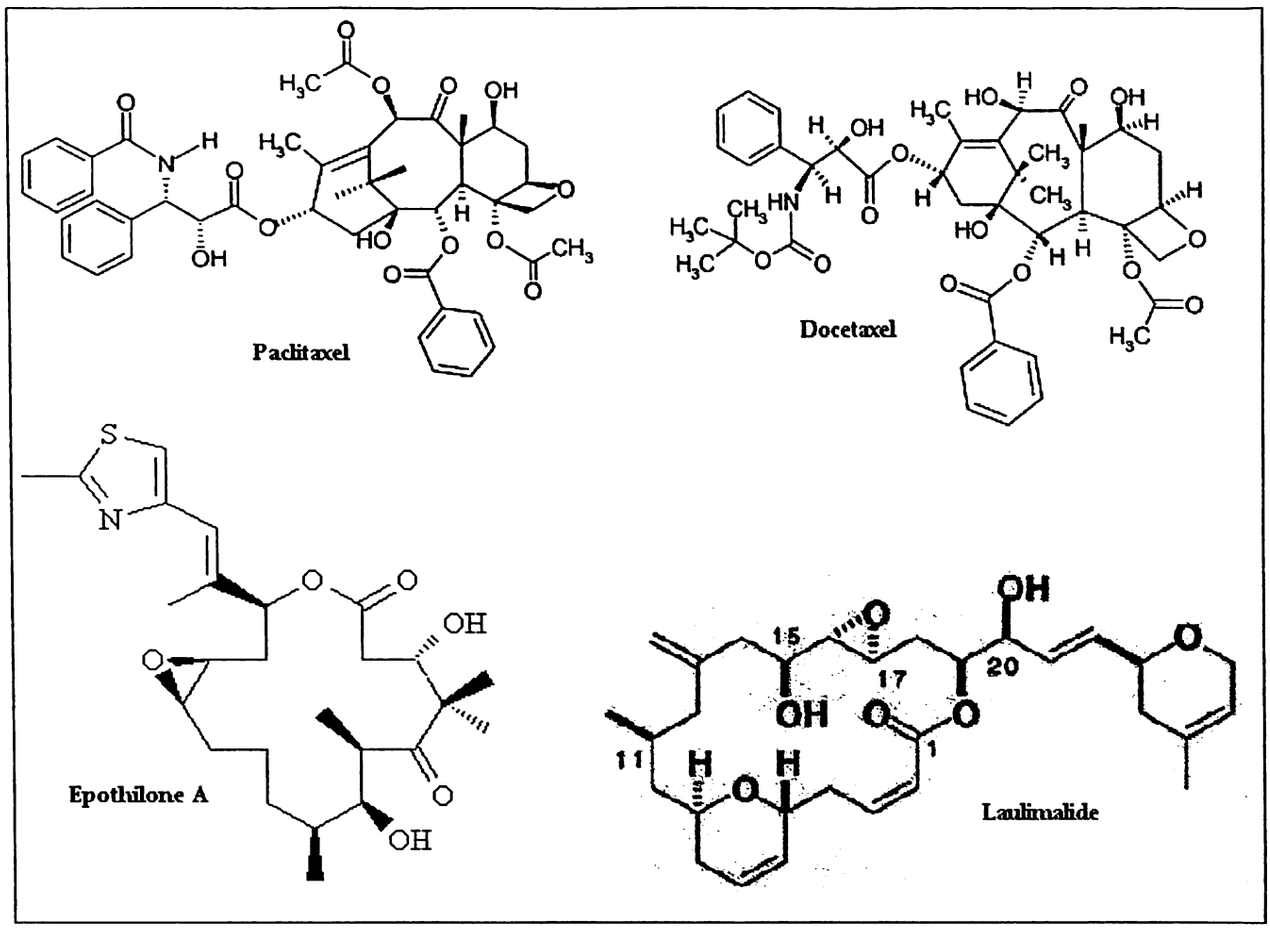

Figure 1.5. Structures of paclitaxel, docetaxel, epothilone A, and laulimalide.
While docetaxel is an analog of paclitaxel, differing at the *N*-benzoyl group, the epothilones and laulimalide do not belong to the taxane family.

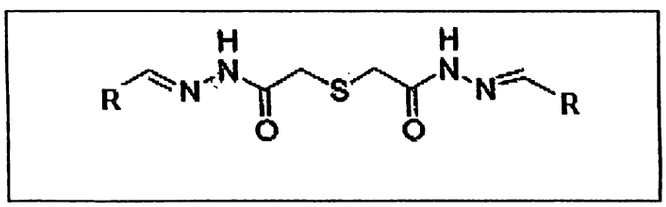

Figure 1.6. Structure of acetic acid 2,2'-thiobis-, bis[(methylene substituted) hydrazide].

DNA Microarray Technology

The Basics

DNA microarray technology is largely based on the so-called central dogma of molecular biology—that gene expression occurs via transcription of DNA into messenger RNA followed by translation of messenger RNA into protein (Alberts et al., 2002). During the microarray process, messenger RNA (mRNA) is extracted from cultured cells or tissues, labeled with a fluorescent dye (often after reverse transcription to DNA), and hybridized to a microarray, an ordered array of tens of thousands of either complete genes or oligonucleotide sequences spotted on a small chip made of a flat, inert substrate such as glass, plastic, or silicon (Schena, 2003). Messenger RNAs hybridize to corresponding genes on the microarray by complimentary base pairing. After hybridization, the microarray is scanned by a laser scanner, which produces a digital image. Each spot glows with an intensity proportional to the number of mRNA molecules hybridized, which according to the central dogma indicates the relative level of gene expression—the amount of protein produced—for each gene (see Figure 1.7). Often, two different samples are compared on the same microarray; in this case, the mRNA from each of the two samples is labeled with a differently-colored fluorescent dye (usually red and green) and hybridized to the same array. In this way, the gene expression associated with two different tissue types or treatments can be directly compared.

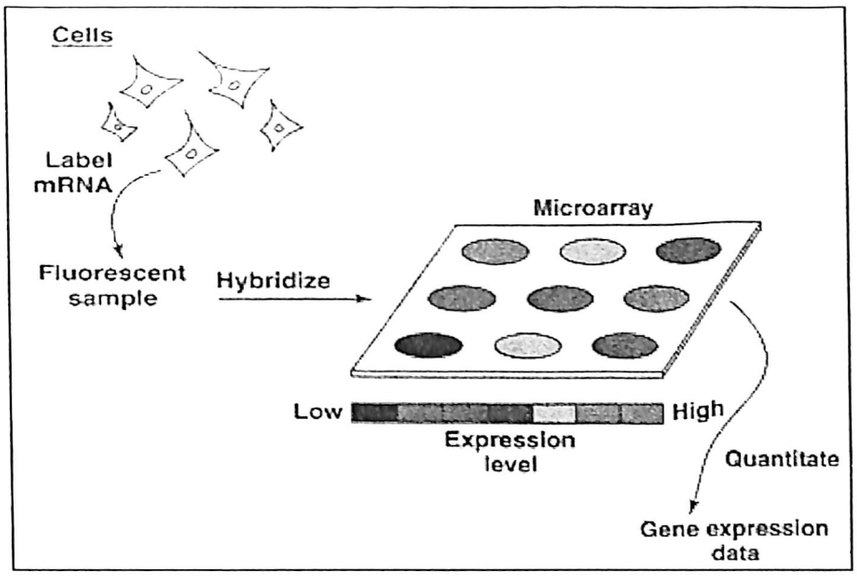

Figure 1.7. Overview of the microarray process.
Messenger RNA is extracted from cells, labeled with fluorescent tags, and hybridized to a microarray containing thousands of gene sequences. When the array is scanned, each spot glows with an intensity proportional to the amount of mRNA bound and thus to the expression of the gene. Quantitative gene expression data are obtained by calculating the fluorescence of each spot.
Source: Schena, M. 2003. Microarray Analysis. John Wiley & Sons, Hoboken, NJ. 630 pp.

cDNA vs. Oligonucleotide Arrays

There are two main types of nucleic acid microarrays used in research today: cDNA arrays, and Affymetrix, Inc.'s oligonucleotide arrays. cDNA arrays are the most commonly used microarrays, accounting for approximately 65% of all microarray publications (Schena, 2003). As its name suggests, this type of array is spotted with complimentary DNAs, or cDNAs, which are molecules derived from mRNA by reverse transcription with retroviral reverse transcriptases (Griffiths et al., 2000). Accordingly, each spot on the array is equivalent to one gene. Oligonucleotide arrays, on the other hand, contain spots of oligonucleotides, or single-stranded molecules composed of 15 to 70 nucleotides (Schena, 2003). On an oligonucleotide array, each gene is represented with 16 to 20 oligonucleotide spots whose sequences generally do not overlap (Ochs and

Godwin, 2003). In deciding which of these array types to use, it is important to consider the advantages and disadvantages of both.

Several important distinctions can be made between cDNA and oligonucleotide microarray technology. First of all, there are inherent differences in the properties of target molecules themselves. Complimentary DNA molecules, which correspond to an entire gene, are much larger than oligonucleotides, which consist of less than one hundred nucleotides (Griffiths et al., 2000). Consequently, cDNA arrays provide longer sequences for hybridization than do oligonucleotide arrays, resulting in a more intense fluorescent signal. However, while the extensive complimentarity afforded by the large size of cDNAs is an advantage in that it leads to considerable probe hybridization, it can also lead to unwanted cross-hybridization of probes closely related to the desired mRNA. Further problems with hybridization in cDNA arrays can ensue due to its double-strandedness; while the cDNA targets are denatured to allow probe hybridization, reannealing can occur, leading to reduced hybridization and loss of fluorescent signal (Schena, 2003). The much shorter target sequences of oligonucleotide arrays, on the other hand, afford increased specificity for probe and decreased cross-hybridization when compared to cDNA arrays. Due to their heightened specificity, oligonucleotide arrays are preferred when studying highly homologous sequences, mRNAs made from the same gene that differ only in splicing or processing, and gene variants differing in just one or very few bases (Lockhart et al., 1996). However, the small size of oligonucleotides leads to differences in hybridization affinity between G:C-rich sequences and A:T rich sequences that affect the signal intensity much more in

oligonucleotide arrays than in cDNA arrays, whose long sequences generally have approximately equal G:C and A:T content. Thus, cDNA arrays may be preferable when studying relatively dissimilar sequences (Schena, 2003).

Oligonucleotide and cDNA microarray technology also differ with respect to the method by which the arrays are synthesized. Complimentary cDNA targets are synthesized by the polymerase chain reaction (PCR) and then robotically spotted onto a slide, while oligonucleotides are synthesized *in situ* on the array by light-directed chemical synthesis (Russo, Zegar, and Giordano, 2003). While PCR is a prevalent and relatively inexpensive technique, phosphoramidite synthesis technology used to make oligonucleotide arrays is complicated and expensive, leading to a substantial difference in cost between cDNA and oligonucleotide arrays. Furthermore, oligonucleotide synthesis requires sequence information before synthesis, while cDNA target molecules can be synthesized directly from tissue samples (Schena, 2003). Thus, cDNA technology allows immediate analysis of the genome of any organism, while oligonucleotide technology is limited to sequences that have already been determined. However, this is becoming less and less of a problem as more and more genomes are sequenced and sequence databases become increasingly expansive (Schena, 2003). In fact, such "sequence-based" arrays are likely to be preferred more and more over PCR-clone based cDNA arrays as the availability of sequence information increases due to the encumbrance of maintaining thousands of cDNA clones for spotting on microarrays (Jordan, 2002).

For this study, the cDNA array was selected for various reasons. As mentioned previously, cDNA arrays cost much less than oligonucleotide arrays; furthermore, our lab had better access to cDNA array equipment than to Affymetrix equipment. More importantly, cDNA array technology allows the direct comparison of two gene expression profiles via the hybridization of two samples to a single array, while oligonucleotide array technology does not. Since the objective of the study was to compare the transcriptional profiles of two drugs, paclitaxel and our lab's novel compound, the cDNA array was preferred (Ochs and Godwin, 2003).

Applications of Microarrays

The ability of a microarray to analyze the expression of tens of thousands of genes at one time has lent the technology to a wide variety of applications. Since the gene expression profile of a cell governs its activity, gene expression data can help to clarify cellular functions, biochemical pathways, and regulatory mechanisms of different kinds of cells (Russo, Zegar, and Giordano, 2003). For example, microarrays have been used to identify gene expression patterns associated with disease and aging. Most of these kinds of studies have been performed with cancer, but other diseases, including diabetes, cardiovascular disease, Alzheimer's disease, stroke, AIDS, cystic fibrosis, Parkinson's disease, autism, and anemia, have also been studied with microarrays (Schena, 2003). By identifying differences in gene expression between normal and diseased individuals, microarrays should allow researchers to better understand the genetic basis and biochemical particularities of these diseases and thus could lead to the development of safer and more effective treatments. As the activity of many drugs

results in a change in gene expression, microarrays can also be used to identify both the mechanism of action of a drug and its associated side effects. Microarrays can be used for toxicity screening, allowing researchers to eliminate toxic compounds before entering them into expensive clinical trials and thus improving the time and cost efficiency of the drug discovery process, and for patient genotyping, imparting the ability to distinguish between patients who are responsive to a drug and those who are resistant (Lee et al., 2003). Yet another promising application of microarrays is their use for genetic testing. As thousands of disease-causing genes are known, it may be possible to screen for several genetic diseases with a single microarray assay. This technology is currently in development and is an exciting possibility for the future. The use of microarrays is by no means limited to medical applications. Microarrays can be also used in agriculture to identify genes associated with improved yield, resistance to disease and osmotic stress, and nutritional composition and thus could accelerate the production of genetically modified crops (Schena, 2003).

Microarrays and Cancer

As mentioned above, microarray technology is particularly useful in the study of cancer, lending itself to myriad applications. As each tumor type has its distinctive gene expression profile, tumor microarrays can be used to both identify new cancer classes and to improve cancer diagnosis. A milestone in this area was the work of Khan and others, which identified thirty-seven genes whose expression was consistently changed across seven different alveolar rhabdomyosarcoma cell lines relative to a reference cell line (1998). Since then, the specific gene expression profiles of several other types of

cancer have been identified (reviewed by O'Neill, Catchpoole, and Golemis, 2003). Eventually, microarrays could become a mainstay in the clinical laboratory, used in conjunction with or even replacing now-conventional histopathological microscopic examination to diagnose cancer. Another promising use of microarrays in cancer research is the identification of possible targets for drug development. By comparing the gene expression profiles of normal and malignant tissue on a microarray, researchers have begun to identify the signaling pathways indicative of carcinogenesis. Two recent studies of this type include the work of Calvo and others (2002), which identified differences in gene expression during the progression of prostate cancer, and the work of Ono and others (2000), which determined the genes involved in ovarian carcinogenesis. This approach may lead drug discovery researchers to new cancer drug targets, leading to a considerable expansion of the chemotherapy repertoire. Yet another approach involves using a microarray to elucidate the gene expression profiles induced by chemotherapeutic drugs. This technique allows researchers to more specifically identify the mode of action of drugs (i.e., the precise biochemical pathways they activate or deactivate) and to better classify them (O'Neill, Catchpoole, and Golemis, 2003). A landmark study in this area was the report of Scherf and others (2000), which was used microarrays to correlate the transcriptional profiles of sixty human cancer cell lines with their response to various anticancer drugs. Another recent significant study is that of Dan and others (2002), which used DNA microarrays to determine the gene expression profiles of thirty-nine human cancer cell lines after treatment with fifty-five anticancer drugs.

This study employed the approach described above—the use of microarrays to compare the gene expression profiles of chemotherapeutic drugs. The intent of this study was to use cDNA microarray technology to compare and contrast the transcriptional profiles of A549 cells, a non-small lung cancer cell line shown to be sensitive to paclitaxel (Torres and Horwitz, 1998; Yang and Horwitz, 2000; Chen and Horwitz, 2002; Hsia et al., 2002; Chen et al., 2003), after treatment with paclitaxel and our lab's novel compound, acetic acid 2,2'-thiobis-, bis[(methylene substituted)hydrazide]. However, microarray work is very complex and requires a substantial amount and variety of equipment and materials, none of which our research group had at the outset of this project. Consequently, extensive preparation of the lab, including ordering, assembling, and organizing equipment and materials and finding protocols, was necessary to initiate a project of this magnitude.

CHAPTER 2: METHODS: SETTING UP A CELL CULTURE AND RNA EXTRACTION LABORATORY (PART I)

At the outset of this project, our lab was not equipped for the drug-screening studies that we wanted to conduct, specifically the comparison of the gene expression profiles of paclitaxel and our lab's novel compound in A549 lung cancer cells. Thus, we divided the large study into Part I, the preparatory steps, and Part II, the experimental steps (see Figure 2.1). I was responsible for Part I, which is described below, as well as the basic design of Part II (see Chapter 3).

In order to carry out the proposed study, it was first necessary to completely assemble the equipment and materials required for cell culture and RNA extraction, techniques that must be executed before microarray hybridization and scanning. Since both techniques are extraordinarily sensitive to contamination (cell culture to fungal and bacterial spores and RNA isolation to RNases, ubiquitous enzymes that degrade RNA), it was crucial maintain as clean an environment as possible. This concern extended both to the selection of materials and to the setup of the laboratory, which are described below.

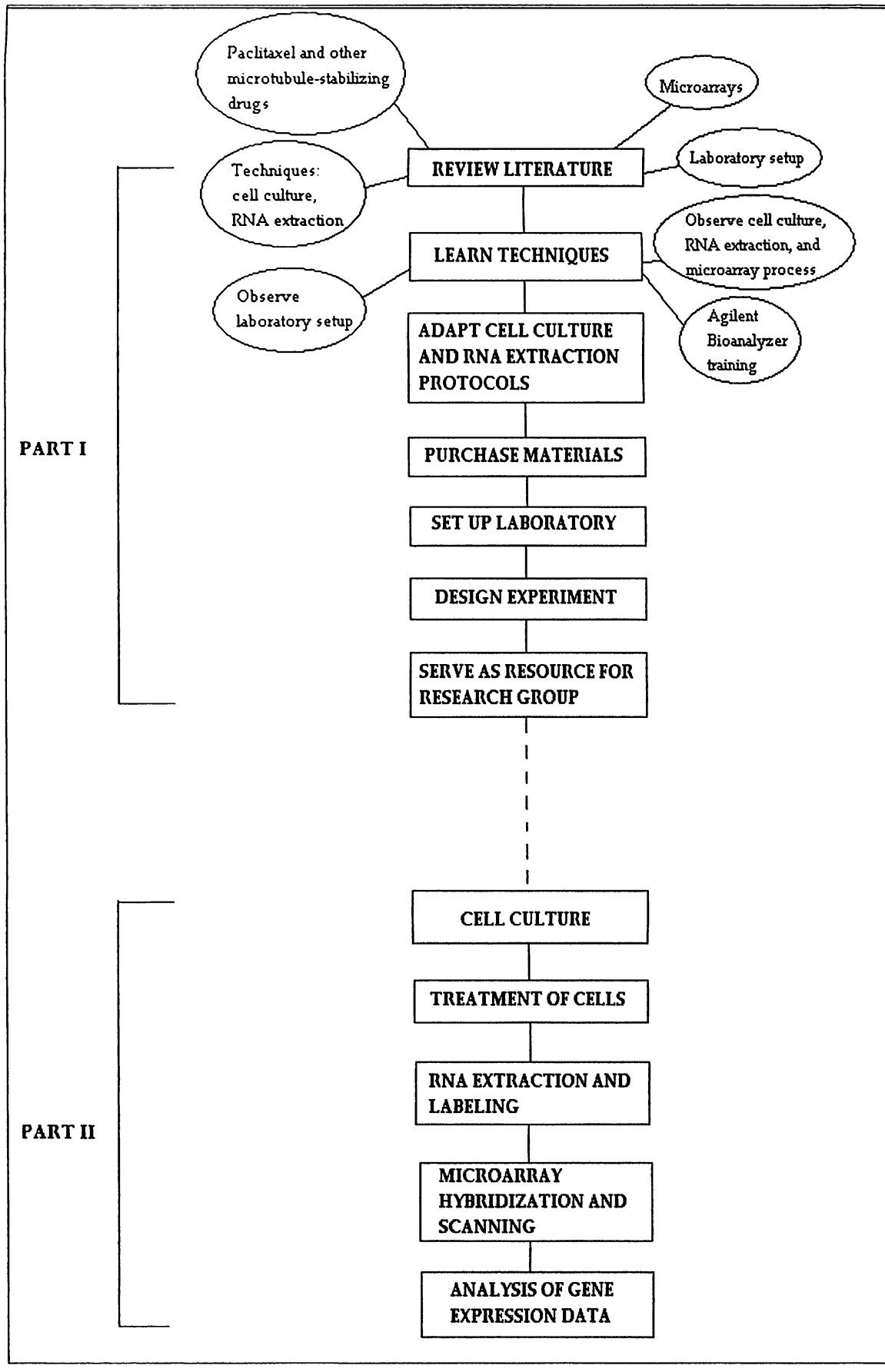

Figure 2.1. Flow chart showing the basic layout of Parts I and II of the study.

Materials

General

Laboratory benches for workspace and equipment and material storage and a water bath for use in several applications, including thawing of frozen serum and other media supplements and temperature control of certain steps in the RNA extraction procedure, were purchased from Fisher Scientific, Inc. As sterile transfer of various solutions and mixtures was fundamental to both cell culture and RNA isolation, many different types of pipettes and pipetters were purchased. For larger scale transfers, various sizes of sterile serological pipettes were purchased from the stockroom, and a Pipetboy (Argos Technologies, Inc), an electronic pipetting aid, was purchased from Fisher Scientific. For smaller volumes, Pipetman® micropipetters of various sizes (Rainin) along with sterile, nuclease-free micropipette tips were purchased from Fisher Scientific.

Cell culture

Cells and media

A549 lung cancer cells (ATCC # CCL-185); F-12K medium, the medium recommended for optimal growth of A549 cells, and fetal bovine serum (FBS), a media supplement necessary for cell growth, were purchased from American Type Culture Collection. Penicillin-streptomycin solution, which is added to media to inhibit bacterial growth; a pH meter (Hanna Instruments), used to ensure that the pH of the media is within an acceptable range for cell growth; and a filtration pump (KNF Laboport™), which provides a vacuum during filter sterilization of media, were purchased from

Fisher Scientific. Sterile filter flasks for sterilization of media were purchased from the pharmacy school stockroom.

Incubation and subculturing of cells

A carbon dioxide (CO_2) incubator (Revco) to provide the proper growth environment for cells was purchased from Fisher Scientific. A carbon dioxide cylinder to supply the incubator was purchased and fitted with a two-stage carbon dioxide regulator (Air Products, Inc.), which controls the flow of the gas into the incubator, purchased from Fisher Scientific. 75 cm^2 tissue culture flasks for the monolayer culture of the cells were purchased from the pharmacy school stockroom.

Various materials were needed for the subculturing or splitting of the cells. 1X trypsin-EDTA solution, which is used to detach cells from surface of culture flask; a centrifuge (Beckman Coulter) for spinning down cells; an inverted phase-contrast microscope (Leica) for monitoring the confluency of cells and assessing their extent of dissociation; and a hemacytometer (Hausser Scientific), used to count cells under the microscope, were purchased from Fisher Scientific, and 15 and 50-mL conical centrifuge tubes were purchased from the pharmacy school stockroom.

Cryogenic storage

Cryogenic storage is an essential component of a working cell culture laboratory, as cultured cells can be saved indefinitely for future applications by storing them in liquid nitrogen (Mather and Roberts, 1998). For the purpose of cryopreservation, a Locator 8 Plus® cryobiological storage system (Barnstead-Thermolyne), Opti-Freeze® Cryopreservation Medium (Fisher BioReagents), 2 mL cryovials (Nalgene), and

Cryogloves® (Tempshield, Inc.) for protection of hands when working with liquid nitrogen were purchased from Fisher Scientific.

RNA extraction

Atlas™ Pure Total RNA Labeling System, a kit that contains all of the necessary chemicals needed to extract total RNA from cells, was purchased from Clontech. Cell scrapers, used to remove cells from the culture flask, were purchased from the pharmacy school stockroom. Sterile, nuclease-free microcentrifuge tubes, used throughout the RNA extraction procedure and for storage of extracted RNA, and two Eppendorf microcentrifuges (one for use in room temperature and one for refrigerator use), which are used in various steps of the extraction procedure, and phosphate buffered saline (PBS; ICN Biomedicals), used to rinse and resuspend the cells prior to RNA extraction, were purchased from Fisher Scientific. To battle the ubiquitous RNases, which degrades RNA (pure, intact RNA is essential for successful hybridization to a microarray), RNaseZap™, a cleansing solution used to remove RNase contamination from work surfaces and lab apparatus, was purchased from Invitrogen. An Agilent 2100 Bioanalyzer, used to assess purity of extracted RNA, was purchased from Agilent.

Laboratory Setup

First, it was imperative to find a laboratory space that would support cell culture and RNA extraction, techniques that are extremely sensitive to contamination and thus require as clean environment that is relatively free of traffic (Mather and Roberts, 1998).

The room to which we were assigned, unfortunately, had previously been used as a storage closet, so we spent a few weeks clearing out the room, cleaning it, and sealing it off from adjacent labs, which were potential sources of cross contamination. Then, new laboratory benches were assembled to provide bench space for work and storage space for materials and equipment. At that time, the laboratory was ready to be set up as a working cell culture and RNA extraction lab.

A biosafety hood, used to ensure a sterile working environment, was placed in the lab, along with materials necessary for its proper use: ethanol and paper towels to clean the hood before and after use, foam alcohol to clean hands before and after use, and a garbage can fitted with a biohazard bag to dispose of biological waste. Since all pipetting would be done under the hood, the Pipetboy and several sterile serological pipettes of each size were placed under the hood as well. The rest of the chemicals and materials that were not temperature sensitive were stored outside of the hood under the new lab benches, the materials pertinent to cell culture near the hood and the materials pertinent to RNA extraction farther down the bench.

Since many of the materials require cool storage, it was necessary to have access to refrigerators and freezers of various temperatures, preferably inside the lab in order to avoid cross-contamination between laboratories. We successfully obtained a refrigerator (approximately 4°C) and freezer (-20°C) for inside the laboratory and purchased a liquid nitrogen storage system. We were not able to obtain a -80°C freezer for inside the lab but located one nearby. The F-12K medium and refrigerator microcentrifuge was stored in the refrigerator, the penicillin-streptomycin solution and

cryopreservation medium in the -20°C freezer, the FBS in the -80°C freezer, and the cells in liquid nitrogen.

The placement of equipment was determined based on the purpose of each device. The filtration pump and CO_2 incubator, which are both used in cell culture, were placed under the biosafety hood for easy access. The incubator was assembled as detailed in the instruction manual and connected to the CO_2 cylinder. A temperature of 37°C, generally considered the best temperature for the growth of human cells because it is the normal temperature of the human body (Mather and Roberts, 1998), and the recommended carbon dioxide level of 5% was set using the incubator's digital controls, and the incubator was allowed to equilibrate. The inverted phase contrast microscope, also used in cell culture was placed near the biosafety hood. The room temperature microcentrifuge and Agilent 2100 Bioanalyzer and computer, equipment used during and after RNA extraction, were placed farther down the lab bench, where the RNA isolation work was to be completed.

Cell Culture and RNA Extraction Protocols

Before starting cell culture and RNA extraction, it was necessary to find reliable protocols for these procedures. I consulted with Dr. Donald Sittman's research group at The University of Mississippi Medical Center to learn these techniques, both observing cell culture and RNA extraction in his laboratory and obtaining copies of their protocols. A brief description of each procedure follows; specific volumes will likely be modified as the protocols are refined through experience.

Cell culture

This protocol is adapted from Dr. Sittman's cell culture protocol and the specific instructions for A549 cells provided by ATCC (Sittman, 2002; American Type Culture Collection, 2004).

Starting cell culture

Growth medium (F-12K medium with 10% FBS and 1% penicillin-streptomycin solution, as recommended by ATCC) is warmed to 37°C, and 15 mL is added to a culture flask. The vial of cells is removed from liquid nitrogen and quickly thawed to 37°C, and its contents are added to the culture flask. The flask is placed in the incubator with its lid loosened one-quarter turn to allow CO_2 flow.

Subculturing

The cells must be subcultured when the growth reaches 80% confluence, according to the ATCC. The media is removed from the culture flask and discarded and replaced with 5 mL of trypsin-EDTA. Once the cells have dissociated, the cells and trypsin-EDTA are removed from the flask and added to a conical centrifuge tube containing 5 mL of F-12K medium. The cells are spun down, the trypsin is decanted, and the cells are resuspended in fresh growth medium. About 15 mL of medium is added to 3-8 new flasks (the optimum subcultivation ratio for A549 cells is 1:3 to 1:8 according to ATCC), and the cells are evenly divided among the new flasks. The flasks are placed in the incubator, their lids loosened one-quarter turn.

Medium renewal

The growth medium must be replaced in between subcultivations—two to three times a week for A549 cells (American Type Culture Collection, 2004). The old medium is simply removed and discarded and replaced with 15 mL of fresh growth medium.

RNA Extraction

The procedure outlined below is a summary of the protocol Dr. Sittman's research group adapted from the procedure in the Atlas™ Pure Total RNA Labeling System User Manual. First, the cells are washed with PBS, scraped into a centrifuge tube, and spun down. Then, the PBS is removed and the cells undergo a series of treatments. First, denaturing solution is added and the cells are homogenized. Then, the homogenate is treated with saturated phenol and chloroform. The upper aqueous phase (phenol), which contains the nucleic acids is retained, while the lower organic phase (chloroform), which contains lipids, and the protein-containing interface are discarded. Subsequently, isopropanol, which precipitates nucleic acid, is added. Finally, the precipitate is treated with DNase, which degrades DNA, leaving pure, intact total RNA.

The Agilent 2100 Bioanalyzer

Once RNA is isolated, its integrity must be assessed. If the RNA is degraded, it will not properly hybridize to the microarray; only pure, intact RNA should be used. The integrity of total RNA is usually assessed by gel electrophoresis, a time-consuming process that requires a relatively large quantity of sample. The relatively new Agilent 2100 Bioanalyzer is a machine that essentially performs electrophoresis on a microchip,

producing both a gel-like image and an electropherogram that can be visualized on a computer screen. The intactness of the RNA can be easily determined by observing the integrity of the 28S and 18S rRNA peaks on the electropherogram. This method of assessing RNA integrity is preferable to traditional gel electrophoresis for the purpose of our study because the bioanalyzer requires only nanogram or even picogram quantities of RNA to perform the analysis, leaving plenty of RNA to perform the microarray hybridization.

CHAPTER 3: THE FUTURE OF THE STUDY (PART II)

Experimental Design

As the preparatory work took longer than expected, we are just now nearing the start of the experimental phase of the project. We intend to conduct the study as follows. First, A549 cells will be cultured as described in the protocol above. Then the cells will be treated with paclitaxel and acetic acid 2,2'-thiobis-, bis[(methylene substituted)hydrazide] using the methods of Chen et al. (2003) as a model. As mentioned in the introduction of this thesis, paclitaxel has differential effects on mitosis and apoptosis depending on the concentration used. Thus, it will be necessary to examine the transcriptional profiles induced by both low and high concentrations of both drugs. Following the protocol outlined in the Chen paper, the cells will be incubated for 18 hours with no drug as a control and with varying concentrations (4, 8, 16, and 45 nM) of each drug. After treatment, total RNA will be extracted from the cells as described in Chapter 2 and its integrity verified using the Agilent 2100 Bioanalyzer. Although most of the published microarray studies include reverse transcription of the total RNA to cDNA, we will likely skip this step, as Dr. Sittman's research group advised us that they have successfully conducted experiments employing fluorescent labeling of the total RNA itself. Since our department at the present time does not have microarray equipment and will likely not have the funds to make such an expensive

purchase in the near future, we will probably send the RNA off for labeling and microarray analysis. We will probably use commercially produced cDNA arrays, which contain a comprehensive representation of the human genome and thus provide many different gene families for comparison.

There are two options for the design of the two-color co-hybridization microarray experiment that we propose to perform: the reference design and the balanced block design (Simon and Dobbin, 2003; see Figure 3.1). In the reference design, each experimental treatment is co-hybridized with a control RNA; thus, in this study, each RNA sample would be hybridized with the untreated control sample, and the fluorescent intensities of the gene spots would be compared across arrays. An advantage of this design is that the fluorescent signal of the reference RNA can be normalized across samples, reducing unwanted variance due to inconsistencies in spot size and shape on different arrays. In the balanced block design, the two experimental treatments are co-hybridized on the same array, allowing a direct comparison between the two treatments. Accordingly, in this experiment, the RNA samples corresponding to the same concentration of the two drugs would be hybridized on the same array (i.e. 4 nM paclitaxel RNA with 4 nM acetic acid 2,2'-thiobis-, bis[(methylene substituted)hydrazide] RNA, 8 nM paclitaxel RNA with 8 nM acetic acid 2,2'-thiobis-, bis[(methylene substituted)hydrazide] RNA, etc.). However, the lack of reference RNA results in noise between the different arrays, preventing an accurate comparison of samples on different arrays. Since this study involves the direct comparison between two classes, the balanced block design seems appealing, but there is a considerable

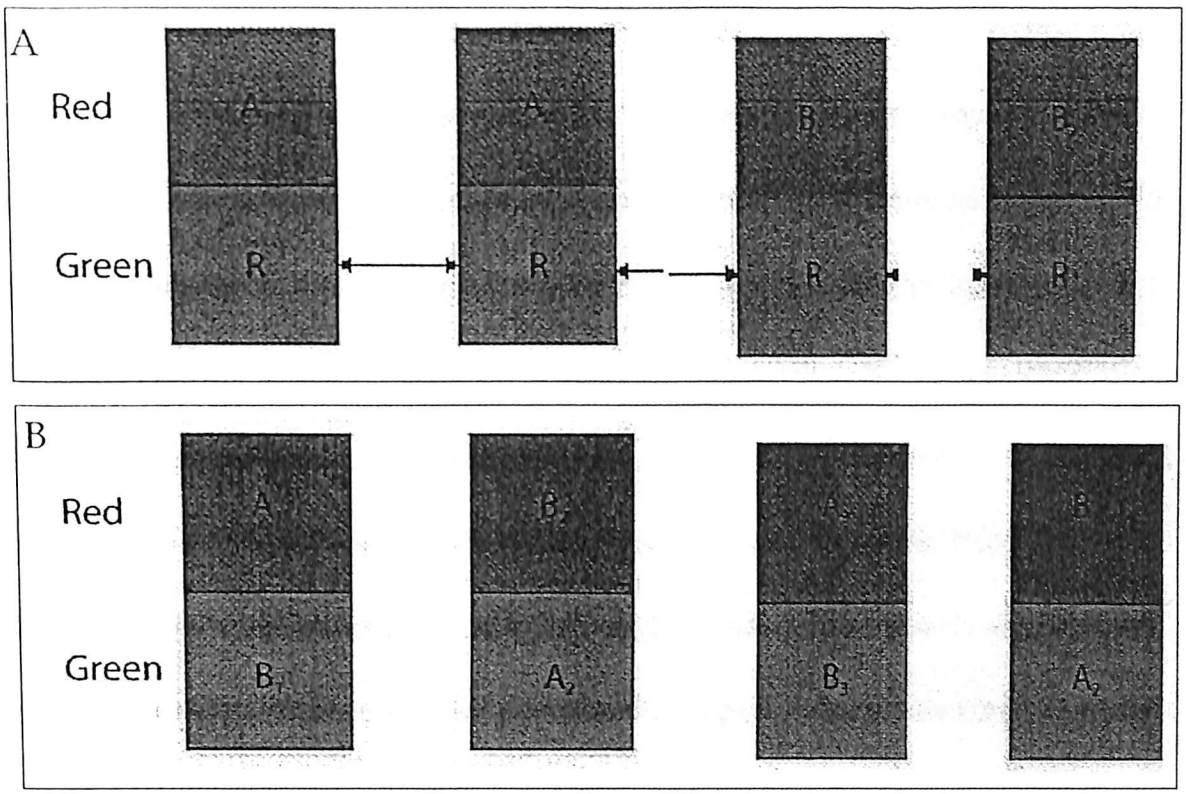

Figure 3.1. Comparison of reference and balanced block design.

A) Reference design. Two different specimens of class A (A_1 and A_2) and two different specimens of class B (B_1 and B_2) are each cohybridized with a reference (control) specimen.

B) Balanced block design. Samples from class A are cohybridized with samples from class B, and a reference sample is not used.

Source: Simon, R.M., and K. Dobbin. 2003. Experimental design of DNA microarray experiments. *BioTechniques*. 34:16-21.

problem with this approach: it is likely that the effects of the two drugs do not correspond exactly at the same concentration; indeed, preliminary work indicates that our group's drug requires a slightly higher concentration to produce the same effects on tubulin as paclitaxel. Thus, we will use the reference design, which will allow a standardized comparison among all treatments.

Since the purpose of this study is to compare the transcriptional profiles induced by the two drugs as a whole, we will not be looking at specific genes. Instead, we will examine the entire spectrum of genes, identifying genes that are either downregulated or upregulated by each drug (compared to control) and comparing these effects across the two treatments.

Hypotheses

The purpose of this study is to identify similarities and differences between the gene expression profiles generated paclitaxel and our group's novel drug. Various experiments have been conducted using microarrays to compare the transcriptional profile of paclitaxel with that of other drugs, with varying results. A study by Brachet and others (2002), for example, compared the gene expression profiles of four anti-cancer drugs: paclitaxel; cisplatin, a DNA synthesis inhibitor; camptothecin, a topoisomerase I inhibitor; and methotrexate, a nucleotide synthesis inhibitor. While considerable overlap was observed in the profiles, significant differences were found as well. On the other hand, the transcriptional profiles of paclitaxel and epothilone B, which both act by stabilizing microtubules, were found to be strikingly similar, despite

differences in their chemical structure (Chen et al., 2003). The distinct results of these two studies indicate that the transcriptional profile of a drug is linked to its mechanism of action. Thus, I postulate that since paclitaxel and acetic acid 2,2'-thiobis-, bis[(methylene substituted)hydrazide] have the same mechanism of action according to previous studies on tubulin (Muri et al., manuscript in preparation), their gene expression profiles will be more alike than different. In fact, we hope that the gene expression signatures of the two compounds will be strikingly similar, confirming that the two drugs indeed share the same mechanism of action. It will be also be interesting to discover any differences in gene expression, which could indicate differential side effects unapparent in the previous studies with tubulin.

CHAPTER 4: THE FUTURE OF MICROARRAYS IN CANCER RESEARCH

Our proposed study and previous similar studies (Brachat et al., 2002; Dan et al., 2002; Fukusaki et al., 2001) demonstrate only a fraction of the vast potential of microarrays in cancer chemotherapy research. While individual microarray studies by themselves are quite useful, the gene expression data obtained from these experiments would be maximally utilized if it could be compiled into databases available for collective use by the scientific community. The potential applications of such databases are numerous, especially in cancer research.

A database containing the gene expression profiles of all cancer types and subtypes would be invaluable as a predictive tool in cancer diagnosis. Instead of relying on histopathology, which often fails to separate closely related cancer subtypes (O'Neill, Catchpoole, and Golemis, 2003), physicians could make a diagnosis of an unknown cancer type by first determining its gene expression profile using a microarray and then matching its profile to its corresponding cancer type in the database. The ability to make such a specific diagnosis would certainly lead to a better prediction of disease prognosis and improved specificity and efficacy of treatment.

Another possible application of a gene expression database involves the formation of a drug sensitivity and resistance database. If the gene expression markers associated with sensitivity and resistance to every cancer drug on the market were

compiled into a database, then a physician could enter the gene expression profile of a patient's tumor into the database and find the treatment that best matches the patient's genetic makeup. Such patient-specific treatment would eliminate the trial-and-error approach to cancer chemotherapy and could possibly increase the cancer survival rate by administering the most effective drug earlier in the course of the disease.

Yet another application of microarray databases, the one most relevant to our proposed study, lies in the field of drug development. The prospect of a database containing the gene expression profiles of thousands of drugs is quite exciting because it would allow researchers to glean information about a novel compound's mechanism of action by matching its gene expression profile with that of a class of compounds in the database. This development would greatly increase the efficiency and lower the expense of drug research by reducing the time, expense, and number of animals involved in preclinical testing (Lee et al., 2003). After obtaining a good general idea of a compound's mechanism of action using such a database, researchers would be able to focus their studies on that compound and expand their research to other compounds instead of spending excessive time, money and personnel performing numerous general assays on a single drug.

As the promising applications above demonstrate, the combination of microarray technology with bioinformatics as a predictive tool has the potential to have a huge impact on cancer research. Unfortunately, there remains a lack of a unifying bioinformatic resource comparable to GenBank, the National Institutes of Health's genetic sequence database that has greatly accelerated work in genomics over the past

few years. Consequently, cancer microarray data remains largely unused beyond the scope of original studies. However, there has recently been a promising development in the area of bioinformatics and microarrays. In January of 2004, Rhodes and others announced their creation of ONCOMINE, a cancer microarray database containing gene expression profiles of several different types of cancer derived from over 4700 microarray experiments (Rhodes et al., 2004). This exciting advance provides hope that other types of databases, such as one for drug development, will be established and expanded in the near future and will eventually reach the scope of databases like GenBank, making it possible for researchers around the world to share microarray data.

With its capacity to quickly provide information about the expression of thousands of genes at once, microarray technology is becoming more and more prevalent in many fields of research, especially cancer research, and is likely to eventually become a mainstay in clinical medicine as well. As microarray technology becomes progressively more widespread, it is increasingly important that technology in the form of an electronic database be developed to share the ever-expanding wealth of gene expression data. The advent of such databases is especially important in my research group's focus—drug discovery—due to the ever-rising resistance of cancers to cancer chemotherapy. Now more then ever, it is imperative that new and effective anti-cancer drugs are discovered and developed quickly, and the development of an anti-cancer drug gene expression database is in my opinion a critical step in accelerating the pace of drug discovery and development.

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