MULTIPLE SCREENING TECHNIQUES: A WAY TO DEVELOP A CHEMICAL-ANIMAL MODEL

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I would like to leave all of you with this famous quote: Every day you may make progress. Every step may be fruitful. Yet there will stretch out before you an ever-lengthening, ever ascending, ever-improving path. You know you will never get to the end of the journey. But this, so far from discouraging, only adds to the joy and glory of the climb.

Sir Winston Churchill

Billy W. Day, Ph.D.

MULTIPLE SCREENING TECHNIQUES: A WAY TO DEVELOP A CHEMICAL-ANIMAL MODEL

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The primary objective and public health relevance of this investigation was to develop a chemical-animal model with a toxicological and therapeutic approach. The results outlined here are developed from the latest techniques being employed in the chemical and biomedical fields. This research outlines a model building approach that progressed from a preliminary agent screening technique (quantitative structure-activity relationship/structure-activity relationship, QSAR/SAR) and in vivo testing using the Chernoff-Kavlock (CK) assay through to in vitro testing in transgenic adenocarcinoma of the mouse prostate (TRAMP) cell lines.

The preliminary investigation involved development of a QSAR/SAR model to predict the teratogenicity of a series of related chemical agents (dopamine mimetics). This QSAR/SAR model was then validated using a complete leave one out cross-validation. The predictivity of a more general QSAR/SAR model of developmental toxicity was then tested experimentally in vivo using the chemical agent retinoic acid.

The second model was based on in vivo animal screening using the CK assay. The CK assay involves the dosing of pregnant animals, either mice or rats, during the organogenesis period of fetal development. This assay quantitatively measures effects on fetal viability and growth, and allows for a more qualitative assessment of teratogenicity by recording obvious malformations.

The third segment of this study was an in vitro evaluation of the effects of a series of microtubule perturbing agents on cell viability, cell death and gene expression of the TRAMP cell lines. This research could contribute to the development of drug treatments that would be more effective against human prostate cancer.

In the first section of my thesis, a mathematical model was generated with experimental data from the literature on a congeneric series of twelve dopamine mimetics. Based on a single physicochemical parameter, the final model is 100% effective at predicting biological activity (teratogenicity) of dopamine mimetics. We also found inconsistencies in the original biological data that might influence the choice of final model.

The second section of my thesis involves the experimental validation of a general QSAR/SAR model that predicted retinoic acid would be positive for developmental toxicity. Retinoic acid was therefore tested in a standard mouse CK assay (the same assay used to generate the data used to generate the model) to test the SAR model prediction. Significant increases in the incidence of both fetal death and intrauterine growth retardation were observed in the offspring of the treated mice. Statistical analysis revealed these effects were dose-dependent. These results demonstrated, in a quantitative manner, the developmental toxic effects of retinoic acid in the mouse, as were predicted by the SAR model and as expected from developmental literature.

The final segment of my thesis dealt with the preliminary in vitro screening of four promising anticancer agents, Analog II, 4-methoxy Analog II, JR oxime I and TDH 169 on the clonal TRAMP cell lines C1A, C2H and C2N. 4-Methoxy Analog II displayed the most promising antiproliferative effects and apoptosis inducing effects. A microarray analysis of mRNA expression in response to 4-methoxy Analog II was conducted to determine agent-

induced expression alterations in the C1A cell line. Upregulation of the apoptosis activating genes Bok and Siva-pending was observed, while the apoptosis inhibiting genes Birc 4, Dad1 and Atf5 were significantly downregulated.

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1 INTRODUCTION

1.1 HISTORY OF THERAPEUTIC AGENTS

To adequately discuss techniques/methods in toxicology and therapeutics, one must first start with the historical approaches used in drug design and discovery. It is next to impossible to trace back the true origin of drug discovery. Many ancient populations reported the medicinal properties of various plant extracts and elixirs, all resulting from a necessarily trial and error search for remedies of specific ailments (Sneader, 1985). The single most important source of drugs or drug precursors has always been and continues to be nature (Verpoorte, 1998). In the 21st century, natural products such as morphine, cocaine, salicylates, atropine, quinine and digitalis are all considered to be ancient. However, these natural products and their derivatives are still useful in therapeutics today, thousands of years after their original discovery. In early civilization, man used elements from his natural surroundings to treat specific ailments. The use of extracts and whole plants as remedies amounted to administration of several entities at once, whose constitution and synergism was unknown (Neamati and Barchi, 2002).

1.2 DEVELOPMENT OF DRUG DISCOVERY

Throughout most of human history there was little to no knowledge regarding how these remedies treated the ailments. The development of therapeutic agents was a true trial and error process that was developed based on experience and passed on from generation to generation. The scientists of the 19th century developed techniques for analyzing and determining the

specific components of the extracts and were thus able to determine the individual components of these ancient therapies.

True drug discovery as we know it today could not begin to develop until the first structures of receptors were found. The first rational drug discovery effort can be traced back to John Langley and Paul Ehrlich, the discoverers of the receptor concept. In 1897, Ehrlich suggested a theory based on what he called side chains or groups on cells that can combine with a particular toxin. Langley had postulated 20 years earlier that alkaloids that caused different salivary flow in cats interacted with specific groups or entities on the nerve endings of the gland cells. Ehrlich actually termed his theory side chain receptors. Without any structural knowledge of the entities transmitting the effect, these may have been the first instances of ligand-receptor interactions observed and partially defined (Neamati and Barchi, 2002).

From ancient times through the work of Langley and Ehrlich little was understood about drug interactions and how they worked inside the human body. However, in the early 20th century the fields of biology, chemistry and pharmacology increased rapidly and theories about drug action and mechanism expanded. In considering the timeframe of the existence of human beings, it's amazing how rapidly the understanding of human disease, preventative medicine, the interactions of chemicals with the human body and the understanding of drug therapies has progressed over the last few decades. Just consider the early history of the United States where therapies such as "blood letting" were still accepted and practiced during the infancy of our nation. Also consider the fact that the average life expectancy of a Caucasian U.S. male born in 1940 was 62.1 years while the life expectancy of a Caucasian U.S. male born in 1994 was 73.3 years (Singh et al, 1996). While there are many factors, such as nutrition and lifestyle, that

contribute to these changes in life expectancy, this change is also attributable to the progress of therapies and medicine.

1.3 Traditional "Modern" Drug Discovery

In traditional medicine, drug discovery is a process that begins with a known disease or ailment in an organism and a therapeutic theory is developed to alleviate or cure the disease or ailment. With the existing knowledge of biological processes and interactions scientists develop a list of therapeutic treatments and begin a screening process to determine the viability of these agents at combating the ailment. These screening techniques typically include in vitro and in vivo testing of each specific agent. This traditional method of drug screening and drug development, while used to develop many of the treatments of today, may or may not lead to an effective treatment. However, even when an effective treatment is achieved it is often through significant failures and the elimination of many countless agents during the screening process.

1.4 Modern Techniques in Drug Discovery

Today, there are many new advancements that are leading to the more proficient development of therapies. For example, the ability to sequence a genome and identify every expressed gene will lead to the identification of thousands of new molecular targets, many of which will be relevant to the onset and persistence of disease. With this genetic information researchers may know the role, function, structure, gene location, biochemical pathway, molecular interactions, and expression levels of each and every protein coded for by a particular genome. These developments in genomic sciences on drug discovery will change the course of this field remarkably. In fact, at present in most major pharmaceutical companies, 10% to 25% of new discovery projects are based on genomics (Caron et al, 2001).

There are several ways to use gene analysis to identify specific molecular targets (Jones et al, 1999). One powerful method for studying differential gene expression is the use of microarrays. Microarrays allow for the rapid analysis of the expression of thousands of genes. In microarrays, the oligonucleotides are attached to glass slides to form arrays and then hybridized with cDNAs from a particular tissue or cell type of interest. A fluorescent detection system allows for the quantitation of interaction of the cloned gene with the cDNA. Using microarrays, gene expression patterns for many different animal tissues can quickly be obtained under different experimental conditions. A typical application is a comparison of cells that are control or drug treated, and hence generate a gene profile of a disease tissue under the stress of a toxin or inhibitor. It follows (but is not always correct) to designate specific proteins encoded by those genes more highly expressed in the diseased state to be a potential targets for therapy (Neamati and Barchi, 2002).

With the development of gene analysis there are many new targets to be evaluated in drug discovery. The large amounts of data that are quickly generated from the genome sequences and functional genomics leads to the dilemma of determining chemical agents that will potentially block the gene(s) associated with the proliferation of the disease or ailment. New techniques have been developed to expedite preliminary drug screenings. These techniques combine elements of biology and chemistry with mathematics, statistics and computer sciences. These techniques are referred to as bioinformatics and chemoinformatics and focus on large datasets such as macromolecular structures, genome sequences, 3D chemical databases and compound libraries. Informatic methodologies rely on a variety of computational techniques (Manly et al, 2001; Luscombe et al, 2001) including sequence and structural alignment, database design and data mining, macromolecular geometry, phylogenetic tree construction, prediction of

protein structure and function, gene searching and expression data clustering, chemical-similarity clustering, diversity analysis, library design, virtual screening and QSAR (Luscombe et al, 2001). Recent advances in informatics include new molecular descriptors, statistical tools and novel visualization methods (Hann and Green, 1999). A major task of informatics in the future is to develop software tools that will provide the means to store, extract, analyze, and display data in a way that chemists can easily understand and appreciate (Heuer, 1999). In attempts to decipher chemical/biological information, computers require the use of molecular descriptors. These descriptors range from simple bulk properties to elaborate three-dimensional formulations and complex molecular fingerprints. A number of studies have been reported that investigate the performance of molecular descriptors and the use of informatic techniques to develop relationships between a series of chemical agents and their anticipated effects on specific gene expression along with the ailment or disease associated with the over- or under-expression of this specific gene (Xue et al, 2000).

1.5 THE IMPORTANCE OF DEVELOPMENTAL TOXICOLOGY TO PUBLIC HEALTH

Developmental toxicology is the field that defines exposures and agents that cause abnormal development. It is estimated that 1 in 3 children are born with birth defects, and birth defects are the leading causes of infant mortality and disability in the world. Many birth defects are the result of chemical interactions resulting from exposure during pregnancy. The effects of many chemical compounds on the outcome of human pregnancies are unknown. To determine the effects of these chemical compounds on pregnancy, researchers utilize clinical studies and animal testing; however, both of these methods have shortcomings. In clinical research, it is difficult to determine all of the chemicals a mother has been exposed to, or whether a combination of these chemicals lead to the reproductive defect. On the other hand, animal testing is time consuming and requires each chemical compound be tested individually. Also, results from animal tests are difficult to apply to humans. Therefore, results obtained by animal testing may or may not apply to the developing human fetus.

1.6 THE IMPORTANCE OF PROSTATE CANCER TO PUBLIC HEALTH

Adenocarcinoma of the prostate is the most common cancer in American men (Gingrich and Greenberg, 1996). Due to public health awareness and improvements in early detection of prostate cancer, the survival rate and treatment of prostate cancer has increased, however, prostate cancer remains the second leading cause of cancer deaths in American men (Parker et al, 1997). In fact, an estimated 29,900 American men lose their lives to prostate cancer each year, with one death occurring every twenty minutes (National Prostate Cancer Coalition, 2003). There are many available treatment options for prostate cancer, including, prostatectomy,

radiation, brachytherapy (radioactive seeds), hormone deprivation therapy, chemotherapy and many other alternative therapies.

In prostatectomy/radical prostatectomy the entire prostate gland is removed with the aim of curing the disease. There are two types of radical prostatectomy, one in which the an incision is made through the abdomen (radical retropubic prostatectomy), and one in which the incision is made in the perineum, the area between the scrotum and the anus (radical perineal prostatectomy). Whether the results are the same from either of these two procedures is currently unknown. With the radical prostatectomy complete tumor clearance is not always accomplished. Approximately 40% of patients that have the surgery are found to have capsular penetrance or positive resection margins. This treatment is not recommended for men with less than 10 years life expectancy. Complications of the surgery include operative mortality, impotence and incontinence, and the risk of mortality of less than 1% (Huland, 1996). Several factors have been shown to influence postoperative sexual function: age, clinical pathological stage and surgical technique). Reported frequencies of impotence range from 20% to 80%. Incontinence is a significant problem for many patients after the surgery. Reported incidences of incontinence range from 4-21% for mild or stress incontinence and from 0-7% for total incontinence (Villers and Rebillard, 2003).

Radiation treatment uses high-energy radiation from x-rays, gamma rays, neutron, and other sources to kill cancer cells and shrink the tumors. Radiotherapy also aims at curing the disease. This treatment is not usually recommended for men with less than 10 years life expectancy (Parker and Dearnaley, 2003). Short-term side effects relate mainly to bowel and bladder problems from the radiation. Longer-term complications include impotence and urinary problems. Reports of impotence range from 25-60% and reports of incontinence range up to 5%.

Approximately 10% of patients have diarrhea/bowel problems requiring treatment and up to 30% have occasional episodes of rectal bleeding (Furst, 1996).

Brachytherapy (radioactive seeds) is a form of radiation treatment whereby small "radioactive seeds" are implanted in the prostate. This procedure does not require a surgical incision. Instead, thin needles are passed into the prostate gland through the skin between the scrotum and the rectum. As the needles penetrate the prostate, they are seen on the screen of the ultrasound machine and can be accurately guided to their predetermined positions within the prostate. When each needle is in its correct position in the prostate, the needle is slowly withdrawn while individual seeds are injected into the prostate gland. While the needles are being inserted, the ultrasound probe is in the rectum. Both the probe and the needles are removed when the procedure is completed. Patients with early-stage, small-volume tumors are the best candidates for this procedure. Treatment with implants alone (either iodine-125 or palladium-103) is usually adequate for an early stage small volume prostate cancer. For larger volume tumors, brachytherapy is usually performed in combination with external-beam radiation (Witt et al, 2003). Incontinence with radioactive isotope seed implantation in this series was noted in 7% of the patients. Impotence was reported in 25%. Impotence was absent in the 50-60 age group, was 15% in the 60-70 age range and increased to 35%+ at ages 70 or greater. Most patients had short-term obstructive or irritative urinary symptoms during the first few weeks following treatment. Late complications included 10% with prolonged urinary blockage symptoms characterized by increased frequency and decreased urinary stream. These symptoms may be treated with Hytrin, an oral medication that relaxes the smooth muscle of the urinary sphincter, or with transurethral incision of the prostate (Reijke and Laguna, 2003).

Hormone deprivation therapy, also known as androgen ablation therapy, is a well established form of treatment for various stages of prostate cancer. This treatment works as follows the basis that prostate cancer grows in response to testosterone. Testosterone is produced in the testicles and the adrenal gland. Testosterone production can be stopped in two ways. The testicles can be removed with a procedure called an orchiectomy. Similarly, testosterone production can be stopped with medications such as leuprolide or goserelin acetate that suppress the pituitary gland and thus decrease production in the testes. This is know as androgen deprivation therapy (ADT). ADT is not a curative therapy, but is reserved for metastatic disease or for patients that will not medically tolerate surgery or radiation. ADT can relieve symptoms from painful bone metastases and slow the overall growth rate of prostate cancer cells. The major disadvantage to this therapy is prostate cancer usually responds to 1 or 2 years of hormone therapy, after which most tumors start to grow again. Once this happens, the treatment goal is to control symptoms. No treatment can cure prostate cancer after hormone therapy stops helping (Trachenberg, 1997).

Chemotherapy is the use of specific drugs that can destroy cancer cells. The drugs circulate throughout the body in the bloodstream and can kill any rapidly growing cells, including potentially non-cancerous ones. Chemotherapy drugs are carefully controlled in both dosage and frequency so that cancer cells are destroyed while the risk to healthy cells is minimized. Often, chemotherapy is not the primary therapy for prostate cancer patients, but may be used when prostate cancer has spread outside of the prostate gland or in combination with other therapies. Because the drugs circulate throughout the whole body, they can affect both healthy and cancerous cells. This can lead to many side effects. The specific side effects will depend upon which drugs and regimens are used. The most common adverse reactions include

hair loss, nausea, vomiting, diarrhea, lowered blood counts, reduced ability of the blood to clot, and an increased risk of infection. Some of these side effects occur only temporarily or are more noticeable when treatment is first started. Most of the side effects disappear when the drugs are stopped. For instance, hair will grow back once chemotherapy is stopped (Oh, 2003).

In addition to these readily used treatments for prostate cancer there are also many other treatments employed. However, the methods described in detail represent the treatments most commonly utilized. The problem with all of these treatments are they all have significant side effects and are all corrective measures to treat patients once the cancer of the prostate has developed. Ideally, the medical community would prefer to develop a treatment or drug that could prevent the onset of prostate cancer altogether. In order to develop a preventative treatment for prostate cancer, research must develop an understanding of the exact cell biology, mechanisms and gene regulations that trigger the onset of prostate cancer.

There are currently many accepted research models for prostate cancer research. One of the most promising models is the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mouse model. In the TRAMP model, mice were genetically engineered to express the SV40 (large T antigen) exclusively in the prostate epithelia and develop prostate cancer. The TRAMP model has advantages over other models, including the prostate cancer progression that mimics the development of prostate cancer in humans, and the ability to conduct research in a mouse that has an intact immune system. In addition, the TRAMP mouse develops prostate cancer with age and provides a model to conduct preventative studies and observe the effects of environment, diet and other factors on prostate cancer development. Therefore, while the majority of existing knowledge and treatment is based on correcting or treating the prostate cancer after it has

developed, the TRAMP model allows research into factors and treatments that may lead to actual prevention of the onset of prostate cancer altogether.

1.7 RESEARCH CONDUCTED

This research is the result of multiple screening techniques and experiments to develop a chemical-animal model building approach. The results appear somewhat unsystematic due to changes in chemical agents. However, the basic approach outlines a model building approach which progresses from an effective preliminary agent screening technique (QSAR/SAR) and in vivo validation (CK Assay), through in vitro testing of resultant compounds optimized for biological effect (TRAMP cell lines).

The preliminary investigation conducted in this study was to develop a QSAR/SAR model to predict the toxic effects of a series of related chemical agents with potential therapeutic value (dopamine mimetics). QSAR/SAR are mathematical models that link chemical structure to biological activity for a defined series of compounds. In this study, a mathematical model on a congeneric series of twelve dopamine mimetics was generated with experimental data from literature. The resulting model is 100% effective at predicting the developmental toxicity of these dopamine mimetics. This QSAR/SAR model was then validated using a complete leave on out cross-validation.

To go beyond statistical validation, a the predictivity of a more general developmental toxicity model was tested in an in vivo system. The in vivo validation involved exposure of timed pregnant mouse study during the organogenesis period of fetal development with the chemical agent retinoic acid and evaluation of developmental effects with the CK assay. This assay quantitatively measures effects on fetal viability and growth, and allows for a more

qualitative assessment of teratogenicity by recording obvious malformations. A series of chemicals not used for the model development was submitted for prediction. One chemical that was predicted to be positive for developmental toxicity was retinoic acid. Retinoic acid is a chemical related to vitamin A that is used extensively to treat acne (Leyden, 1988). It has shown to affect the expression of genes in the developing embryo (Hart et al, 1990). Retinoic acid was utilized in a standard mouse CK assay to test the SAR model prediction. Significant increases in both the incidence of fetal death and in intrauterine growth retardation were observed in the offspring of the treated mice. Furthermore, statistical analysis revealed these effects were dose-dependent. These results demonstrated, in a quantitative manner, the developmental toxicity of retinoic acid in the mouse, as was predicted by our SAR model and as was expected from developmental literature.

A similar in vivo assay would have been conducted on the dopamine mimetics, however, at this point the direction of the research changed. Additional experimentation progressed in vitro with a different series of compounds. However, the final testing conducted would be the same type of in vitro experimentation used to develop a chemical animal model.

The in vitro experimentation was conducted on a series of four microtubule perturbing agents that have been classified as promising antiproliferative cancer agents (Analog II, 4-methoxy Analog II, JR oxime 1 and TDH 169). Research was conducted with these four agents to determine their effects on cell viability, cell death and gene expression of the TRAMP cell lines C1A, C2H and C2N. The four antiproliferative cancer agents are Analog II, 4-methoxy Analog II, JR oxime I and TDH 169.

Preliminary screening using the MTT assay revealed that 4-methoxy Analog II treatment displayed the most promising antiproliferative effects. Continuing with just 4-methoxy Analog

II, an ELISA assay was conducted on all TRAMP cell lines. This revealed the minimum apoptosis-inducing levels of 4-methoxy Analog II on each cell line. Additional experiments were then limited to the C1A TRAMP cell line, because they showed the most promising results. Additional studies (PARP cleavage and flow cytometry) were performed to confirm and quantify apoptosis due to 4-methoxy Analog II on the C1A cell line. Finally, a microarray study was conducted to determine alterations in gene regulation of the C1A cell line due to treatment with 4-methoxy Analog II. Changes in gene regulation due to treatment were found in the apoptosis activating genes Bok and Siva-pending (found to be upregulated) and the apoptosis inhibiting genes Birc 4, Dad1 and Atf5 (found to be downregulated).

Further research would include in vivo testing of the agents identified by the TRAMP cell lines experiments that produced the best therapeutic results. The potential benefits from this research could include the development of drug treatments that are more effective at combating human prostate cancer.

2 DOPAMINE MIMETICS

2.1 QSAR/SAR MODEL

The leading causes of infant mortality and disability in the world are birth defects. Many birth defects are the result of chemical interactions resulting from exposure during pregnancy. The effects of many chemical compounds on the outcome of human pregnancies are unknown. Currently, the accepted method of determining the effects of a compound on the fetus and pregnancy outcome is through clinical studies and animal testing. These methods both have major drawbacks. In clinical research, it is impossible to determine all chemicals a mother has been exposed to, or whether a combination of these chemicals led to the reproductive defect. The other method of determining the effects of a compound on the fetus and pregnancy outcome is animal testing. This method is very expensive, time consuming and requires each chemical compound to be tested individually. Results from animal tests are also difficult to apply to humans, since mechanisms and their outcomes in biological systems vary from species to species. Therefore, the results obtained by animal testing may or may not apply to the developing human fetus.

As an alternative to these types of studies, Quantitative Structure-Activity Relationships (QSAR) have been developed to predict the effect of chemicals on biological systems, including the reproductive system. QSARs are mathematical models linking chemical structure to biological activity for a series of compounds (Hansch and Fujita, 1964; Kubinyi, 1993). QSAR models have been extensively used to analyze the toxic effects of compounds. To identify chemicals that result in birth defects and reproductive difficulties, a computer-based model is

utilized to implement a Structure-Activity Relationship (SAR) and QSAR. First, a set of chemicals with known biological activity are analyzed using computer software ("learninbg set"). In this work, the Molecular Modeling Pro™ program calculates the compound's physicochemical properties. A compound's physicochemical parameters can be classified into three categories: electronic, hydrophobic, and steric (Hansch and Leo, 1995; Kubinyi, 1993). These properties govern how the molecule will (or will not) interact with the biological system. The relationship between the compound's physicochemical parameters and the biological activity can, after statistical analyses, then be expressed by a mathematical model. The model is developed using methods such as linear discriminate analysis. Based on the analysis, specific physicochemical parameters that are statistically significantly correlated to the outcome of biological response are determined. With the generated model and critical physicochemical parameters, other compounds with unknown biological activity can be analyzed. By calculating the physicochemical parameters of these other compounds, and applying the mathematical model, the biological activity of these unknown compounds can be predicted.

The models generated using computer modeling and statistical methods may, and, in fact, should be verified through animal testing. For reproductive effects, this validation technique is the Chernoff-Kavlock assay. This in vivo assay measures postnatal growth and survival (Chernoff and Kavlock, 1982) of several groups of animals, at various doses, to develop a dose-response to a specific compound.

Developmental toxicology deals with exposures to agents that cause abnormal development (Shepard, 1986; Mattison et al, 1989; Schardein, 1985; Stein et al, 1984). A developmental toxicant can be a chemical agent (i.e., therapeutic agents such as thalidomide), a

biological agent (i.e., infections such as rubella virus), a physical agent (i.e., radiation exposure), or a deficiency state (i.e., lack of vitamin A and/or E).

2.2 DEVELOPMENTAL ENDPOINTS

There are four adverse developmental endpoints: (1) embryonic, fetal or neonatal death, (2) growth retardation, (3) malformations, and (4) functional defects. There are many examples of the endpoints of developmental toxicity. The fetus is susceptible to the endpoint of death throughout the entire pregnancy. Death refers to fetal death, early and late; stillbirth, the delivery of a dead conceptus that is older than 20 weeks of gestation, and perinatal death, which refers to death of a conceptus between 20 and 28 weeks of gestation, and the 7^{th} day after birth.

The growth retardation endpoint includes intrauterine growth retardation. A fetus may be symmetrical, meaning uniform from side to side but smaller than usual, or asymmetrical, meaning smaller on one side more so than the other. Malformations include both major and minor structural defects. Major malformations clearly affect the health, function and/or survival of the individual (i.e., malformed heart, valves or vessels). Minor malformations are less severe, not life-threatening, and easily repaired (i.e., extra digits). Functional defects include mental retardation, learning disabilities and developmental disabilities. These include visual and hearing impairment. The fetus is most susceptible to developing malformations during organogenesis. Organogenesis is the time when the major organs are being developed. In humans this is three to eight weeks from the beginning of gestation. The fetus is most susceptible to growth retardation and functional defects from week eight to birth. This is the period of gestation after all organs are developed and the fetus is growing. The fetus is susceptible to death through all of gestation.

2.3 ANIMAL ASSAYS FOR DEVELOPMENTAL TOXICITY

The human species would be the most accurate to study the effects of an exposure to a development toxicant. Ethical reasons obviously make this impossible. Fortunately, there are several experimental animal models that can be used to test a developmental toxicant. Three well known animal assays that can be utilized to test a developmental toxicant are the (1) Segment I, (2) Segment II, and (3) Chernoff-Kavlock (CK) assays.

In the Segment I assay, the test substance is administered to the experimental animal (both males and females) prior to and during the period in which the animal mates. Administration of the drug to the female subjects is continued up to mid gestation and sometimes even up to the moment the young are weaned. This assay gives information on fertility and reproduction. The drawback is that since both parents and offspring are dosed, it is sometimes impossible to attribute the adverse effect to the reproductive or developmental stages.

In the Segment II assay, the pregnant animals are dosed during the period of organogenesis. The mother is examined during the entire pregnancy for signs of toxicity. Data such as weight increase and general health are monitored. Just before the young are born, the dams are killed to allow in utero inspection. This test provides information about substance, embryonic or fetal toxicity.

In the CK assay, pregnant animals are dosed during organogenesis. The dam is examined during the entire period for signs of toxicity. Data such as weight increase and general health are monitored. The dams are allowed to deliver the pups. The pups are measured for growth retardation, fetal death, morphological and behavioral alterations. This assay is a more qualitative with respect to defects, and more quantitative with respect to death.

2.4 QSAR/SAR Model for Developmental Toxicity

Another screening tool that can be utilized in studying a developmental toxicants relationship is the computational approach that gives Quantitative Structure Activity Relationships (QSAR)/Structure Activity Relationships (SAR). QSAR and SAR differ in that QSAR yield quantitative differences between the biological potency of active compounds, whereas SAR only qualitatively discriminates active and inactive compounds. The underlying assumption in QSAR/SAR is that the biological activity is a function of the chemical structure. After the chemical descriptors are calculated, the QSAR/SAR model can be developed mathematically. The QSAR/SAR model is a statistical association relating the physico-chemical properties of the compounds tested to the biological activity recorded.

2.5 Steps in Building a QSAR/SAR Model

To develop a QSAR/SAR model, these five steps are often conducted: (1) conformational analysis; (2) generation of physico-chemical properties; (3) preliminary model development; (4) linear discriminant analysis; and (5) choice of the final candidate model.

Conformational analysis is a process where single (i.e., sp^3C-sp^3C) bonds are rotated in an effort to determine the global low energy conformation of a molecule. In nature, the lower energy conformations are more likely to occur. Another reason to do conformational analysis is that some of the properties calculated are dependent on the final three-dimensional structure of the molecule. The final three-dimensional structure represents the lowest energy (i.e., in terms of steric strain) arrangement of atoms comprising a molecular structure (i.e., the global minimum energy conformation). Table 1 shows the calculated physical-chemical parameters generated from a typical QSAR/SAR model.

Table 1. Physical-Chemical Parameters from a Typical QSAR/SAR Model

Steric Properties	Electronic Properties	Transport Properties
Molecular Weight	Hansen Dispersion	Log P
Molecular Volume	Hansen Polarity	Hydrophilic-Lipophilic Balance (HLB)
Density	Hansen Hydrogen Bonding	Solubility Parameter
Surface Area	Hydrogen Bond Acceptor	Percent Hydrophilic Surface
	Hydrogen Bond Donor	Water Solubility
	Dipole Moment	
	Highest Occupied Molecular Orbital (HOMO)	
	Lowest Unoccupied Molecular Orbital (LUMO)	

Physiochemical properties are classified into three categories: steric properties; electronic properties; and transport properties. Steric properties describe the size or shape of the compound. Electronic properties give metrics of the molecule including electronic aspects of repulsion and attraction within and with other molecules that might affect potential binding sites. Transport properties are surrogate measures of how compounds cross membranes in the body and how they are transported through the body.

There are three preliminary model development tools used to build the model. These are: (1) histograms; (2) correlation matrices; and (3) all possible regression analysis.

The histograms are generated to determine the normality assumption needed for discriminant analysis. A correlation matrix is made for all possible independent variables to determine information redundancy. All-possible regression is used as a tool to explore all of the potential models. The criteria used to determine the best models from the group of candidate models is the r^2 value. The r^2 is an analysis technique used to determine the amount of variation of measured versus computationally-predicted activity in each model.

Linear discriminant analysis is used to generate the difference between two or more groups with respect to several variables simultaneously. Linear discriminant analysis is used to

generate functions for the classification of chemicals into actives versus inactives. Discriminant analysis can be then used to predict the classification of new observations.

2.6 QSAR/SAR Final Candidate Model

For the choice of the final candidate model, a complete leave one out cross validation procedure is often employed. These are defined steps taken to measure the performance of the discriminant model. The complete leave one out cross validation is a technique where each compound is removed once from the total data set and the remaining compounds are used to train and build a new model. The new model on the reduced training set is then applied to the single compound that was removed during the generation of the current model. This procedure repeats until models with each of the individual observations removed are generated. Using this method, the sensitivity, specificity and squared distance (deviation) are calculated. The sensitivity is the proportion of the experimental positives that were predicted positive by the model. Specificity is the proportion of the experimental negatives predicted negative by the model. The squared distance shows the separation between the two groups.

In this study, a twelve compound database consisting of a series of dopamine mimetics was analyzed to create a model to predict actives versus inactives. Figure 1 is the structure of the parent compound dopamine, and Table 2 shows the twelve variations from the parent compound.

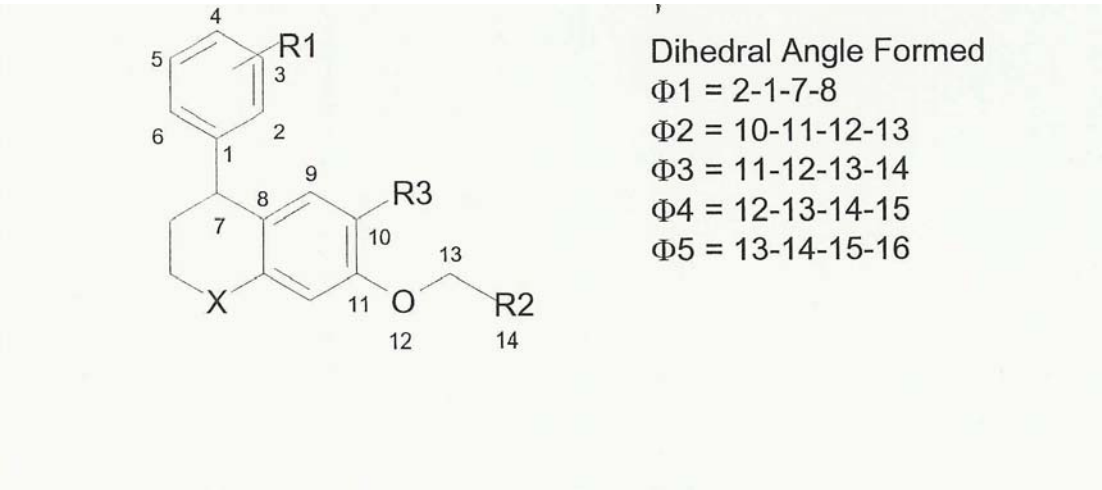


Figure 1 Parent Compound of Dopamine Mimetics

Table 2. Twelve Dopamine Mimetics

NAME	X	R1	R2	R3	Φ1	Φ2	Φ3	Φ4	Φ5
Compound 1	O	3-CF3	-CH2N(CH3)2	------	340.785	270.579	180.088	161.113	------
Compound 2	O	4-CF3	-CH2N(CH3)2	------	343.248	91.1753	181.453	159.337	------
Compound 3	O	------	-CH2N(CH3)2	------	340.669	89.4998	180.631	170.441	------
Compound 4	O	------	-CHOHCH2NHCH3	------	343.547	90.2517	178.993	290.297	179.5
Compound 5	O	4-F	-CHOHCH2N(CH3)2	------	340.444	89.7288	178.741	290.386	179.0
Compound 6	O	------	------	-OCH2CH2N(CH3)2	340.996	90.642	180.272	158.987	------
Compound 7	O	3-CF3	-CH2N(CH3)2	-----	342.363	269.989	179.868	160.011	------
Compound 8	O	CH=CH-CH=CH-	-CH2N(CH3)2	-----	5.10591	89.6349	179.658	160.9	------
Compound 9	C=CH2	4-Cl	-CH2N(CH3)2	-----	60.896	179.801	179.858	170.318	------
Compound 10	C	4-CL	-CH2N(CH3)2	-----	58.8397	271.843	180.045	169.618	------
Compound 11	C	4-CF3	-CH2N(CH3)2	-----	51.7162	270.561	180.402	159.525	------
Compound 12	O	CH=CH-CH=CH-	-CHCH2NHCH3	-----	342.403	90.2388	179.895	289.654	180.1

The modeling in this study was based on data published by SmithKline Beecham Pharmaceuticals. Their work consisted of a series of dopamine mimetics developed as potential therapeutic agents. The envisioned population that might use these agents included fertile female humans, and reproductive toxicity was therefore a concern. The dopamine mimetics were tested in rats, who were treated orally by gavage from days 6 to 15 post coitum. A qualitative assessment of developmental toxicity was examined (Ridings and Baldwin, 1992). After determining the teratogenic effects in rats, the authors used the data from the twelve dopamine mimetics and applied QSAR techniques (Ridings et al, 1992). From this excercise a model was established that would predict biological activity in rats. For their model, fifty-six physico-chemical parameters were analyzed for each dopamine mimetic. Twenty of their descriptors were based on specific atoms. The authors chose a correlation coefficient upper limit of 0.75 to eliminate redundant information. The final result was a 22 parameter model predicting biological activity of these dopamine mimetics.

In the present study, the original rat study data of these dopamine mimetics, was used to construct a hopefully equally accurate but mathematically simpler biological activity predictive model, using molecular modeling, physico-chemical properties, and statistical techniques. The outcome of this re-evaluation was a two independent single parameter models of similar productivity described by the molecules' HLB (hydrophilic lipophilic balance) and density.

2.7 MATERIALS AND METHODS FOR DOPAMINE MIMETIC QSAR

Computational Methods. The Molecular Modeling Pro™ (Version 3.14) and Molecular Analysis Pro™ (Version 2.0) published by ChemSW™ Inc. were used for computational analysis. Structures for the twelve dopamine mimetics were manually entered and molecular mechanics-based conformational analysis was performed on each model to generate its global minimum energy conformer. These lowest energy conformers were used to ensure that analyses using the semi-empirical Complete Neglect of Differential Overlap (CNDO) method would reach conformational convergence. CNDO analyses were performed with each model to identify eighteen molecule-specific parameters.

After completing the calculations, Minitab for Windows (Release 11.12) published by Minitab Inc. was used to perform statistical analysis. Histograms of all parameters were generated. It was seen from these metrics that diastereomers created a non-normality. There were four compounds that are diastereomers. The diastereomers display different electronic parameters such as HOMO, LUMO and dipole moment. The diastereomers were eliminated to achieve sufficient normality in the learning set.

The next step was to use Pearson's correlation matrix of all the parameters associated with the dopamine compounds, excluding the diastereomers. A correlation coefficient with an

upper limit of 0.9 was utilized to eliminate parameters that were highly correlated. Table 3 lists the parameters with correlations above 0.9. When two parameters are highly correlated, the final predictive model should not include both parameters.

Table 3. Correlations between parameters for Dopamine Mimetics

PARAMETERS	r-Value
molecular weight and surface area	0.951
molecular volume and surface area	0.962
Hansen dispersion and density	0.934
Hansen dispersion and solubility parameter	0.940
LUMO and molecular volume	-0.971
Hansen hydrogen bonding and Hansen polarity	0.931
H bond donor and Hansen polarity	0.923
percent hydrophilic surface and H bond donor	0.963
LUMO and MR	-0.909

The parameters with highest correlation coefficients were plotted against each other. Analysis of such plots was used to determine if the coefficient in the correlation matrix was falsely inflated by a certain outlier. This was not the case with any of the r-values generated. Therefore, the correlation between the parameters was a true correlation. Using the seventeen variables and eight compounds (the four diastereomers excluded), a best subset regression was performed using Minitab. The criterion used to chose the best model was the adjusted correlation coefficient, R-Sq (adj). Based on the R-Sq (adj), density and HLB were the variables with the greatest predictive capacity for the biological activity of the dopamine mimetics shown in Table 4.

Table 4. R-Squared values of candidate models

CANDIDATE MODELS	
Parameter	**R-Squared**
HLB	0.615
Density	0.587
Hansen Dispersion	0.519
Hydrogen Bond Acceptor	0.329
Molecular Weight	0.327
Dipole Moment	0.323
% Hydrophilic Surface	0.263
Hydrogen Bond Donor	0.220
LUMO	0.160
Solubility Parameter	0.143

Discriminant analysis was then performed using all twelve molecules and the parameters HLB and density. Discriminant analysis creates a final predictive model and evaluates model performance. The two final predictive models were:

Biological Activity of Dopamine Mimetics = 4.45 - 0.528 HLB

and

Biological Activity of Dopamine Mimetics = -7.30 + 7.14 density

Cross Validation. The leave one out cross validation technique was then applied to the two final predictive models. The results of this analysis are listed in Table 5.

Table 5. Leave one out cross validation results

Model	Sensitivity	Specificity	Proportion Correct	Squared Distance
HLB	1.000	0.857	0.917	5.482
Density	0.857	0.800	0.833	4.888

2.8 RESULTS OF DOPAMINE MODEL BUILDING

2.8.1 Modeling overall developmental toxicity based on the original author's evaluations

Compounds 1 (BRL 16644; 2-[[3,4-dihydro-2,2-dimethyl-4-[3-[trifluoromethyl]phenyl]-2*H*-1-benzopyran-7-yl]oxy]-*N*,*N*-dimethylethanamine; CAS 59257-24-8) and 2 (BRL 16657) were shown to be potent inhibitors of monoamine uptake in vitro and were developed as possible antidepressant agents (Ainsworth et al., 1982; Johnson et al., 1983). A series of congeneric compounds were generated based on their similar chemical structures (Figure 1), and all but compound 6 were found to have monoamine potentiating properties (Ridings and Baldwin, 1992). The dopaminergic potentiating properties of compounds 1 and 2 had been implicated with teratogenic activity (Baldwin and Ridings, 1986), however, so the potential for developmental toxicity was evaluated in vivo for the entire series (Ridings and Baldwin, 1992).

BRL 16644 (2-[[3,4-dihydro-2,2-dimethyl-4-[3-(trifluoromethyl)phenyl]-2*H*-1-benzopyran-7-yl}oxy}-*N*,*N*-dimethylethanamine) (Figure 1) was developed as a potential anorexigenic and antidepressant agent. Compound 1 is a potent monoamine uptake inhibitor that acts mainly as an agonist in the dopaminergic and somewhat in the serotonergic and noradrenergic systems. BRL 16657 (compound 2) is another dopamine mimetic, differing from compound 1only in the position of the trifluoromethyl moiety on the 4-phenyl ring. Compounds 1 and 2 have similar pharmacological properties (Ridings and Baldwin, 1992).

The 12 compounds chosen for evaluation of development toxicity potential are based on a similar chemical structure with alternate substitutions as given in Figure 1. To determine the physicochemical parameters necessary for modeling it was necessary to first determine the lowest free energy conformation. Bond lengths and angles corresponding to these conformations are given in Table 2. Analysis of the physicochemical parameters generated from these structures showed that several were highly correlated within this set of chemicals, especially lowest unoccupied molecular orbitals (LUMO) and molecular volume (Pearson's correlation = 0.971), percent hydrophilic surface and hydrogen bond donor (0.963), molecular volume and surface area (0.962), molecular weight and surface area (0.951), Hansen dispersion and solubility parameter (0.940), Hansen dispersion and density (0.934), Hansen hydrogen bonding and Hansen polarity (0.923) and LUMO and molar refractivity (0.909).

Ridings and Baldwin concluded that they had evidence for developmental toxicity in the testing of five chemicals: compounds 1, 2, 7, 8 and 11. In their discussions of the results of each chemical they considered three aspects of developmental toxicity: lethality, as indicated by post-implantation fetal loss, intrauterine growth retardation (IUGR), as indicated by fetal weight, and teratogenicity, i.e., incidence of structural malformation. Evidence of significant changes in any of these three endpoints was considered sufficient to establish the compound as a developmental toxicant. The three endpoints were highly correlated, with compounds 1, 2 and 7 positive for all all three, compound 11 positive for decreased fetal weight and incidence of malformation, and compound 11 positive only for teratogenicity (concordances of 83 – 92%).

Computational analysis of the physicochemical parameters and toxicological activity of these 12 dopamine mimetics yielded a number of single parameter models highly predictive for overall developmental toxicity. The most predictive factors are given in Table 6, along with the

results of the leave-one-out validations of their models. They are ordered by correlation coefficient, but this did not always perfectly correlate with predictivity, although the five single-parameter models given were not significantly different in predictivity. The "best" model, based on hydrophilic/lipophilic balance (HLB), misclassifies only compound 6, which is not a true dopamine mimetic. The physicochemical parameters these five models are based on are not themselves highly correlated, suggesting that they are classifying the compounds based on different properties. This is supported by the fact that the models misclassify different subsets of the input compounds.

Table 6. Single parameter models for predicting overall developmental toxicity using the original author's determinations.

	Correlation coefficient	Concordance	Sensitivity	Specificity	Misclassified compounds
HLB	0.784	0.917	1.000	0.857	6
Density	0.766	0.833	0.800	0.857	5, 8
Hansen dispersion	0.720	0.833	0.800	0.857	8, 12
hydrogen bond acceptor	0.574	0.667	0.571	0.800	4, 5, 9, 12
molecular weight	0.572	0.750	0.429	1.000	5, 9, 12

In this study, the original rat study data of these dopamine mimetics was used to construct an equally accurate, but mathematically simpler biological activity predictive model, using molecular modeling, physico-chemical properties, and statistical techniques. The outcome of this re-evaluation was a pair of single parameter models described by the molecules' HLB (hydrophilic lipophilic balance) and density. The models are:

Biological Activity of Dopamine Mimetics = 4.45 - 0.528 HLB

and

Biological Activity of Dopamine Mimetics = -7.30 + 7.14 density

Through statistical methods these models were validated and the results of the analysis show that, individually, the parameters HLB and density effectively predicted biological activity of all compounds except for compound 6. Compound 6 is, however, not truly a dopamine mimetic. Compound 6 is without any monoamine potentiating properties. Therefore, these two parameters were 100% effective at predicting the biological activity of the true dopamine mimetic compounds analyzed, and 91.7% effective at predicting the biological activity of all twelve compounds. The squared distance was 5.48, which showed that the means of the two groups were 5.48 standard deviations apart. This showed there was very little overlap between the groups when using ± 3 standard deviations per group. This also showed that the predictions were divided into two distinct groups (biologically active or inactive). Table 7 summarizes the results of this analysis and lists the five single parameters that individually are most associated with biological activity. In addition, this table provides the sensitivity and specificity of each of these physicochemical parameters when applied to the twelve dopamine mimetics as single parameter models.

Table 7. Best 5 Sin gle Parameter Models for Predicting Fetal Weight/Malformations- (compounds 1,2,7,11 categorized positive)

	Correlation coefficient	Concordance	Sensitivity	Specificity	Misclassified compounds
Density	0.926	1.000	1.000	1.000	
Hansen dispersion	0.860	0.917	1.000	0.875	12
dipole moment	0.796	0.917	1.000	0.875	12
hydrogen bond acceptor	0.743	0.750	1.000	0.625	4, 5, 12
HLB	0.699	0.917	1.000	0.875	8

Modeling individual developmental endpoints based on the original author's determinations

As mentioned above, developmental toxicity can be broken down into a number of specific effects that may or may not be mechanistically related. Ridings and Baldwin (1992) speculated that the dopaminergic potentiating properties of these compounds were directly related to their teratogenic activity, which is consistent with the high concordance of the three developmental toxicity endpoints of fetal death, IUGR and malformation found in this series (as well as with the misclassification of compound 6, which has no dopaminergic biological activity, in the HLB model above). While incidence of any of these harmful toxic effects would be sufficient to preclude the pharmacological use of a compound, pooling the developmental toxicity endpoints may not be the best way of modeling the phenomenon. As it stands, the models given in Table 6 essentially describe the endpoint of malformation, since all of the positive compounds were teratogenic. Adjustments to the database must be made, however, if fetal death and growth inhibition are considered separately.

Compound 8 was positive only for malformation, so modeling of IUGR can be done simply by considering it as negative (Table 8). Once again, there is no significant difference in the predictivity of the 5 single-parameter models given in Table 8, but the "best" model, based

on density, successfully predicts the toxicity of all 12 compounds. Indeed, the parameters in these models are very similar to those found in modeling malformation or overall developmental toxicity, except that the order has been changed and molecular weight has been replaced by dipole moment. All five models had perfect sensitivity, and compound 12 was misclassified as positive by three of the models. Compound 11 was positive for both teratogenicity and growth inhibition, but negative for induction of fetal death. Modeling fetal loss as an endpoint, with only compounds 1, 2 and 7 as positive, yields the single-parameter models given in Table 9. The parameters reiterate those from Table 8 with some changes in the order, and, once again, all 5 models have perfect sensitivity. Interestingly, compound 11 was consistently misclassified by all five models.

Table 8. Single parameter models for pred icting IUGR using the orgin al author's determinations.

	Correlation coefficient	Concordance	Sensitivity	Specificity	Misclassified compounds
Density	0.926	1.000	1.000	1.000	
Hansen dispersion	0.860	0.917	1.000	0.875	12
dipole moment	0.796	0.917	1.000	0.875	12
hydrogen bond acceptor	0.743	0.750	1.000	0.625	4, 5, 12
HLB	0.700	0.917	1.000	0.875	8

Table 9. Single parameter models for predicting induction of fetal death using the original author's determinations.

	Correlation coefficient	Concordance	Sensitivity	Specificity	Misclassified compounds
hydrogen bond acceptor	0.700	0.833	1.000	0.778	5, 11, 12
Density	0.680	0.917	1.000	0.889	11
dipole moment	0.614	0.917	1.000	0.889	11
HLB	0.587	0.833	1.000	0.778	8, 11
Hansen dispersion	0.576	0.833	1.000	0.788	5, 11

Next, the endpoints fetal death, fetal weight and malformations were used to generate predictive models for these specific endpoint. The original data was utilized to categorize the compounds as active or inactive for these specific endpoints. For this evaluation the endpoints fetal weight and malformations had exactly the same active and inactive compounds. Therefore only one model was run to predict both of these endpoints. The results of this analysis provided results similar to the other analyses, with the same five physicochemical parameters being associated with these endpoints. The results of the model development with the endpoint of fetal death produced two single variable models based on density and dipole moment. The resulting predictive equations are:

$$\text{Fetal Death} = -5.76 + 5.56 \text{ density}$$

and

$$\text{Fetal Death} = -0.381 + 0.284 \text{ dipole moment}$$

The sensitivity is 1.00 and the specificity is 0.889 for both of these models. Table 9 provides the sensitivity and specificity for the 5 best single parameter models associated with fetal death. The result of the model development with the endpoint of fetal weight/malformations produced a single variable model based on density that has a sensitivity is 1.00 and the specificity is 1.00. The study based on the endpoint of fetal death produced two models based on density and dipole moment that were 91.7% correct at predicting the impact of the twelve compounds on fetal death. Both of these fetal death models misclassified compound 11. Using the fetal death model a compound with a density of approximately 1.22 or greater or a dipole moment greater than 4.86 indicates the compound is active for fetal death.

Table 10 Maternal toxicity

Compound	Dose (mg/kg)	No. mated	No. pregnant	No. died	No. stereotypy	Bodyweight gain (g)
control		70	63	0	0	55.5
1	10	14	12	0	0	57.2
	20	28	26	0	0	43.6
	40	14	10	4	5	13.3
2	10	14	14	0	0	31.6
	20	42	36	4	8	6.4
	35	14	7	4	10	-3.0
3	2	14	11	0	0	56.8
	4	14	14	0	0	46.4
	8	14	12	0	0	35.0
	10	12	9	1	2	34.4
	12	14	8	3	9	25.3
4	5	14	14	0	0	53.3
	10	14	13	1	0	36.4
	14	14	13	1	3	26.1
5	1.2	14	10	1	0	45.1
	2	12	7	1	2	25.1
6	30	14	12	1	0	43.2
	100	14	14	0	0	32.3
7	25	14	12	0	0	25.2
	50	14	9	2	5	-3.2
8	20	14	11	2	0	39.1
	30	14	10	2	2	42.4
	50	14	4	7	13	20.0
9	1	14	13	0	0	37.3
	2	14	12	0	2	39.9
	3	14	10	2	4	35.6
10	4	14	5	5	11	22.5
11	2	14	14	0	0	40.9
12	20	14	12	0	0	31.4
	30	14	6	3	11	25.0

Table 7 lists the 5 best single parameter models associated with the endpoints fetal weight and malformations and the sensitivity and specificity of each.

The resulting predictive equation for these endpoints is:

Fetal Weight/Malformations = -8.59 + 8.25 density

Applying the fetal weight/malformation model a density value of 1.16 or greater is indicative of a compound that is active for growth retardation and/or malformations.

2.8.2 Modeling developmental toxicity after reevaluation of the raw data

The previous modeling has been based exclusively on the conclusions drawn by the authors of the original report containing the data on the possible developmental toxicity of this set of compounds. In some cases, the authors provided enough raw data to allow for an independent assessment of their results, as summarized in Table 10. This allows us to not only confirm for ourselves the conclusions they reported, but also go beyond those results by exploring different methods of data evaluation. To be confident in establishing a toxic effect, the experimental data on a compound should ideally fulfill two criteria: 1) one or more doses should exhibit a significant change in the toxic endpoint, and 2) there should be evidence of dose-dependency. In the current data, only the former criterion was considered, indeed, compounds 10 and 11 were analyzed with a single dose, precluding the possibility of establishing dose dependency. In addition, the data in Table 11 was pooled from five original sets of experiments; in the original report, each experiment was compared only to the concurrent control. We have determined that the controls for the five experiments were not statistically different from one another, and have therefore pooled them—comparison to this pooled control group allows for greater power in subsequent analyses.

Table 11. Summary of develop mental toxicity data from Ridings and Bal dwim (1992) (pooled from five experiments).

Compound	Dose (mg/kg)	No. litters	Litter size	Perinatal loss (%)	Fetal wt (g)	No. fetuses	No. malformed
control		62	11.0	4.3	5.4	681	1
1	10	12	9.7	4.9	5.8	116	0
	20	25	10.9	2.8	5.5	272	38
	40	8	8.9	27.2	4.8	71	57
2	10	14	10.1	4.0	5.6	141	6
	20	33	9.5	10.9	5.1	313	288
	35	4	9.8	9.2	5.1	39	39
3	2	11	9.3	10.1	5.6	102	0
	4	14	11.2	3.8	5.6	157	0
	8	11	12.7	2.7	5.3	140	0
	10	9	11.0	2.7	5.6	99	0
	12	7	11.6	2.4	5.4	81	0
4	5	14	11.2	5.1	5.5	157	0
	10	13	12.2	3.3	5.3	159	0
	14	10	11.9	6.1	5.4	119	0
5	1.2	10	10.8	0.9	5.7	108	0
	2	7	10.9	3.6	5.4	76	0
6	30	12	11.7	2.6	5.2	140	0
	100	14	11.7	5.6	5.4	164	0
7	25	11	7.6	37.3	5.0	84	66
	50	6	4.8	60.1	4.4	29	29
8	20	11	12.2	2.4	5.5	134	0
	30	9	12.2	4.5	5.5	110	0 (1)
	50	2	9.5	10.0	5.5	17	0 (1)
9	1	13	9.9	6.2	5.6	129	0
	2	10	10.6	5.4	5.5	106	0
	3	10	11.5	3.1	5.6	115	0
10	4	4	9.5	8.8	5.8	38	0
11	2	14	10.4	3.2	4.9	145	2
12	20	12	10.4	2.6	5.4	125	0
	30	6	11.0	11.8	5.3	66	0

Enough raw data was provided in the original report (or could be regenerated from data given in the original report) to reevaluate two of the three toxic endpoints used to establish overall developmental toxicity: incidence of post-implantation fetal death and incidence of malformations. Variabilities were not provided for the fetal weights measured in these experiments, therefore a rigorous reevaluation of the IUGR data cannot be performed. Some general observations can be made, however. Evidence for statistically lower fetal weights were presented for four compounds, compound 1 (in 1 experiment at the highest dose), compound 2 (in 2 of 3 experiments at the intermediate dose), compound 7 (in 1 experiment at each of two doses) and compound 11 (in 1 experiment at the only dose tested). There is no evidence of dose response in the data from compound 1 (P = 0.23), since the weight of the fetal controls was lower than that observed at the lower doses of the compound. There is also no evidence for dose-dependency in the data from compound 2 (P = 0.27); the fetal weight at the highest dose is the same (although non-significantly lower than controls) as that at the intermediate dose, despite the fact that several other compounds induce lower weights (so the weight attained does not represent a lower limit). There is a trend towards dose-dependency in the data from compound 7 (P = 0.09), and no opportunity to assess dose-dependence for compound 11. Thus, if dose-dependency were required to establish toxicity, none of these data would be sufficient.

Evidence for teratogenicity, induction of fetal malformation, was presented for five compounds. The number of term fetuses with specific malformations was significantly increased by compound 1 at the two higher doses, by compound 2 at all three doses and by compound 7 at both doses. Indeed, the dose-dependence of the first two compounds approached significance (both P = 0.053), but not that of compound 7 (P = 0.31). Compound 8, however, as shown in Table 11, had no malformed fetuses at term at any dose and should therefore have been considered negative for teratogenicity. It was classified as a teratogen because malformations were noted in two of the dead fetuses “lost” post-implantation, one at each of the upper doses of

this chemical. It is not clear whether similar analyses were performed on the lost fetuses of all of the other negative experiments, or of the controls. Moreover, even if the malformed dead fetuses are accepted as relevant data, they fail to establish significant increases in the frequency of malformation at either dose: for the 30 mg/kg dose a single malformed fetus added to the total number of fetuses is not significant ($P = 0.24$), regardless of whether the additional "lost" fetuses are considered and assumed to be normal. If the frequency is compared only that of to the specific concurrent control (no malformed fetuses out of 110 term, 115 total animals) the results are even less compelling ($P = 0.38, 0.43$, respectively). The single malformed fetus at the 50 mg/kg dose occurred in a much smaller population, but also occurred in the experiment where there was also a malformed fetus among the control progeny (out of 141 term, 151 total fetuses), such that the increase is not significant ($P = 0.20$). Only if this frequency is compared with that of the pooled controls (one malformed fetus out of 681 term, 712 total fetuses) does it begin to approach significance ($P = 0.076, 0.053$, respectively). Pooling the results of the 30 and 50 mg/kg doses still does not infer a significant increase in malformation, whether or not the other dead fetuses are taken into account when compared to either concurrent ($P = 0.19$) or pooled controls ($P = 0.063, 0.062$). If this compound is reclassified as negative for teratogenicity, physicochemical modeling yields the same series of models as those given in Table 8.

When compound 8 was classified as a negative and also when eliminated the analysis yielded predictive models based on density that properly classified all of the compounds used to generate the models. Finally, the assessment of fetal weight/malformations generated a model based on density that was 100% correct in classifying all twelve compounds.

Next, new models were generated with compound 8 categorized as both totally eliminated from the data set and as a negative (Table 12). These new models with a recategorized compound 8 were generated due to the questionable call of compound 8 being biologically active. When the models were run with compound 8 as a negative the five

physicochemical properties associated with biological activity remained the same with the exception of molecular weight, which was replaced by dipole moment when compound 8 was categorized as a negative. Additionally, when compound 8 was categorized as a negative the discriminant analysis results revealed the physicochemical parameter density produced sensitivity and specificity both of 1.00. The results of the discriminant analysis with compound 8 eliminated provided the same list of crucial parameters as when compound 8 was categorized as a negative, and identical sensitivity and specificity as the model when compound 8 was listed as a negative. These results display that the original categorization of compound 8 as a positive biologically active agent are suspect and that the determination that compound 8 is biologically active based on 2 dead malformed pups is a questionable call. The predictive model generated with compound 8 categorized as a negative is:

Biological Activity of Dopamine Mimetics = -8.59 + 8.25 density

and the predictive model generated with compound 8 eliminated is:

Biological Activity of Dopamine Mimetics = -8.55 + 8.21 density

Finally, the application of the predictive models was completed to determine values of the specific parameters in the models that would indicate biological activity of unknown compounds with similar structure to those used to develop the model. Using the model generated from the original teratogenicity calls and compound 8 eliminated a density value of 1.16 or greater is indicative of a compound that is teratogenic.

Table 12. Single parameter models for predicting induction of malformation after removal of compound 8 from the data set.

	Correlation coefficient	Concordance	Sensitivity	Specificity	Misclassified compounds
Density	0.922	1.000	1.000	1.000	
Hansen dispersion	0.854	0.909	1.000	0.857	12
dipole moment	0.788	1.000	1.000	1.000	
HLB	0.777	0.909	1.000	0.857	6
hydrogen bond acceptor	0.729	0.727	1.000	0.571	4, 5, 12

The classification of compound 11 as a teratogen is based on the occurrence of 2 malformed fetuses in the term population of the only dose tested, 2 mg/kg. This incidence of malformation is also not significantly elevated over either the concurrent controls (once again, the one experiment where a malformed fetus was observed amongst the controls) (P = 0.38) or the pooled controls (P = 0.076). Models based on this data set with both compounds 8 and 11 classified as negative for toxicity have already been presented in Table 9 (and consistently predict that compound 11 should be positive for toxicity).

The original authors found only three compounds to induce fetal loss: compound 1, with a significant increase in post-implantation loss at the highest drug concentration; compound 2, with a significant increase in fetal death in 1 of 2 experiments performed at the intermediate dose; and compound 7, with significantly increases in fetal death at both doses examined. Reanalysis with pooled data confirms that these observations are highly significant ($P < 0.0001$). The data for compounds 1 and 2 are essentially one point curves, however, and there is no evidence of dose-dependency for any of the three compounds (P = 0.15, 0.24 and 0.22, respectively). The highest concentration of compound 2 does induce an increase in post-implantation loss that approaches significance (P = 0.089). Several other experiments also reach significance when compared to the pooled controls, however; there was a great deal of range amongst the five controls in this endpoint, from a low of 1.6% loss to a high of 6.5%, giving different experiments different abilities to detect an increase in fetal death. The lowest concentration of compound 3 tested now also has a significantly increased frequency of post-implantation loss (P = 0.013), as well as the higher concentration of compound 12 (P = 0.007), while several other experiments approach significance: the lowest concentration of compound 9 and the only concentration tested of compound 10, P = 0.089 and 0.085, respectively). Thus, by the criterion that any significant change is sufficient basis for activity, compound 12 should be

considered positive for toxicity, despite the fact that there is no evidence of dose-dependency (P = 0.67), and although there is not enough evidence to establish compounds 9 and 10 as positive, it might be prudent to remove them from the analysis as equivocal. Compound 3 presents a much more difficult problem (akin to that of compound 9), however: the significant point is the lowest in the concentration curve, and, indeed, instead of evidence of increased fetal loss with increased dose, the regression approaches significance in the opposite direction, that increased dose results in less fetal loss (P = 0.085). Even if a single experiment is sufficient to establish toxicity in the absence of evidence of dose-dependence, it is difficult to allow a single experiment to establish toxicity in the face of evidence against dose-dependence.

The only other factor to be considered in the evaluation of this data primarily concerns those chemicals determined to have no evidence of developmental toxicity. To be considered as negative for activity, these compounds must have been adequately tested, which in the field of developmental toxicity means they must have been tested to the point of maternal toxicity. A summary of the pertinent maternal toxicity data from Ridings and Baldwin (1992) is given in Table 9. Three types of maternal toxicity were measured, of which only the first truly meets the requirement for demonstration of significant maternal toxicity: maternal death. No females died in any of the five control groups, whereas death was observed in the animals treated with all twelve compounds. The original authors did not test these data for significance, however, and not all of these incidences are significant if compared to the pooled controls. The following compounds were found to induce significantly increased frequencies of death: compound 1 at the highest dose (P = 0.0005), compound 2 at the 2 highest doses (P = 0.02 and 0.0005, respectively), compound 3 at the highest dose (P = 0.004), compound 7 at the highest dose (P = 0.03), all three doses of compound 8 (P = 0.03, 0.03 and < 0.0001, respectively), the highest dose of compound 9 (P =0.03), compound 10 at the only dose tested (P < 0.0001) and compound 12 at the higher of the two doses tested (P = 0.004). Of these compounds, there was evidence of

dose-dependence in the maternal toxicity of only two, compounds 2 (P = 0.048) and 8 (P = 0.047), with two others showing a trend towards dose-dependence, compounds 3 (P = 0.062) and 4 (P = 0.097) (despite the fact that no single dose exhibited a significant rate of maternal loss). Determining the adequacy of the testing is most important for chemicals not shown to have activity in the toxicity tests, therefore these data confirm the validity of subset of the "negative" tests that excludes those involving compounds 4, 5, 6 and 11.

Besides death, two other indicators of toxicity were determined in the treated females in these experiments. First, characteristic behavioral changes, or "stereotypy" were observed in a subset of animals (Table 10). The incidence of stereotypy was highly correlated with maternal death ($P < 0.0001$, R = 0.91), and every experiment but one that showed a significantly increased incidence of maternal death also showed a significant increase in stereotypy (no such behavior was observed in any of the control groups). A number of experiments were significantly increased for stereotypy in the absence of fetal death, including the second highest dose of compound 3 (P = 0.02), the highest dose of compound 4 (P = 0.004), the highest dose of compound 5 (P = 0.02) and the intermediate dose of compound 9 (P = 0.026). None of these effects showed clear evidence of dose dependency, although several came close: compounds 2 (P = 0.055), 3 (P = 0.079) and 9 (P = 0.054). If an increase in stereotypy is accepted as representative of maternal toxicity, compounds 4 and 5 may then be considered as negative compounds for developmental toxicity. Compounds 6 and 11 are therefore the only compounds negative for some or all types of developmental toxicity that may be considered to not to have been adequately tested.

3 RETINOIC ACID CHERNOFF-KAVLOCK (CK) ASSAY

3.1 BACKGROUND

13-cis retinoic acid (cis RA) or isotretinoin is a drug commonly used in the treatment of severe cystic acne (Chan et al, 1996). In humans, cis RA exposure during pregnancy is established as being a potent teratogen (No authors listed, 1989). Clinical observations evidence suggests that cis RA is characteristic of heart and craniofacial malformations including ear and palatal defects (Goulding and Pratt, 1986). In animal testing, several studies have indicated that cis RA is embryopathic in the monkey, mouse, chick embryo, hamster, rat and rabbit, causing malformations and fetal death (Wei, 1999; Hummler, 1990; Mallo, 1997; Hart, 1990; Eckhoff, 1997; Ward, 1995; Tzimas, 1994). Malformations of the ear were observed in monkey fetuses exposed to cis RA (Wei et al, 1999). Malformations and fetal death were assessed in the cynomolgus monkey with cis RA. Malformations that included both external ears, hypo- or aplasia of the thymus and ventricular septal defects of the heart (Hummler et al, 1990). In the mouse, malformations in the middle ear were observed (Mallo, 1997). The effects of cis RA in the chick embryo occurred in mesenchymal tissues derived from the cranial neural crest cells. There were also craniofacial and cardiovascular malformations following the treatment with cis RA (Hart et al, 1990). In whole rat embryos, a study of all trans-, 13-cis- and 9-cis retinoic acid was conducted and malformations seen were limb reduction defects associated with short term rise in embryonic retinoid levels (Ward et al, 1995). Cis RA treatment significantly increased fetal resorptions and malformations in rabbits at a dose of 15 mg/kg/day (Eckhoff et al, 1994). Testing for developmental toxicants can be performed utilizing SAR modeling and animal

assays. Through modeling the compound 13-cis retinoic acid was predicted to be positive for developmental toxicity.

3.2 QSAR/SAR Validation Using the CK Assay

The validation of this model was then tested by an animal assay. The animal assay utilized in this study was a modified version of the Chernoff-Kavlock (CK) assay. In this test, pregnant female mice were orally gavaged with 13-cis retinoic acid. The dosing of this compound was during the period of major organogenesis. There were three dosage levels of retinoic acid which induce a certain amount of maternal toxicity. The mother is examined during the entire pregnancy for signs of toxicity. The dams are allowed to deliver the pups. The endpoints evaluated in this modified version of the Chernoff-Kavlock assay were fetal death and growth retardation. To determine growth retardation, pups were tagged and weighed on postnatal day one and three. To determine fetal death the number and status of all pups born was monitored. After postnatal day three the dams were killed and determination of implantation sites were conducted.

3.3 Materials for Retinoic Acid CK Assay Validation

Chemicals. Cis-Retinoic acid was obtained from Fisher Scientific. Diphenylhydantoin, corn oil, ammonium sulfide was obtained from Sigma -Aldrich.

Animals. Time-pregnant outbred Swiss, Hia® mice, were obtained from Hilltop Lab Animals (Scottdale, PA). The experiment was performed at Magee-Women's Research Institute (MWRI). MWRI provided animal technicians to assist with animal care and all procedures were approved by the Institutional Animal Care and usage Committee.

3.4 METHODS FOR RETINOIC ACID CK ASSAY VALIDATION

Computational Methods. Molecular Modeling Pro™ (Version 3.14) and Molecular Analysis Pro™ (Version 2.0) were used for QSAR/SAR calculations.

Group Assignment for the Modified Chernoff/Kavlock Assay. On gestation day (GD) 3, body weights were recorded and animals were ranked from lightest to heaviest. Animals were excluded based on physical condition or extremes in body weight. Selection of a permutation (i.e., 1, 2, 3) was given to each animal. The permutations had as many numbers as there were groups (i.e., dosing levels). The permutations were rotated to provide an equal weight distribution in all groups.

Modified Chernoff/Kavlock Assay. The mice were divided into 20 mice per group based on the group assignment. The groups consisted of a negative control (corn oil), positive control (diphenylhydantoin), high (100%), medium (50%), and low (25%) of the 10% lethal dose of retinoic acid. The animals were housed individually in cages with wood shavings for bedding and were given standard mouse chow and water. The room was kept on a 12-hour light/dark cycle with constant temperature at 70° F. Dosing of the animals started on gestation day six. Dosing was done by oral gavage. The animals were dosed in the morning at the same time each day. Treatment continued until day 14 of gestation. Animals were allowed to deliver. Some animals delivered prematurely on gestation day 15. None of the dams that delivered prematurely survived. The majority of the dams delivered on day 19. All living pups were tagged and weighed on postnatal days one and three. The pups and dams were sacrificed on postnatal day three. The uterus of each dam was examined for implantation sites both before and after staining with ammonium sulfide.

Uterine Staining with Ammonium Sulfide. After the third day, postnatal pups and dams were humanely sacrificed by cervical dislocation. Dams were necropsied and the uterus

excised. The uterus was placed in a petri dish. A solution of 10% ammonium sulfide staining solution was prepared in a fume hood. Each uterus was treated at room temperature with approximately 5 ml of the 10% ammonium sulfide solution. After 15 minutes, each uterus was examined for darkly-stained implantation sites. The number of positively stained implantation sites for each uterus was recorded. The implantation sites that were evident prior to staining were also recorded.

3.5 Results of Retinoic Acid QSAR/SAR Validation Using the CK Assay

The endpoints of interest were maternal toxicity, growth retardation and fetal death. Even though the primary endpoint of interest was developmental toxicity, maternal toxicity was also examined to evaluate drug dosing.

3.6 Maternal Toxicity

A slight maternal toxicity relationship was demonstrated for all three dose groups of cis RA at 25%, 50%, and 100% of the 10% lethal dose. To evaluate maternal toxicity, a plot of change in weight from gestation day 4 to gestation day 8 versus dose was generated (grams versus dose mg/kg/day) Figure 2.

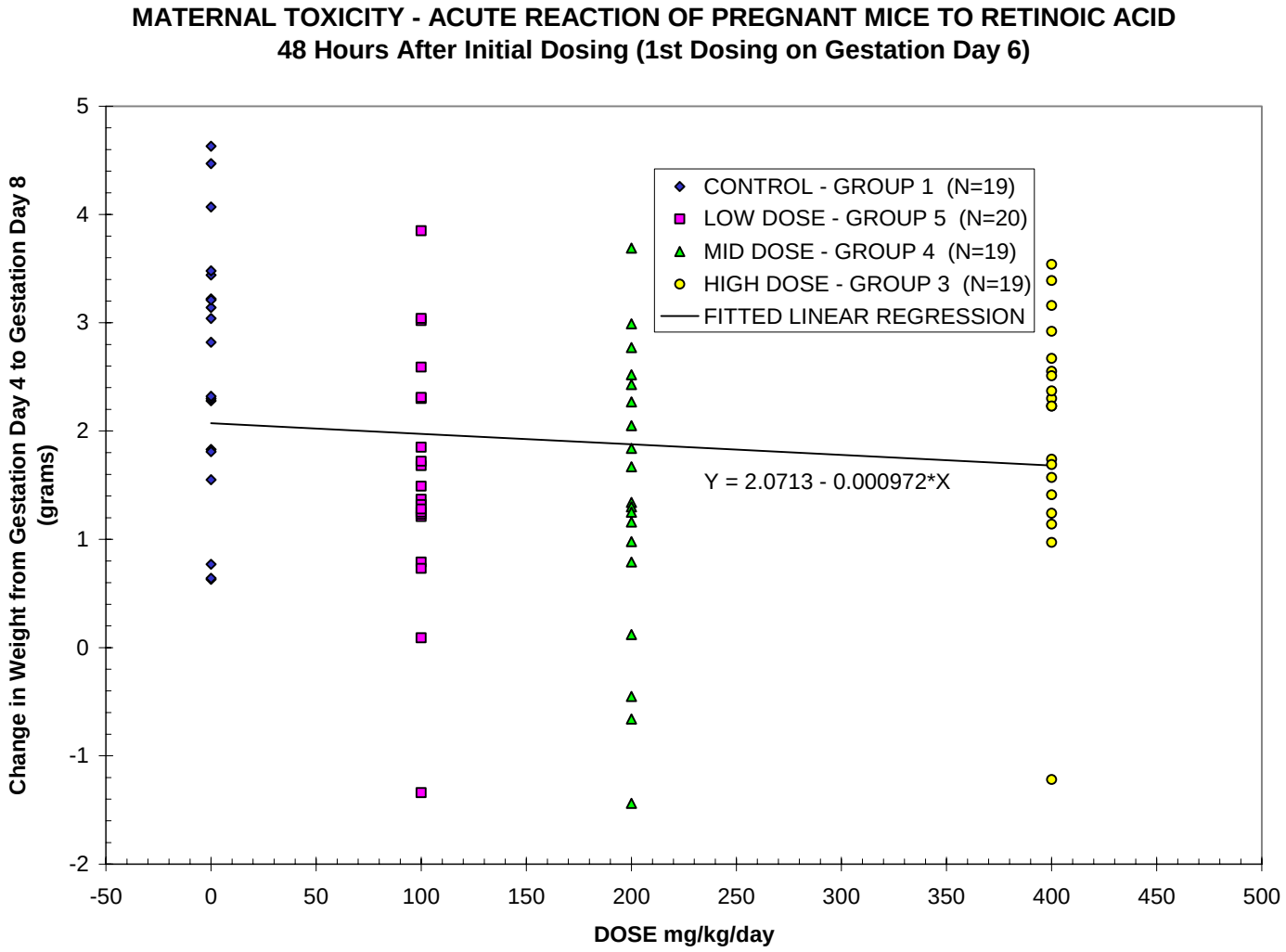


Figure 2 Maternal Toxicity- Acute Reaction of Pregnant Mice to Retinoic Acid

This plot was considered to be an acute reaction of the pregnant mice to cis RA at 48 hours after the initial dosing. In this case, a statistically significant maternal toxicity relationship was not observed, although there was a slight reduction in weight gain of dams as the dose increased. To further analyze maternal toxicity, another plot was generated displaying the chronic reaction of pregnant mice to cis RA at 216 hours after initial dosing Figure 3.

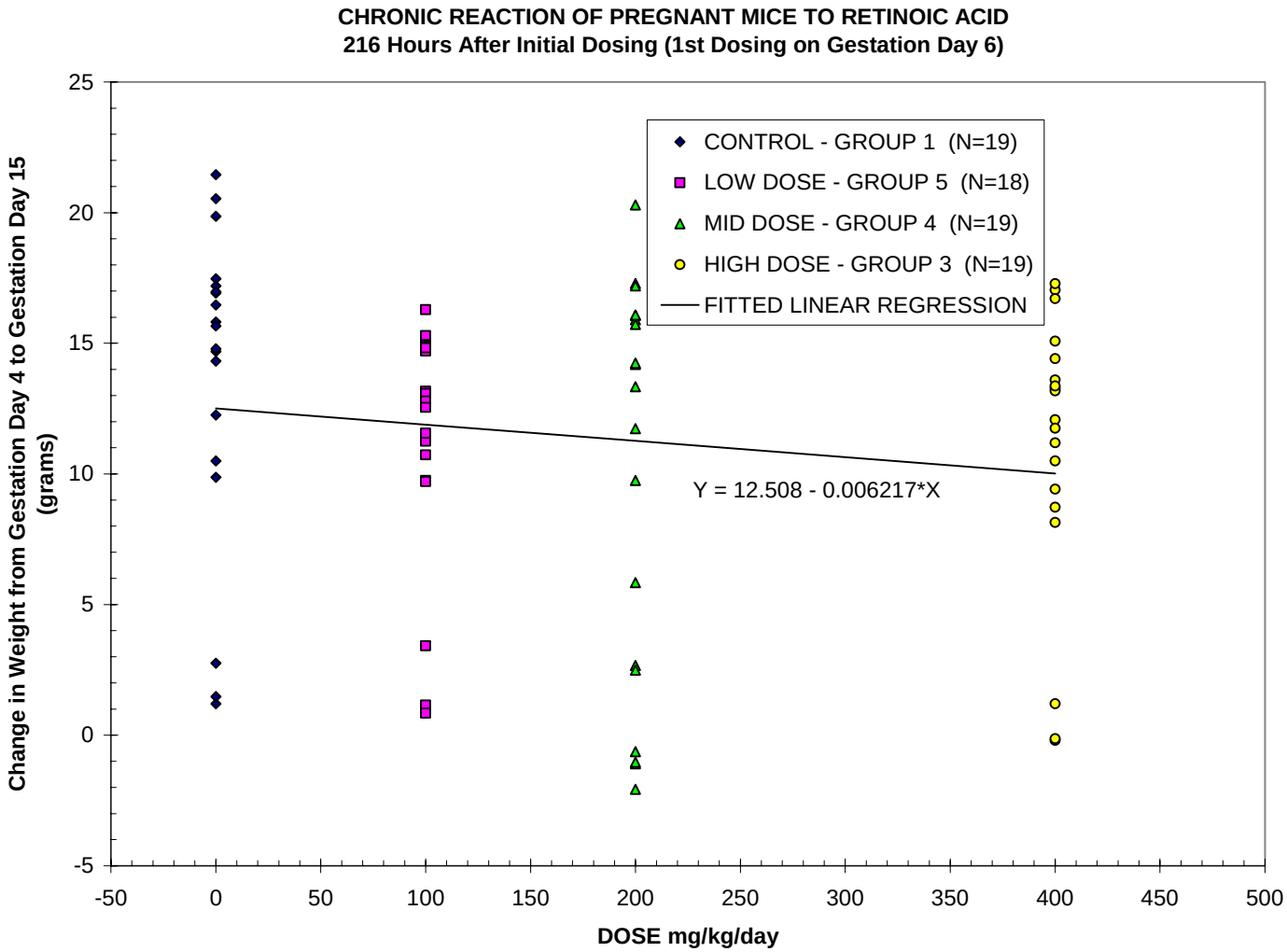


Figure 3. Maternal Toxicity- Chronic Reaction of Pregnant Mice to Retinoic Acid

For this plot, the dams change in weight from gestation day 4 to gestation day 15 was measured and plotted against dose (grams versus dose mg/kg/day). Again, there was a slight reduction in weight gain as the dose increased; however this was not statistically significant.

3.7 PUP GROWTH RETARDATION

Another endpoint of interest was growth retardation. To evaluate growth retardation, the pups were tagged and weighed on postnatal day 1 and postnatal day 3. A plot of the change in weight (grams) versus dose as a percentage of LD_{10} was generated and is shown in Figure 4.

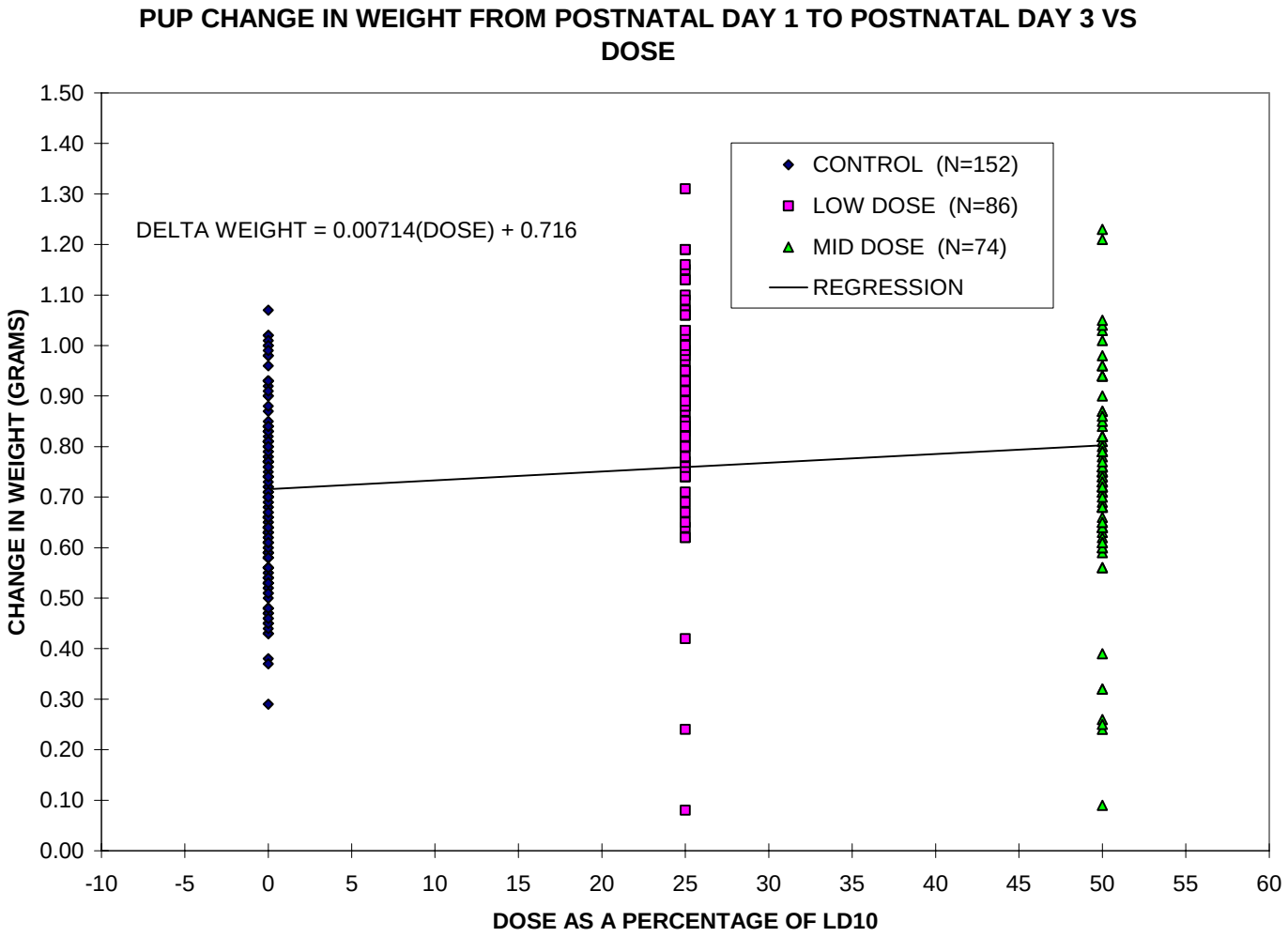


Figure 4. Growth Retardation- Pup Change in Weight from Postnatal Day 1 to Postnatal Day 3 Versus Dose

There was no high dose data included in Figure 4 because only 2 pups were born alive to a dam and neither of the pups survived to postnatal day 3. This plot shows a slight upward trend in pup weight gain as the dose of cis RA increased.

3.8 PUP DEATH

The next step was to examine fetal death. Fetal death was classified into four categories: prenatal; neonatal; postnatal; and total death. For this study, prenatal death was determined as the percentage of implantation sites that never developed into a fetus. A plot of the percentage of implantation sites that never developed into a fetus versus dose as a percentage of LD_{10} is shown in Figure 5.

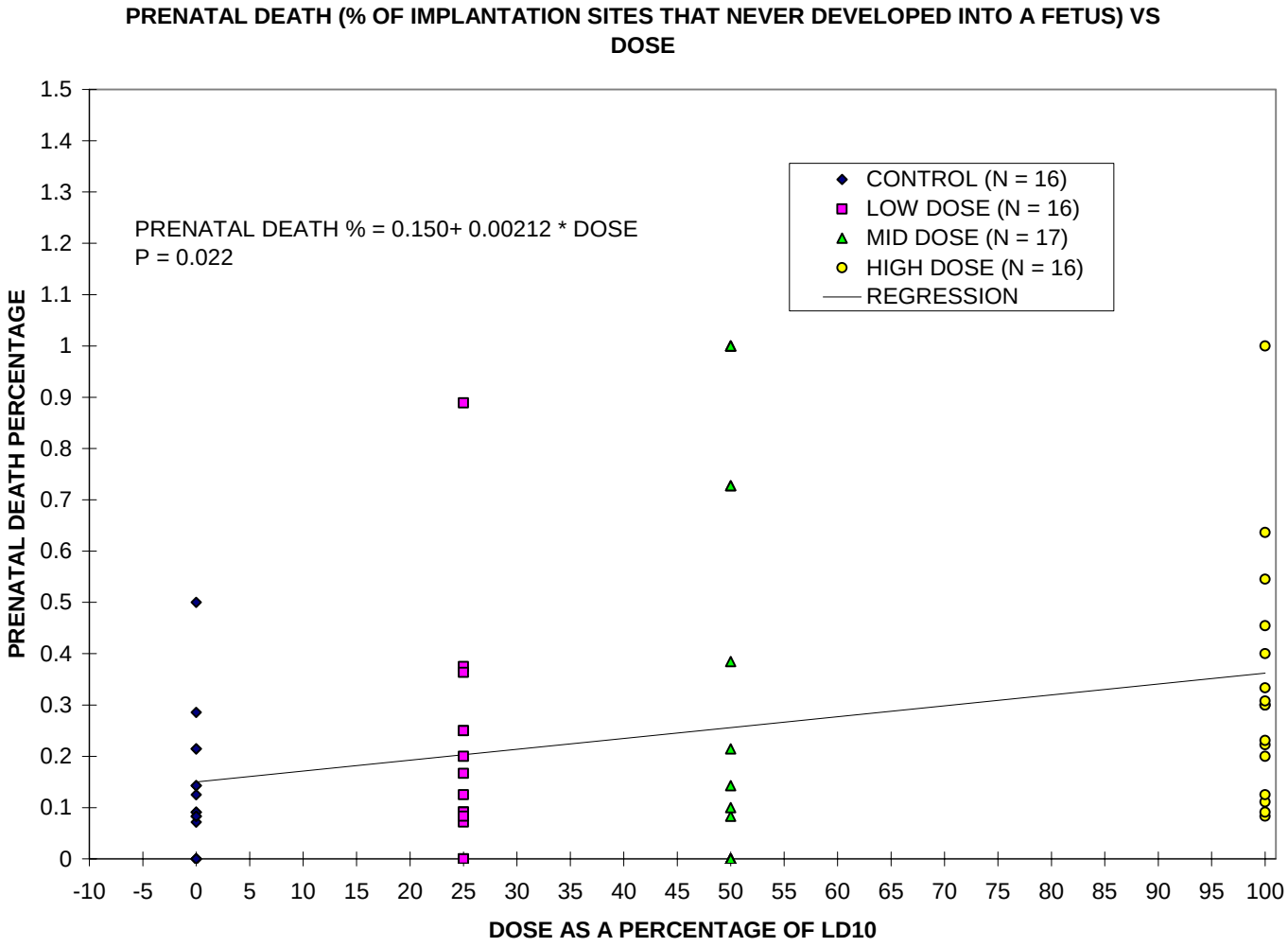


Figure 5. Prenatal Death- Percent of Implantation Sites That Never Developed Into a Fetus Versus Dose

In this case, nonpregnant dams were excluded and, as the dose increased, so did prenatal death. The p value was 0.022, which is statistically significant.

Neonatal death was determined to be the percentage of pups born dead as a proportion of total births. To evaluate neonatal death, a plot of the percentage of pups born dead as a proportion of total births versus dose as a percentage of LD_{10} was generated Figure 6.

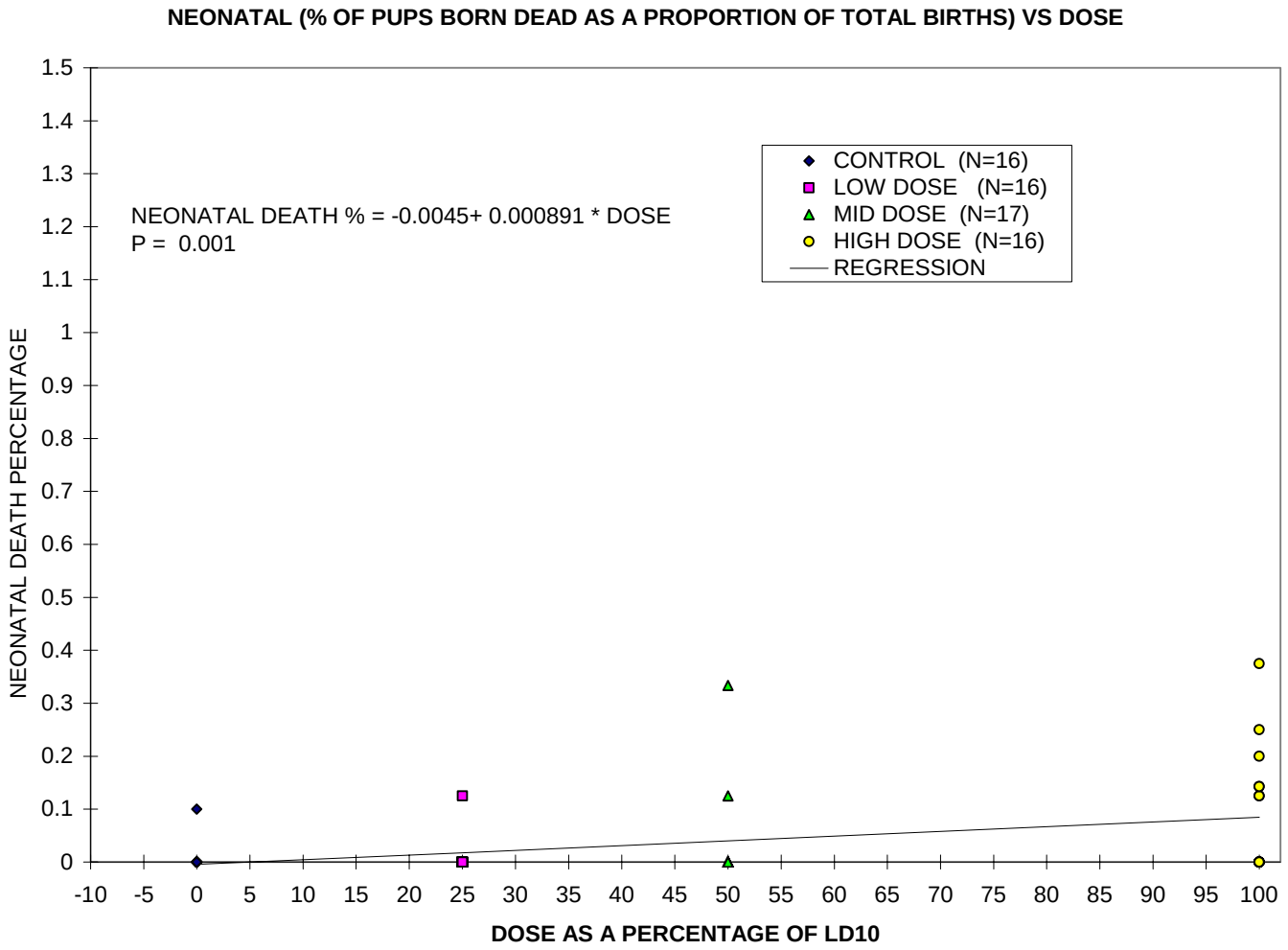


Figure 6. Neonatal Death- Percent of Pups Born Dead as a Proportion of Total Births Versus Dose

The nonpregnant dams and dams that never gave birth to live pups were excluded from this analysis. This evaluation showed that as the dose increased, so did neonatal death. The p value was 0.001, showing a statistically significant relationship.

Postnatal death was determined as the percentage of pups that were born alive and died before postnatal day 3. To evaluate postnatal death, a plot of percentage of pups that were born alive and died before postnatal day 3 versus dose as a percentage of LD_{10} was generated and shown in Figure 7.

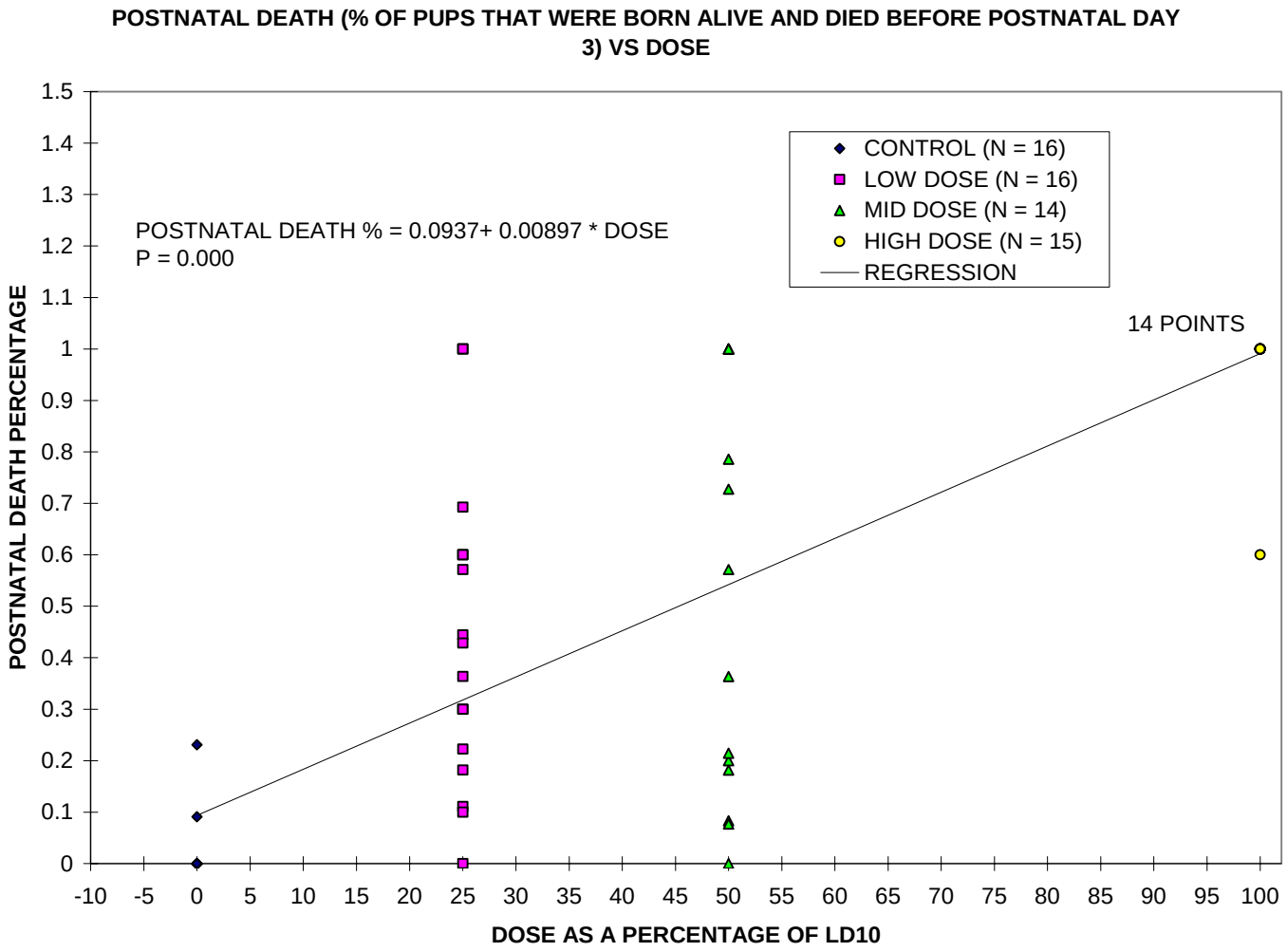


Figure 7. Postnatal Death- Percent of Pups That Were Born Alive and Died Before Postnatal Day 3 Versus Dose

In this study, the nonpregnant dams and dams that never gave birth to live pups were excluded. As the dose of cis RA increased, postnatal death also increased. Based on statistical evaluation, this relationship was significant, with a p value of <0.001. In considering the graph of this information, it should be pointed out that there appears to be an influential point in the high dose group. However, this is not the case, because this point actually represents 14 dams. In the high dose group there were 14 dams that had complete loss of litter postnatally, and only one dam that delivered two pups that survived postnatally.

Total death was determined as the percentage of implantation sites that did not develop into surviving pups. A plot of the percentage of implantation sites that did not develop into surviving pups versus dose as a percentage of LD_{10} was generated and is shown in Figure 8.

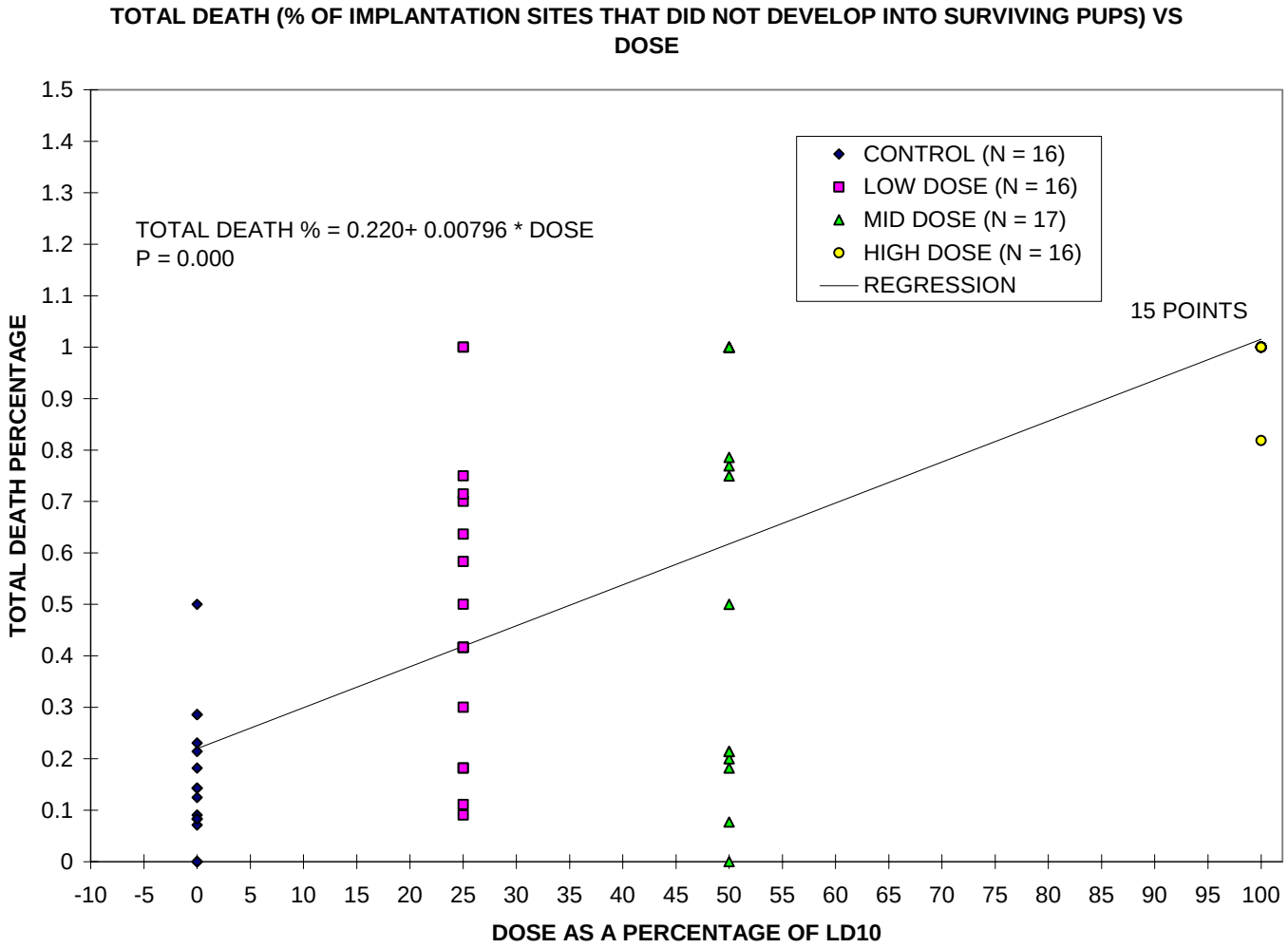


Figure 8. Total Death- Percent of Implantation Sites That Did Not Develop Into Surviving Pups Versus Dose

In this evaluation, nonpregnant dams were excluded. This evaluation showed that as dose increased, total death increased, and this relationship was statistically significant. The p value of this relationship was < 0.001. In the high dose group there were 15 dams that had complete loss of litter (one more than the previous evaluation because one pregnant dam in the high dose group did not deliver any pups) and one dam had two surviving pups.

3.9 RETINOIC ACID CHERNOFF-KAVLOCK (CK) ASSAY DISCUSSION

The assay used quantitatively measures effects on fetal viability and growth. In our assay, malformations were not examined. Instead, death was examined because malformed pups are more likely to be transformed into fetal death. This happens because malformed pups and pups with functional defects are more likely to die and more likely to be killed by their mother.

In this study, maternal toxicity was slightly evident but not significant. Maternal toxicity would be critical if a developmental toxicity relationship was not observed, because this would raise the question of whether the dose used was high enough. In this case, the lack of maternal toxicity was not critical since a significant developmental toxicity relationship was observed.

No effect on fetal growth as the dose increased was observed. In fact, the reverse was seen with a slight increase with change in weight. This seems contradictory, as it seems plausible that a toxic chemical should cause less weight gain. A possible explanation for this is that there were fewer surviving pups as dose increased, and therefore the survivors in the higher dose groups had less competition for nutrients from the mother.

The endpoint that was influenced by cis RA was fetal death. This can be seen in Figures 5,6,7 and 8. In all instances, when the dose increased the number of surviving pups decreased. In all categories of fetal death that were evaluated, the relationship between increased dose and increased death was statistically significant, giving a p value of 0.05.

4 TRANSGENIC ADENOCARCINOMA OF THE MOUSE PROSTATE MOUSE MODEL

4.1 BACKGROUND

Adenocarcinoma of the prostate is the most common cancer in American men. (Gingrich and Greenberg, 1996). Due to public health awareness, early detection of prostate cancer has improved; for example, the implementation of widespread PSA-based screening programs has enhanced the ability to diagnose prostate cancer at an early stage (Foster et al, 1997). Regardless of such awareness, prostate cancer remains the second leading cause of cancer deaths in American men (Parker et al, 1997).

The implementation and utilization of newly developing cancer drugs and models is key to arresting or curing this commonly diagnosed disease. The use of an animal model that closely mimics prostate cancer development in humans is an ideal way of studying initiation, progression and metastasis. The Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mouse model was developed to study a wide variety of issues in prostate cancer. The TRAMP model was genetically engineered so that each mouse will develop prostate cancer with age. Many other mouse models use human prostate cancer cell grafts onto the prostate to produce the disease. However, in this model, the mice usually have a defective immune system that prevents rejection of the graft. This results in an unnatural situation in which researchers cannot evaluate the normal interplay between cancer, its native environment and the immune system (SoRelle, 2003). The progression of the disease in the TRAMP model closely mimics what is seen in

humans, and since the cancer is developed within the TRAMP mouse, preventative studies can also be conducted.

The TRAMP mouse model was developed by taking a minimal probasin promoter containing 426 bp of 5' flanking sequence and 28 bp of 5' untranslated sequence of the rat probasin gene to target expression of SV40 large T-antigen to the epithelium of the mouse prostate. In the TRAMP model, prostatic disease progresses from mild to severe intraepithelial neoplasia, to focal adenocarcinoma that metastasizes to the lymph nodes, lungs, and occasionally to the bone, kidney, and adrenal glands (Gingrich et al, 1996). TRAMP mice develop prostatic intraepithelial neoplasia by 8-12 weeks of age that progress to adenocarcinoma with distant metastases by 24-30 weeks of age. Cell lines were derived from the prostatic adenocarcinoma of a 32 week old C57BL/6 TRAMP mouse. From this single animal, three cell lines were developed: TRAMP-C1; TRAMP-C2; and TRAMP-C3. C1 and C2 are tumorigenic and C3 is nontumorigenic. From C1 and C2, six clonal cell lines were created by three rounds of limiting dilutions. The C1 line produced the C1A and C1D lines. The C2 line produced C2D, C2G and C2N lines. C1A, C2G, C2H and C2N cells are tumorigenic when grafted into syngenic C57BL/6 male hosts (Foster et al, 1997).

The previous section discussed the utilization of an animal model as a way of studying the progression of prostate cancer development. In this section, four promising investigational antiproliferative cancer agents will be discussed. These agents are Analog II, 4-methoxy Analog II, JR oxime 1 and TDH 169. These agents inhibit tubulin polymerization by binding at the colchine site of the protein. Tubulin polymerization is critical for the separation of sister chromatids in mitosis and is a proven antitumor target.

Analog II is a cyclopropyl stilbene derivative first found to be antiestrogenic in the mouse (Magarian and Benjamin, 1975). Several in vitro and in vivo studies have shown Analog II to

have activity against both MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) breast cancer cells, an effect not mediated by the estrogen receptor (Jain et al, 1997). In breast cancer cell lines, Analog II disrupts microtubules and causes apoptosis (Balachandran et al, 1999). Analog II at low concentrations has been shown to block PSA production and cell proliferation in the human androgen-responsive prostate cancer cell line LNCaP (Balachandran et al, 2000).

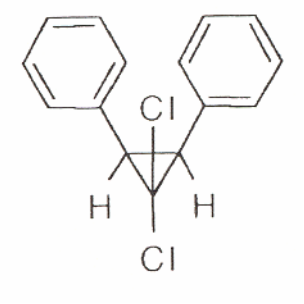


Figure 9. Struture of Analog II

4-methoxy Analog II is a derivative of Analog II. It is a more potent inhibitor of tubulin perturbation and cell proliferation. This compound has been shown to inhibit prostate specific antigen production and to induce apoptosis in both LNCaP and androgen independent human prostate cancer PC-3 cell lines (Balachandran et al, 2000).

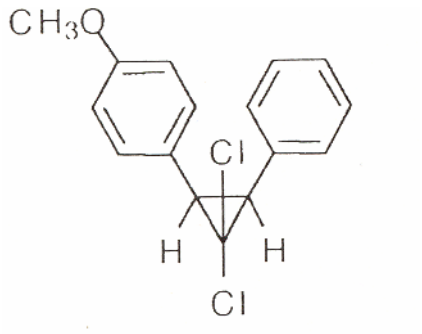


Figure 10. Structure of 4- Methoxy Analog II

JR oxime 1 (3,4,5-trimethoxybenzaldehyde O-(8-hydroxy-5-methyl-8-thiophen-2-ylocta-2,4-dienyl)oxime) is an analog of the potent tubulin polymerization inhibitor curacin A. Curacin A is derived from the cyanobacterium (blue green algae) *Lyngbyna majuscula*, found off the coast of Curacao (White et al, 1997). JR oxime 1 is less lipophilic than its parent compound curacin A. JR oxime 1 is more potent at inhibiting tubulin assembly than curacin A, and JR oxime 1 is only slightly weaker at inhibiting cell growth than curacin A (Wipf et al, 2002).

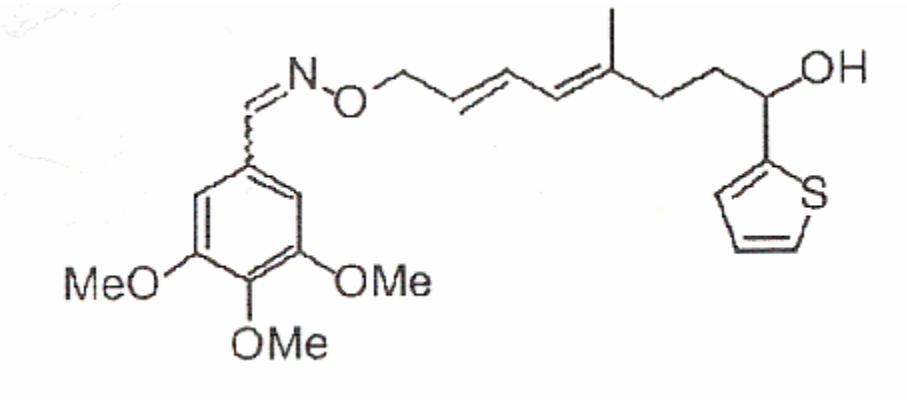


Figure 11. Structure of JR Oxime 1

TDH 169 (5'-hydroxy-4'H-spiro[1,3-dioxolane-2,1'-naphthalen]-4'-one) is a member of a library of compounds originally prepared as thioredoxin inhibitors. Subsequent studies have shown it to have effects on tubulin assembly. It is antiproliferative at low micromolar concentrations against human breast, prostate and ovarian cancer cells (Wipf et al, 2001).

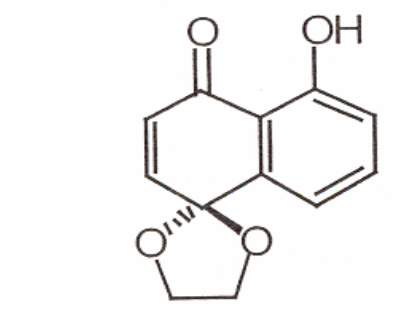


Figure 12. Structure of TDH 169

The goal of this portion of the studies was to test the effects of the four promising antiproliferative agents against TRAMP cell lines. One of the aims of the study was to identify at least one of the compounds as a potential chemopreventative agent for prostate cancer. To accomplish this, at least one of the agents has to have low toxicity and effectively reduce the proliferation of the metastasis of the prostate cancer cell.

4.2 MATERIALS AND METHODS FOR TRAMP CELL LINE SCREENING

4.2.1 Materials

Chemicals. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) phenol free high glucose with L-glutamine and without sodium pyruvate media obtained from Gibco. The media was supplemented with 10% fetal bovine serum obtained from Hyclone, 5 μg/ml insulin obtained from Sigma, 25 u/ml penicillin-streptomycin obtained from Gibco, and $1X10^{-8}$ M dihydrotestosterone obtained from Sigma. Media was changed every three days. Cells were split when they reached confluency by rinsing in Ca++, Mg ++-free Hanks balanced salts solution obtained from Gibco. The cells were then detached with 0.25% trypsin until cells were released. The test agents were synthesized in the labs of Professors Billy Day and Peter Wipf, University of Pittsburgh. The compounds were of >99% purity as determined by ^{1}H and ^{13}C NMR and high resolution mass spectrometry.

Cell lines. The clonal cell lines were isolated from cell lines TRAMP-C1 and TRAMP-C2 by three rounds of limiting dilution. Clonal cell lines C1A were isolated from TRAMP-C1 and C2H and C2N were isolated fromTRAMP-C2. The cells were obtained from Dr. Barbara Foster, Roswell Park Cancer Institute.

Doubling Time. Doubling times of the cell lines in vitro were determined by plating the cells in 96 well plates at 1200 cells per well and counting cells in triplicate wells every 12 hours using the MTT assay. Doubling times were calculated from the log phase of the growth curves. The doubling times were calculated to be approximately 16 to 18 hours.

MTT Stock Solution. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) stock solution (10 mg/ml) was prepared as follows: 0.3 g MTT dissolved in 30 ml of PBS was filtered with a Steriflip 50 ml disposable vacuum filtration system through a 0.22 μM Millipore Express™ membrane. The stock solution of MTT was stored at –20°C. Each MTT stock solution was stored for a maximum of one month before being replaced.

4.2.2 Methods

Growth Inhibition Studies. The C1A, C2H and C2N cells were seeded at 1200 cells/well in 96 well plates and in 100 μl of complete culture media. After 24 hours the cells were treated with various concentrations of agents dissolved in DMSO (final concentration 0.2%). The concentrations for the first screening was prepared using progressive five-fold dilutions to generate seven concentrations (640 pM to 10 μM). The concentrations used for the second screening was a two fold dilution, and was centered around the GI50 from the first screen. The control for each cell line contained DMEM media and 0.2% DMSO.

The effect of the agents, Analog II, 4-methoxy Analog II, TDH 169, JR oxime 1 on viability of the cells was determined using the MTT assay. The MTT assay is a colorimetric assay that measures the reduction of MTT by mitochondrial succinate dehydrogenase. MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored formazan product. Since reduction of MTT occurs in metabolically active cells, the level of activity is a measurement of the viability of the cells. The quantity of formazan product is directly proportional to the number of living cells in culture. Time points of continuous agent

exposure considered were 0, 24, 48 and 72 hours. MTT was added to each well and incubated for 3 hours. After incubation, 100 μl of 20% SDS was added to each well and the plate was placed back in the incubator overnight. The following morning, the plate was read with a microplate reader (BioRad). The absorbance at 595 nM was recorded for each well.

Calculation of GI_{50}, TGI and LC_{50}. The GI_{50} is defined as the concentration of agent that gives a 50% reduction in the net cell increase as compared to the control cells during the period of incubation. The equation for GI_{50} is 50= [100 X (1-((average cell count of treatment group - average cell count of time 0)/(average cell count of control group - average cell count of time 0))]. The total growth inhibition or TGI is defined as the agent concentration that gives 100% reduction in the net cell increase as compared to the control cells during the incubation. The equation for TGI is (average cell count of treatment) = (average cell count of time 0). The LC_{50} is defined as the concentration of the test agent resulting in a 50% reduction in the measure of cell growth at the end of the drug treatment as compared to that at the beginning. The equation for LC_{50} is –50=[100 X ((average cell count of treatment group -average cell count of time 0) / (average cell count of time 0))] (Boyd and Paull, 1995). After finding the GI_{50}, TGI, and LC_{50} the slope of the line is calculated using at least two points that surround the GI_{50}, TGI, or LC_{50} and solving with the equation y=mx+b. Using this slope of a line, equations and the following values for y, 50 (for GI_{50}), 100 (for TGI) and –50 (LC_{50}).

Apoptosis Assay ELISA. Cells were plated in 6-well plates at 20,000 cells/well. Cells were treated with 4-methoxy Analog II at concentrations based on the MTT assay. The concentrations used were the calculated GI_{50}, GI_{70} and TGI of 4-methoxy Analog II. The cells were allowed to attach for 24 hours and then the test agent was added. Twenty-four hours after the test agent was added the ELISA (Roche Diagnostics, Indianapolis, IN) assay was performed. The ELISA assay measures small DNA fragments and histones, with a mixture of anti-histone

and anti-DNA-POD. During this incubation period, the anti-histone binds to the histone-component of the nucleosomes and simultaneously captures the immunocomplex to the coated microtiter plate. Additionally, the anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes. The unbound antibodies are removed by washing. There is quantitative determination of the amount of nucleosomes by the POD retained in the immunocomplex. The amount of nucleosomes is determined spectrophotometrically.

Protein Extraction and Western Blot Analysis. The mouse prostate cell line C1A was plated and cultured in complete media and allowed to attach for 24 hours, followed by the addition of the TGI concentration of 4-methoxy Analog II (1.5 μM) and incubated for 0, 24, 48, 72 hours. Control cells were incubated in the media with 0.2% DMSO using the same time points. After incubation, the cells were harvested by scraping from the culture dishes and collected by centrifugation. A cell count was then taken using a Coulter Counter. Cells were resuspended in a 100 μl protease inhibitor cocktail containing 1ml lysis buffer and 20 μl of 50X protease inhibitors (PharMingen). A freeze-thaw method was then applied using ethanol and dry ice. The protein concentration was then determined using the Bio-Rad Protein Assay Reagent. For each sample, 50 μg of total proteins was resolved on 10% SDS Page and transferred to a nitrocellulose membrane. The membrane was incubated with primary monoclonal PARP rabbit antibody (1:1000) purchased from Cell Signaling Technologies for 1 hour and washed two times in Tween/PBS. Secondary anti-rabbit antibody from ECL Western blotting system (Amersham) was added. After 1 hour, the membrane was washed three times with Tween/PBS. The substrate from the ECL kit was then added and protein bands were detected using Kodak film. Films were scanned using a Kodak X-Ray Developer, model number X-OMAT 2000 Processor, vendor Baldwin.

Flow Cytometry. AnnexinV (PharMingen, San Diego, CA) staining and flow cytometry were conducted to detect apoptotic cells. C1A cells were treated with 1.5 μM 4-methoxy Analog II for 0, 12, 24, 48 and 72 hours, or with 0.2% DMSO (control). There were four controls used to set up the flow cytometric parameters. These were: blank plus 100 μl of annexin buffer, 5 μl of annexin V and 100 μl of annexin buffer, 5 μl of propidium iodide (Sigma) and 100 μL of annexin buffer. Samples were prepared with annexin v and propidium iodide and divided into two groups; stained versus unstained. The samples were then analyzed on an EPICS-XL benchtop cytometer (Beckman Coulter) and analyzed using EXPO 32 software.

RNA Isolation. The TRIZOL reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues consisting of a mono-phasic solution of phenol and guanidine isothiocyanate (Chomczynski and Sacchi, 1987). Addition of chloroform followed by centrifugation separates the solution into aqueous and organic phases. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample are recovered by sequential precipitation (Chomczynski, 1993). Precipitation with ethanol yields DNA from the interphase and an additional precipitation with isopropyl alcohol yields proteins from the organic phase (Chomczynski, 1993). Copurification of the DNA is useful for normalizing RNA yields from sample to sample. This technique works well with small quantities of tissue (50-100 mg) and numbers of cells ($5 \text{ X } 10^6$).

Cells were lysed directly in the 3.5 cm culture dish by adding 1 ml of TRIZOL reagent and passing the cell lysate several times through a pipette. The homogenized samples were incubated for 5 min at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform (0.2 ml per 1 ml of TRIZOL reagent) was added and tubes were vigorously shaken by hand for 15 seconds, then incubated at 30°C for to 3 minutes.

The samples were centrifuged at 12,000 X g for 15 minutes at 8°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained in the aqueous phase.

The aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol per 1 ml of TRIZOL reagent used for the initial homogenization. Samples were incubated at 30°C for 10 minutes, then centrifuged at than 12,000 X g for 10 minutes at 8°C. The RNA precipitate, formed a gel-like pellet on the side and bottom of the tube.

The supernatant was removed and the RNA pellet was washed once with 1 ml of 75% ethanol per 1 ml of TRIZOL reagent used for the initial homogenization. The sample was mixed by vortexing and centrifuged at 7,500 X g for 5 minutes at 8°C.

At the end of the procedure, the RNA pellet was vacuum-dried for 5-10 minutes. The RNA was dissolved in 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 60°C. RNA can also be redissolved in 100% formamide (deionized) and stored at -70°C (Bracete et al, 1998).

DNA Microarray. These studies were carried out in collaboration with the University of Pittsburgh Genomics and Proteomics Core Labs. The Affymetrix Genechip® system is a unique microarray technology utilizing a patented photolithographic process to manufacture 25-mer oligonucleotides (probes) directly on the array surface. Each mRNA or EST sequence is represented by 11 probe pairs (the probe set for this gene.) Each probe pair consists of one feature containing a perfect match probe (PM) and an adjacent feature containing a mismatch probe (MM). The sequences of the two probes differ by one base in the central position.

Gene expression levels in tissues or cultures are measured from total RNA isolated from the sample of interest and processed to create biotinylated cRNA. Five to forty micrograms of

total RNA was used as template in a reverse transcription reaction using oligo$(dT)_{24}$ primers attached to a T_7 RNA polymerase promoter sequence (1X Invitrogen 1st strand buffer, 5 μM T7-$(dT)_{24}$ primer, 1mM DTT, 500 μM each dATP, dGTP, dCTP, dTTP, 200-1000 U Superscript II, incubated for 1 h @ 42^{o}C). This single stranded cDNA was transformed into double stranded cDNA by addition of 10 U DNA Ligase, 40 U DNA Polymerase I, additional dNTPs to 200 μM each, 2U RNAse H and 2nd strand buffer to 1X, followed by incubation at 16^{o}C for 2 hours. Ten units of T_4 DNA Polymerase were added and the reaction incubated at 16^{o}C for an additional 5 minutes. The reaction was stopped by the addition of EDTA to 0.03 M and applied to an Affymetrix cDNA clean-up column. At the end of the second strand reaction, the cDNA sample was mixed with DNA binding buffer and this mixture applied to the column. The column was spun in a microfuge to bind the cDNA to the membrane. DNA wash buffer supplied with the kit was used to wash the membrane, which was then dried by centrifugation. The cDNA was eluted with the kit-provided elution buffer. An aliquot of the ds-cDNA equivalent to 5-7 μg of starting RNA was added as template to an in vitro transcription reaction as per the ENZO BioArray high efficiency RNA transcript labeling kit. The resulting biotinylated cRNA was purified cleaned using an Affymetrix RNA clean up column. The procedure was identical to that for the DNA clean up using appropriately modified membranes and buffers as supplied. After elution, the cRNA was quantified by reading the OD_{260} of a 1:100 dilution on a spectrophotometer. An aliquot of 20 micrograms of cRNA was incubated at 94^{o}C in fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc) for 35 min to break the RNA into segments of 35 to 200 bases. A 1 μl aliquot of the sample was run on an Agilent Bioanalyzer to verify that fragmentation resulted to give in RNA of the desired size distribution.

Fifteen micrograms of the fragmented RNA was added to a final volume of 300 μl hybridization cocktail (1X hybridization buffer, 100 μg/ml Herring sperm DNA, 50 μg/ml

acetylated BSA, 50 pM Affymetrix Control Oligo B2, 1X Affymetrix Eukaryotic Hybridization Control the 1X hybridization buffer containeded 100 mM MES, 1M Na+, 20 mM EDTA, 0.01% Tween 20.) An appropriate volume of this sample was applied to the GeneChip® and the chip was incubated overnight at 45^{o}C with rotation. Following hybridization, the sample was removed and the GeneChip cassette filled with non-stringent wash buffer. The chip was loaded onto an Affymetrix Fluidics station for washing and staining. Wash and stain protocols were the double stain protocols developed by Affymetrix for use with the Affymetrix Fluidics Station. To remove unbound sample, arrays were first washed with non-stringent wash buffer (6X SSPE, 0.01% Tween 20) followed by a stringent wash in 100 mM MES, 0.1M Na+, 0.01% Tween 20. The GeneChips® were then stained for 10 minutes in streptavidin-phycoerythrin (SAPE) solution (1X MES stain buffer, 2 mg/ml acetylated BSA, 10 μg/ml SAPE; 1X MES stain buffer contained 100 mM MES, 1M Na+, 0.05% Tween 20). Non-stringent buffer was used to wash off the first stain solution. Signal amplification was achieved by 10 minutes of incubation with biotinylated anti-streptavidin (1X MES stain buffer, 2 mg/ml acetylated BSA, 0.1 mg/ml normal goat IgG, 3 μg/ml biotinylated anti-streptavidin) followed by a second 10 min incubation with SAPE. The chip was washed and filled with non-stringent wash buffer before being removed from the fluidics station and scanned using the GeneArray® scanner.

Basic absolute analysis was performed using the Microarray Analysis Suite (MAS) 5.0 with each chip scaled to a median signal intensity of 150. The signal from each probe set was calculated from the intensity levels measured for each PM and MM probe pair in that set. Signal levels reflected the abundance of expression of a given gene in the sample. In addition, MAS 5.0 calculated a detection p value. This parameter provided a measure of the probability that the gene is present in the transcriptome of the sample and therefore a measure of the reliability of the calculated signal value.

4.3 RESULTS OF TRAMP CELL STUDY

To test the effects of these four compounds; 4-methoxy Analog II, Analog II, JR oxime 1 and TDH 169, on cell growth, three different cell lines, C1A, C2H, C2N,were treated with 0.2% DMSO as a control, and a double screening method was used to calculate the GI_{50}, TGI and LC_{50}. Colchicine was used as a positive control. The first screen consisted of test agent concentrations in a five fold dilution series (640 pM to 10 μM). The second screening was centered around the GI_{50} of the first screen, using 2- fold dilutions (0.625 to 1 μM). In the first screening, Analog II displayed GI_{50}, TGI and LC_{50} greater than the maximum concentration used (10 μM on all three cell lines); therefore, Analog II was eliminated from the second screening. Tables 13, 14 and 15 summarize the results of the first and second screenings on all three cell lines, and show that 4-methoxy Analog II was the most promising compound to carry forward. 4-methoxy Analog II was the most potent of the four agents screened, and behaved similarly across all three cell lines. 4-Methoxy Analog II also exhibited the most dramatic concentration dependent inhibition on growth as compared to the other drugs screened (Figures 13-21).

Table 13. GI_{50} TGI and LC_{50} of Test Agents Against the C1A Cell Line

Compound	GI_{50} (μM)	TGI (μM)	LC_{50} (μM)
4-methoxy Analog II	0.29 ± 0.06	1.5 ± 0.70	5.3 ± 1.1
Analog II	> 10 μ M	> 10 μ M	> 10 μ M
JR oxime 1	5.4 ± 1.3	> 10 μ M	> 10 μ M
TDH 169	2.0 ± 0.30	3.3 ± 0.0	3.3 ± 0.0
Colchicine	0.04 ± 0.01	> 10 μ M	> 10 μ M

Table 14. GI50, TGI and LC50 of Test Agents Against the C2H Cell Line

Compound	GI_{50} (μM)	TGI (μM)	LC_{50} (μM)
4-methoxy Analog II	0.32 ± 0.06	1.7 ± 0.68	4.0 ± 3.5
Analog II	> 10 μ M	> 10 μ M	> 10 μ M
JR oxime 1	5.8 ± 0.95	> 10 μ M	> 10 μ M
TDH 169	2.9 ± 0.29	4.8 ± 0.89	8.96 ± 0.0
Colchicine	0.04 ± 0.008	> 10 μ M	> 10 μ M

Table 15. GI50, TGI and LC50 of Test Agents Against the C2N Cell Line

Compound	GI_{50} (μM)	TGI (μM)	LC_{50} (μM)
4-methoxy Analog II	0.36 ± 0.06	1.6 ± 0.90	3.7 ± 1.1
Analog II	> 10 μ M	> 10 μ M	> 10 μ M
JR oxime 1	5.4 ± 2.1	> 10 μ M	> 10 μ M
TDH 169	0.78 ± 0.23	1.2 ± 0.0	1.1 ±0.82
Colchicine	0.08 ± 0.02	> 10 μ M	> 10 μ M

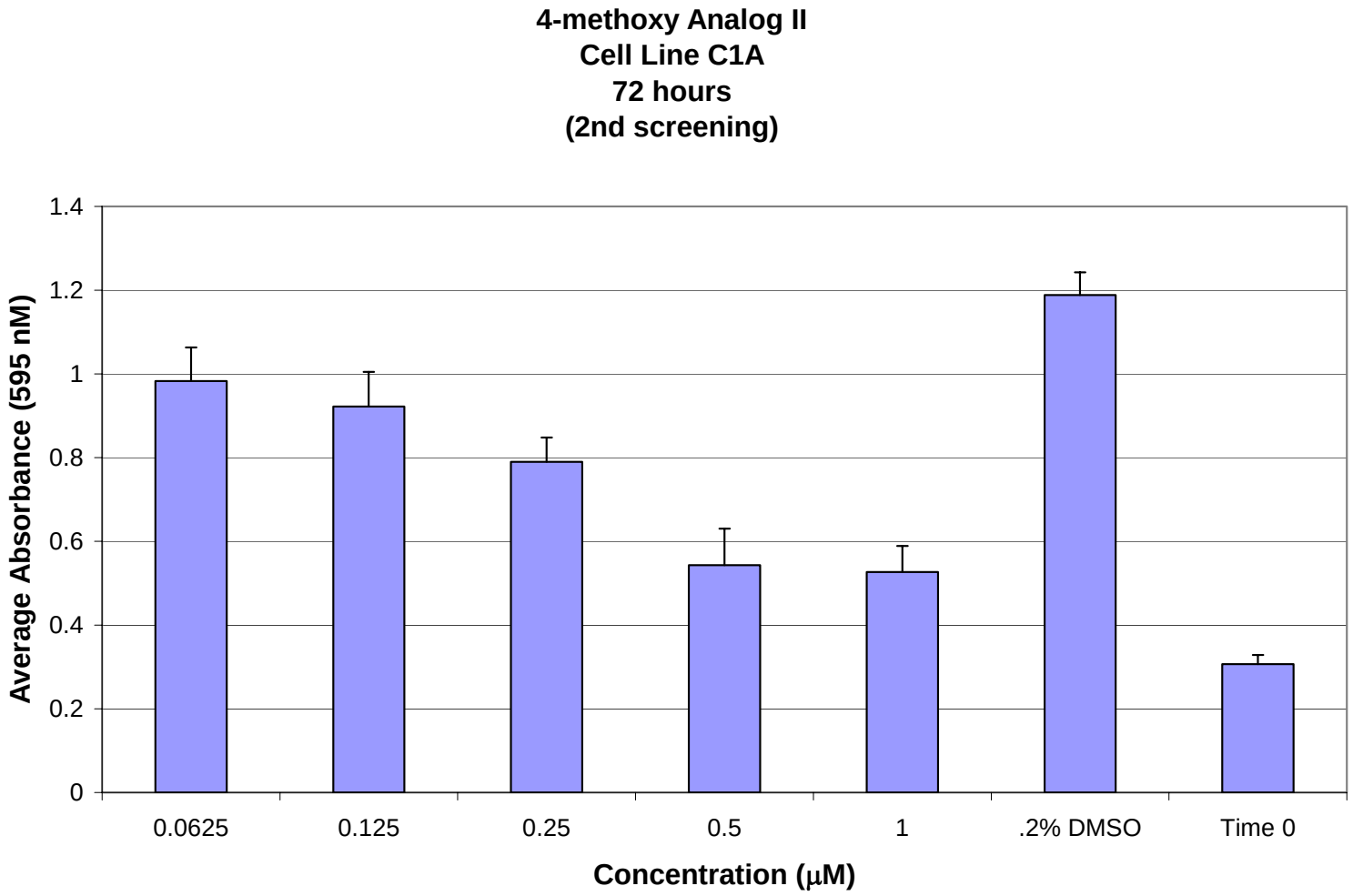


Figure 13. Concentration-Dependent Growth Inhibition of 4-Methoxy Analog II on the TRAMP C1A Cell Line

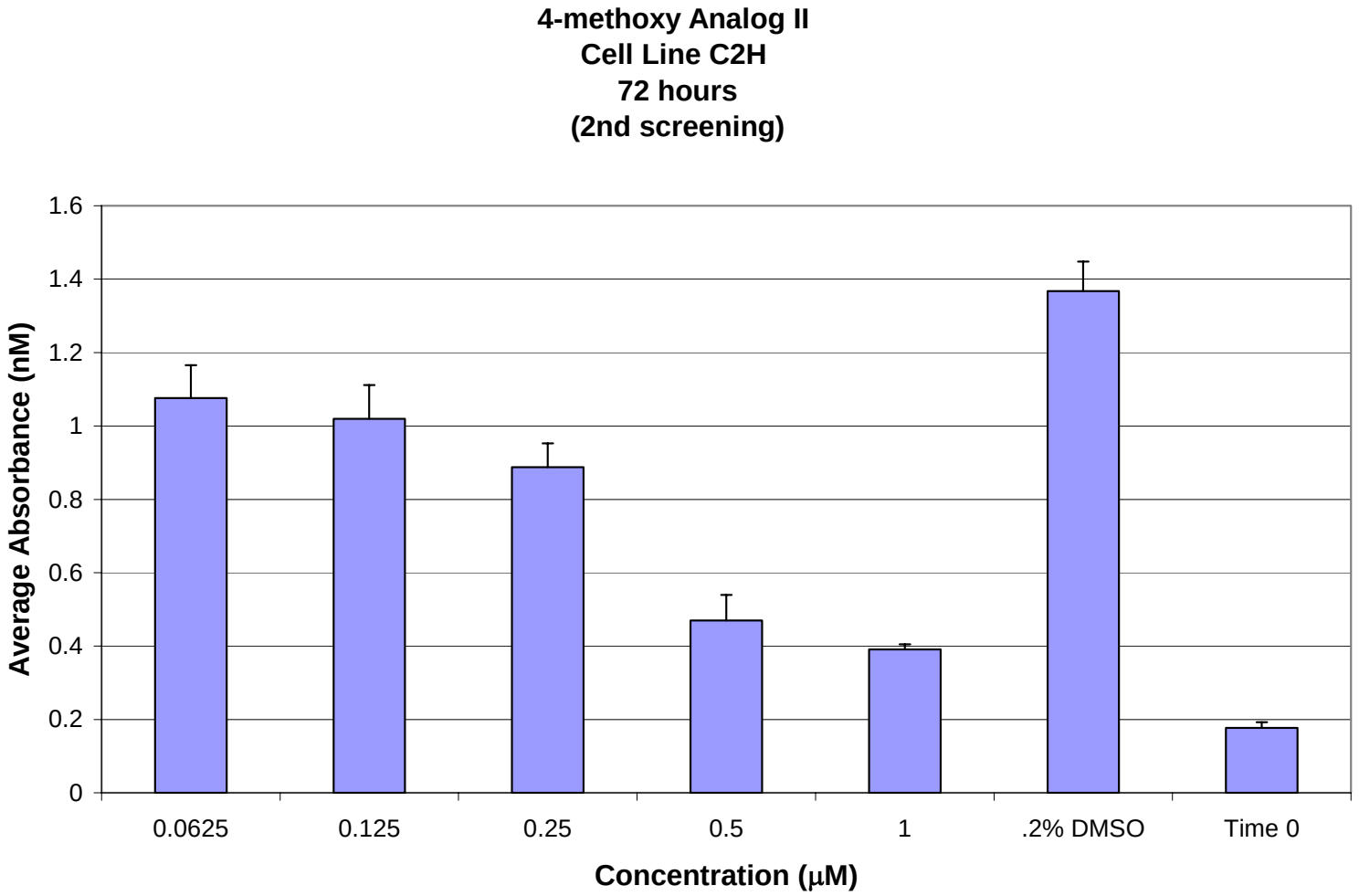


Figure 14. Concentration-Dependent Growth Inhibition of 4-methoxy Analog II on the TRAMP C2H Cell Line

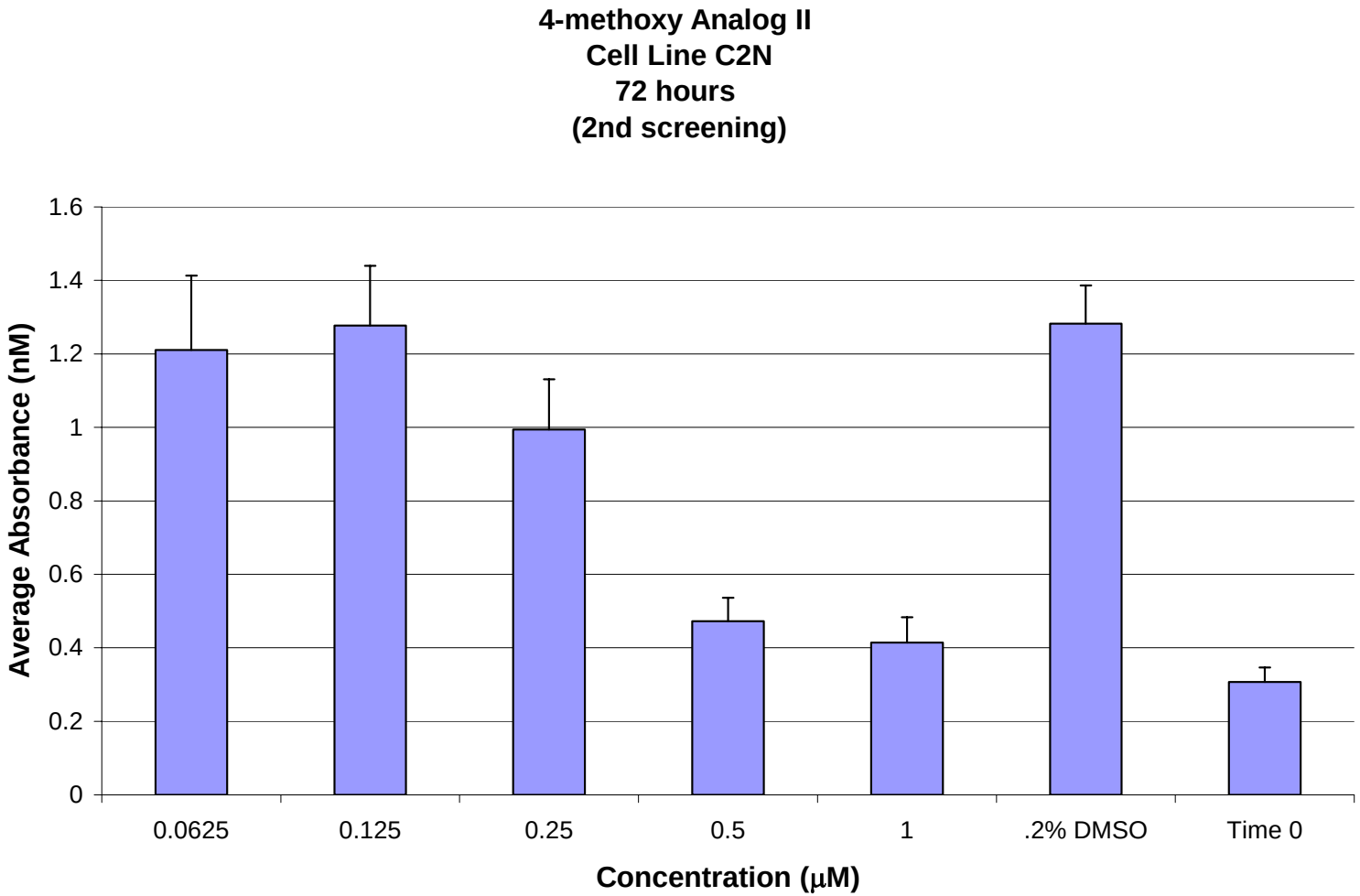


Figure 15. Concentration-Dependent Growth Inhibition of 4-methoxy Analog II on the TRAMP C2N Cell Line

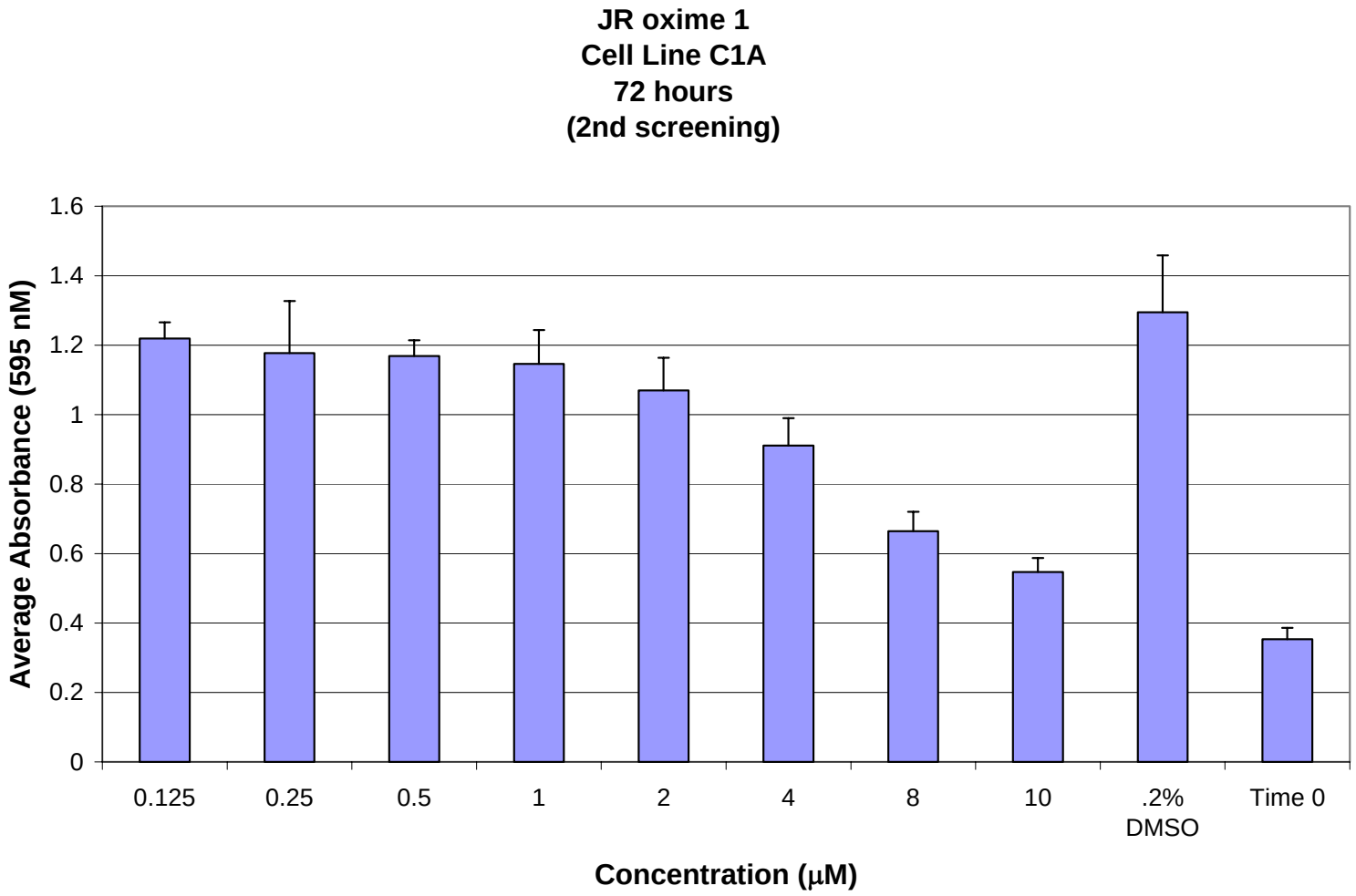


Figure 16. JR oxime 1 Concentration-Dependent Growth Inhibition on C1A Cell Line

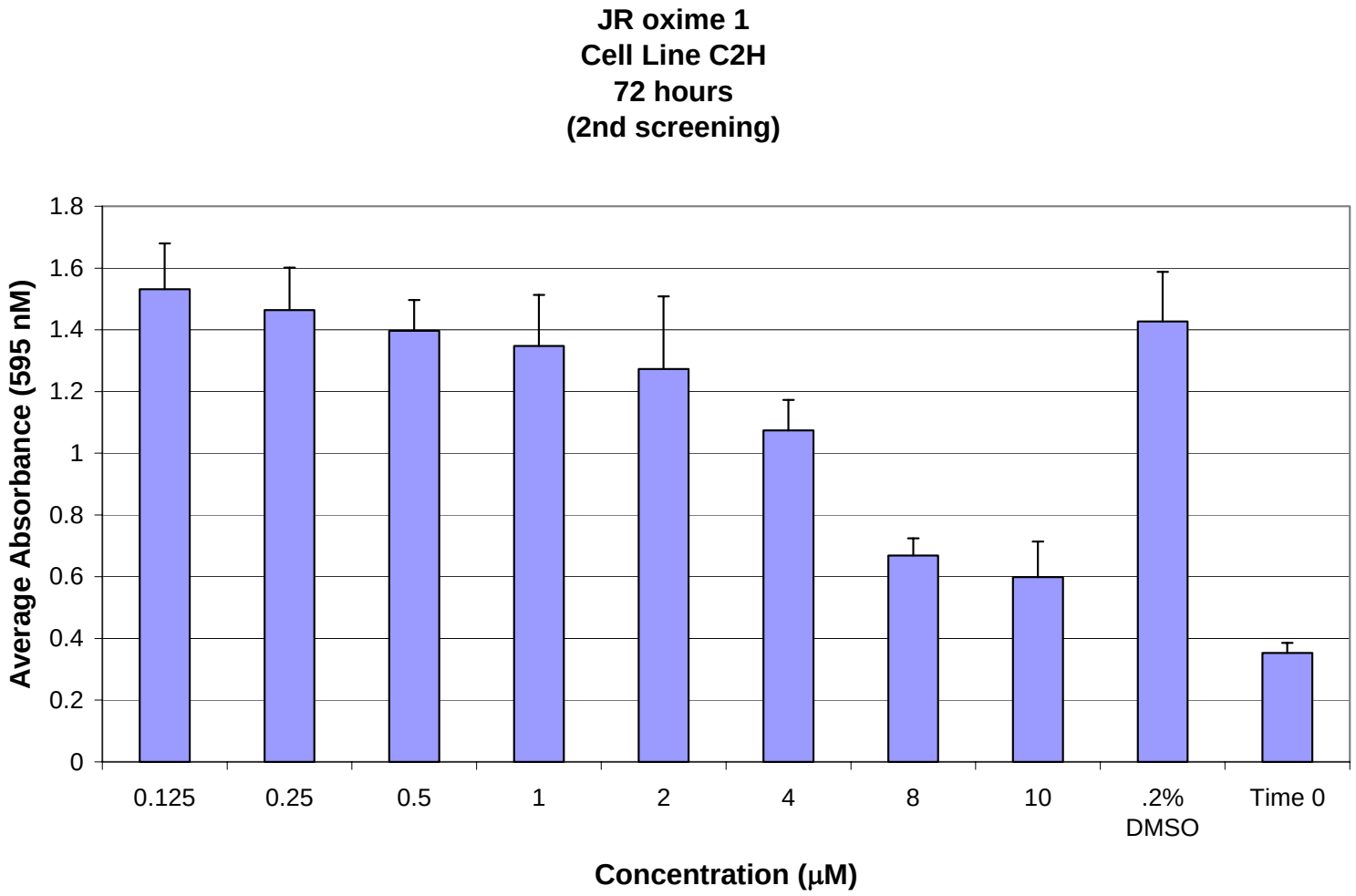


Figure 17. JR oxime 1 Concentration-Dependent Growth Inhibition on C2H Cell Line

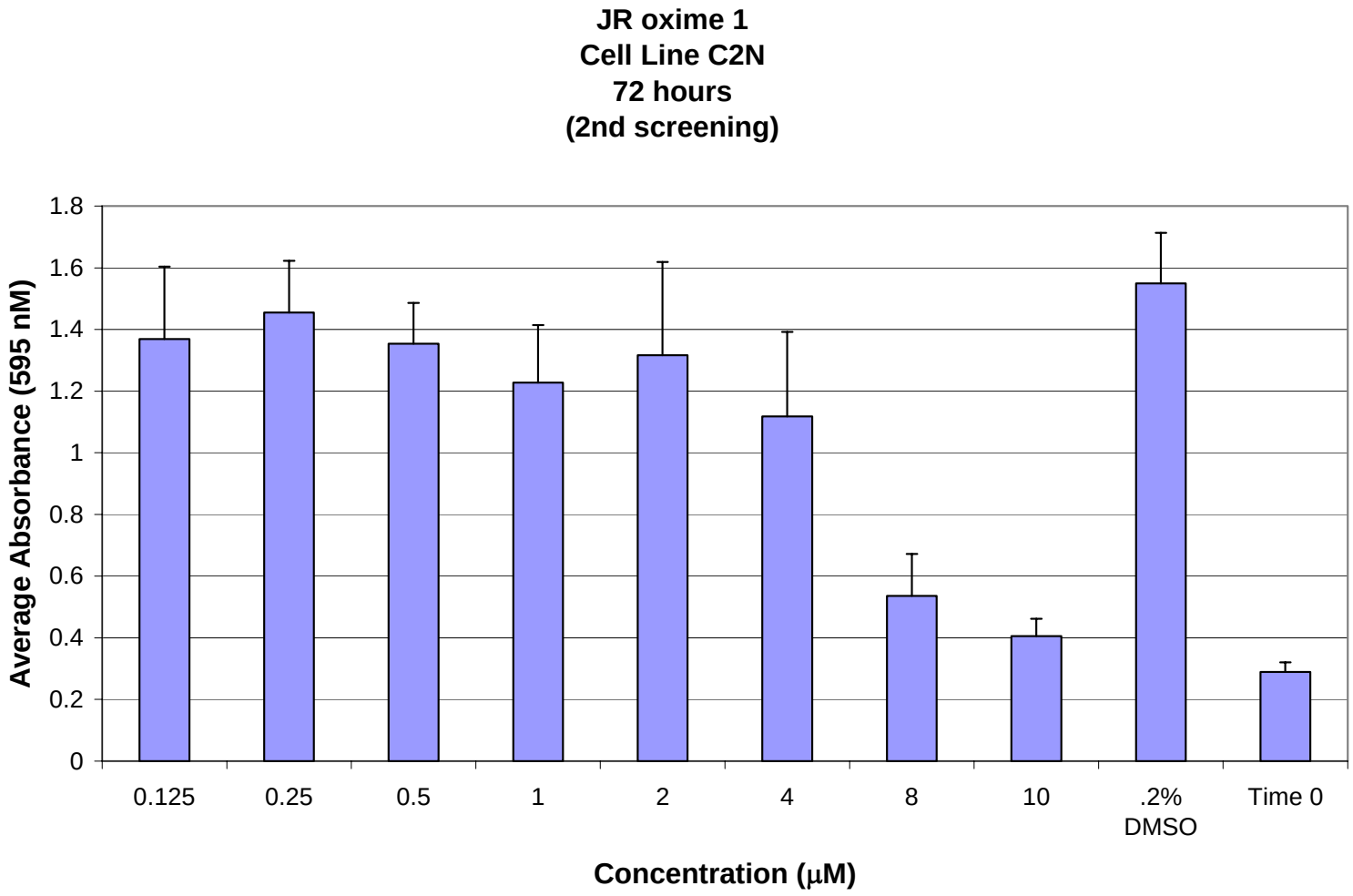


Figure 18. JR oxime 1 Concentration-Dependent Growth Inhibition on C2N Cell Line

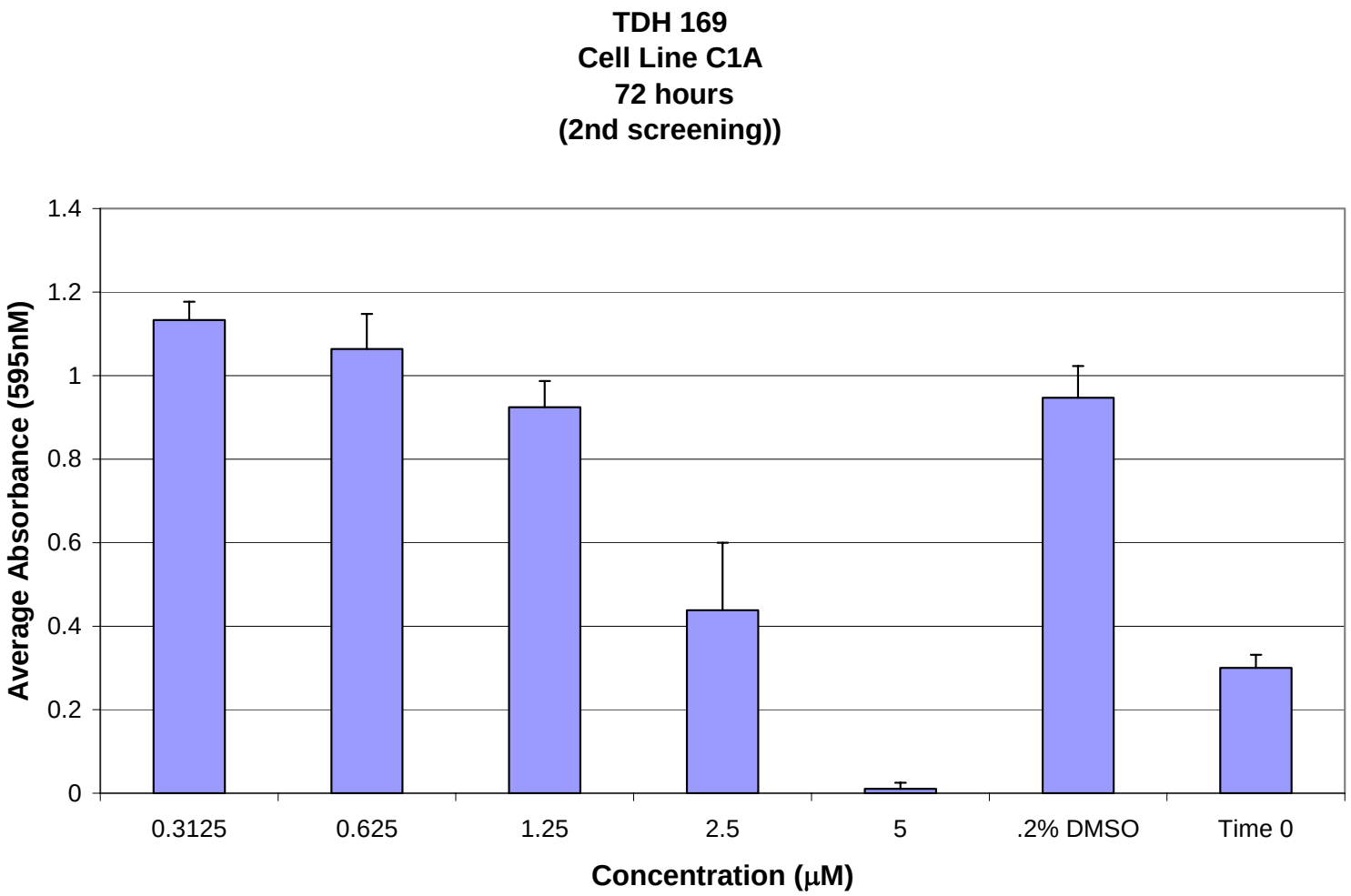


Figure 19. TDH 169 Concentration-Dependent Growth Inhibition on C1A Cell Line

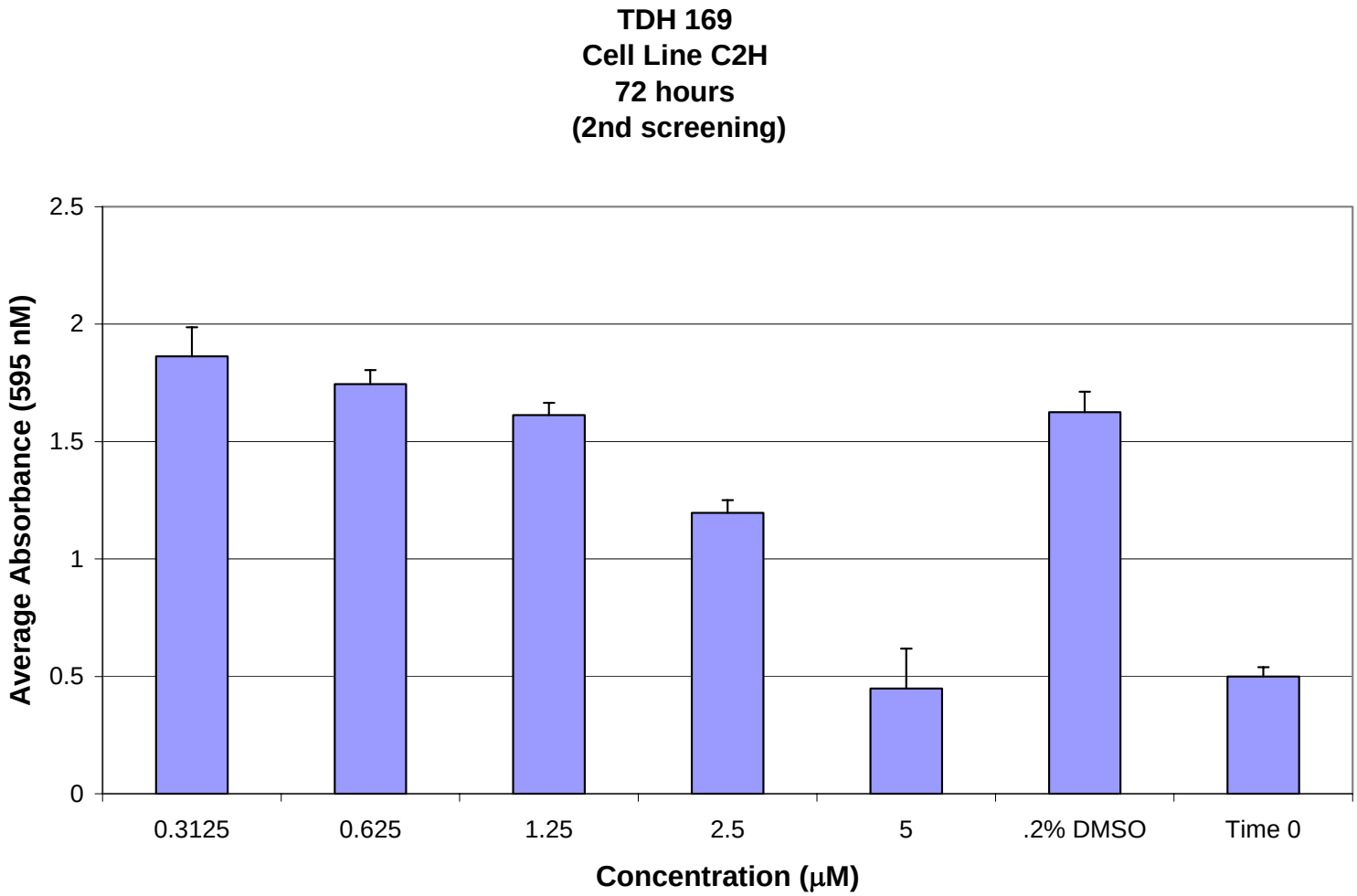


Figure 20. TDH 169 Concentration-Dependent Growth Inhibition on C2H Cell Line

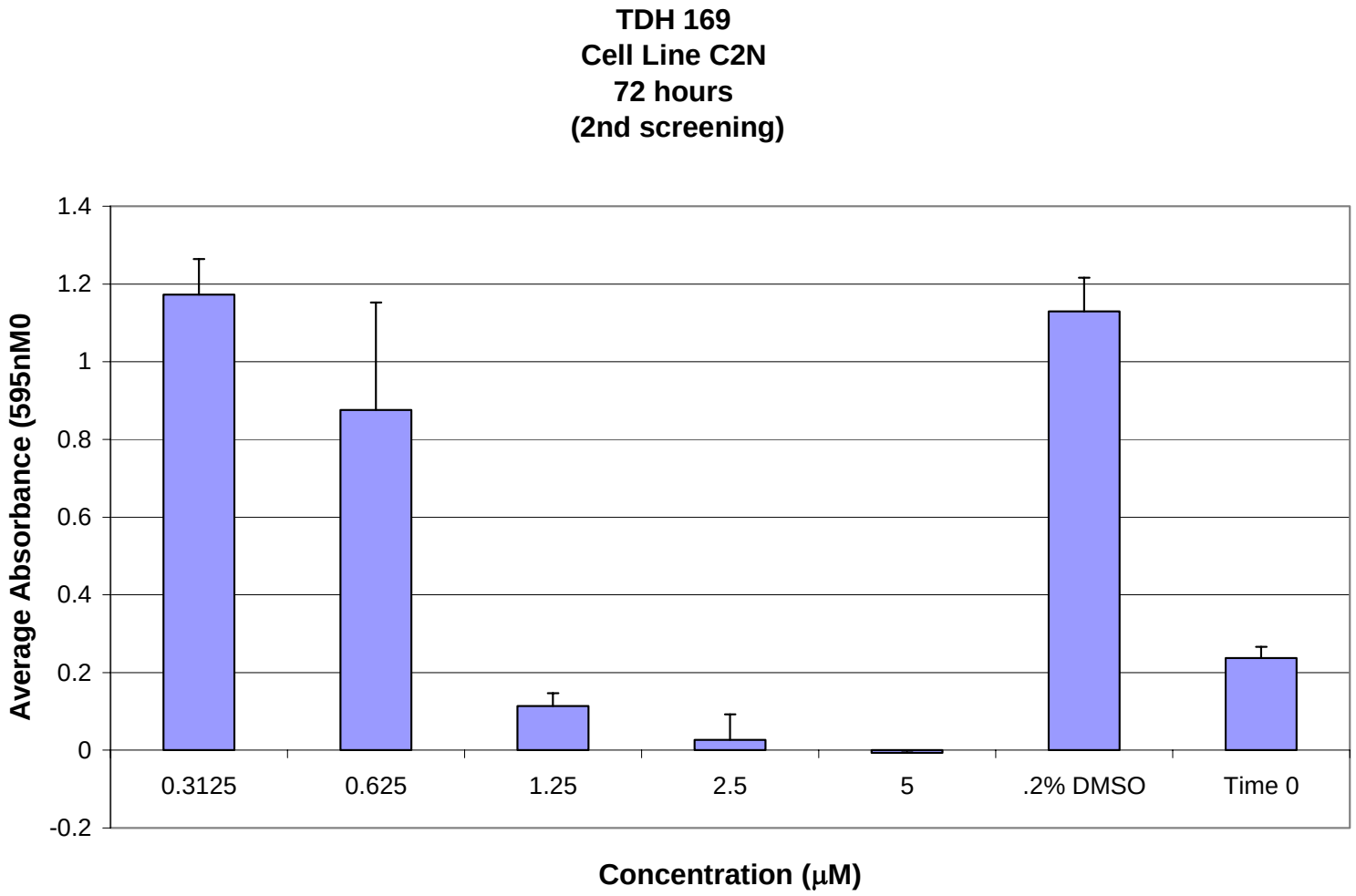
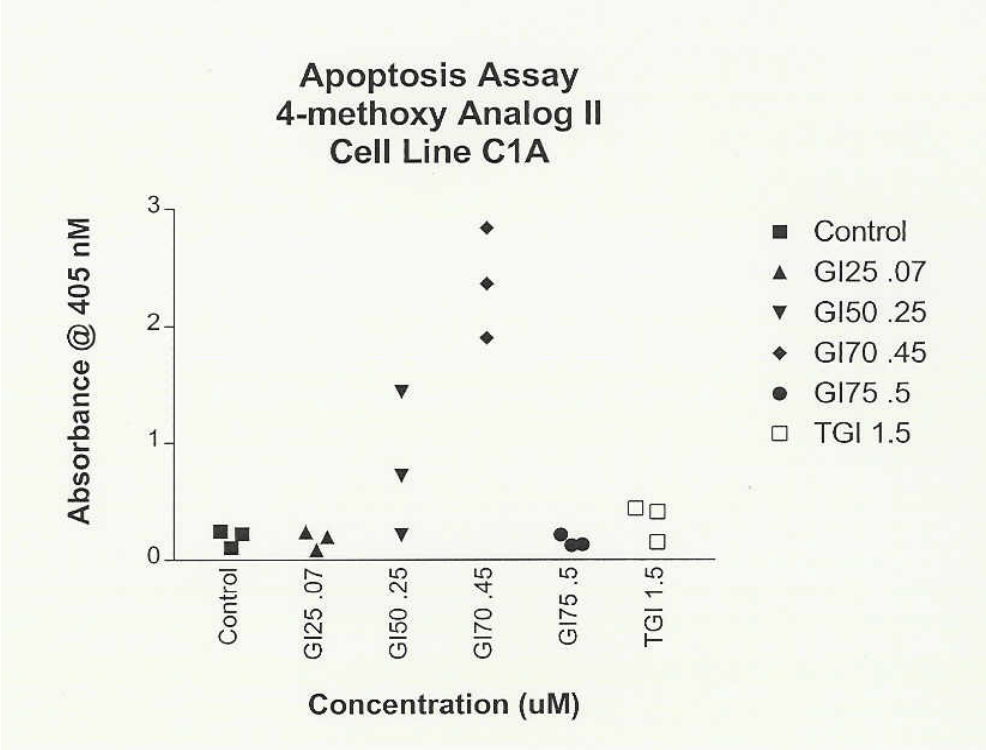


Figure 21. TDH 169 Concentration-Dependent Growth Inhibition on C2N Cell Line

It was then hypothesized that the 4-methoxy Analog II concentration-dependent inhibition could be due to programmed cell death (a.k.a. apoptosis). In order to test this hypothesis, an ELISA assay was conducted to quantify apoptosis and determine the minimum apoptosis-inducing concentrations of 4-methoxy Analog II on each cell line. The results are shown in Figures 22, 23 and 24. In each of these graphs, an increase is seen in apoptosis as the concentration of 4-methoxy Analog II was increased. On cell line C1A, 4-methoxy Analog II caused an increase in apoptosis up to the GI_{70} concentration. Beyond the GI_{70}, the cytotoxicity increased to the extent that the apoptosis was limited due to competing cell death mechanisms, likely necrosis. Table 16 lists the calculated minimum apoptosis-inducing concentrations of 4-methoxy Analog II on each cell line.



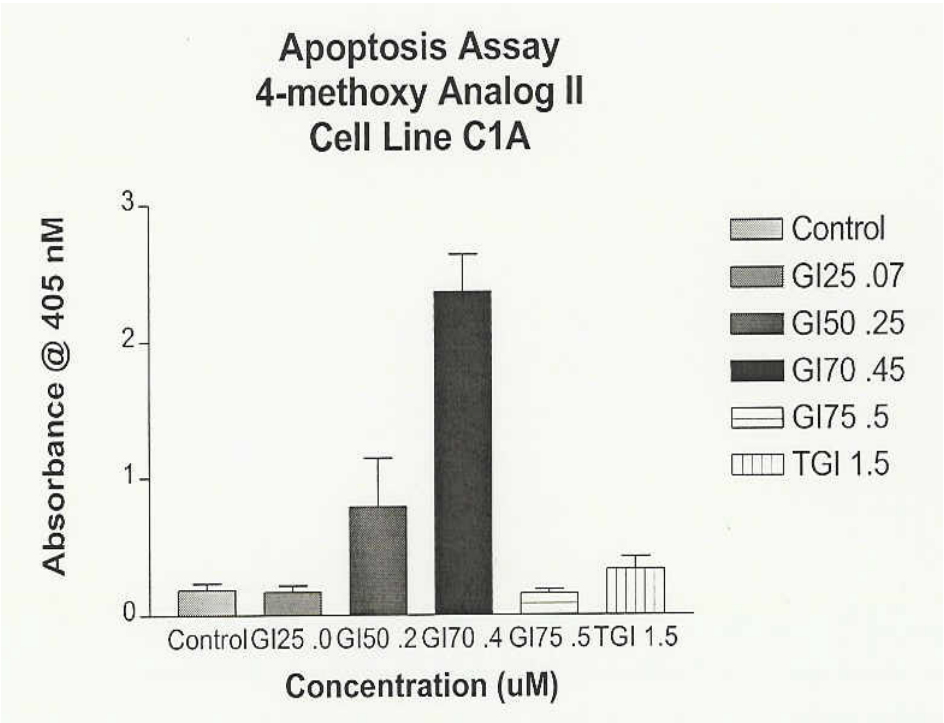
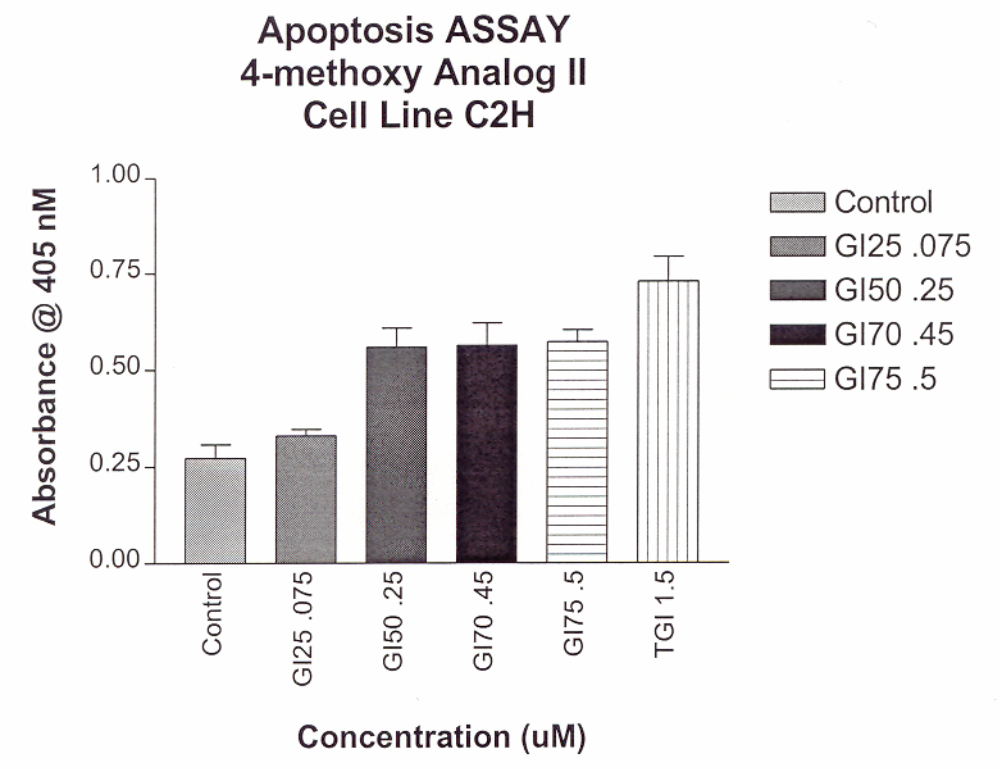


Figure 22. Apoptosis due to 4-methoxy Analog II in the C1A Cell Line



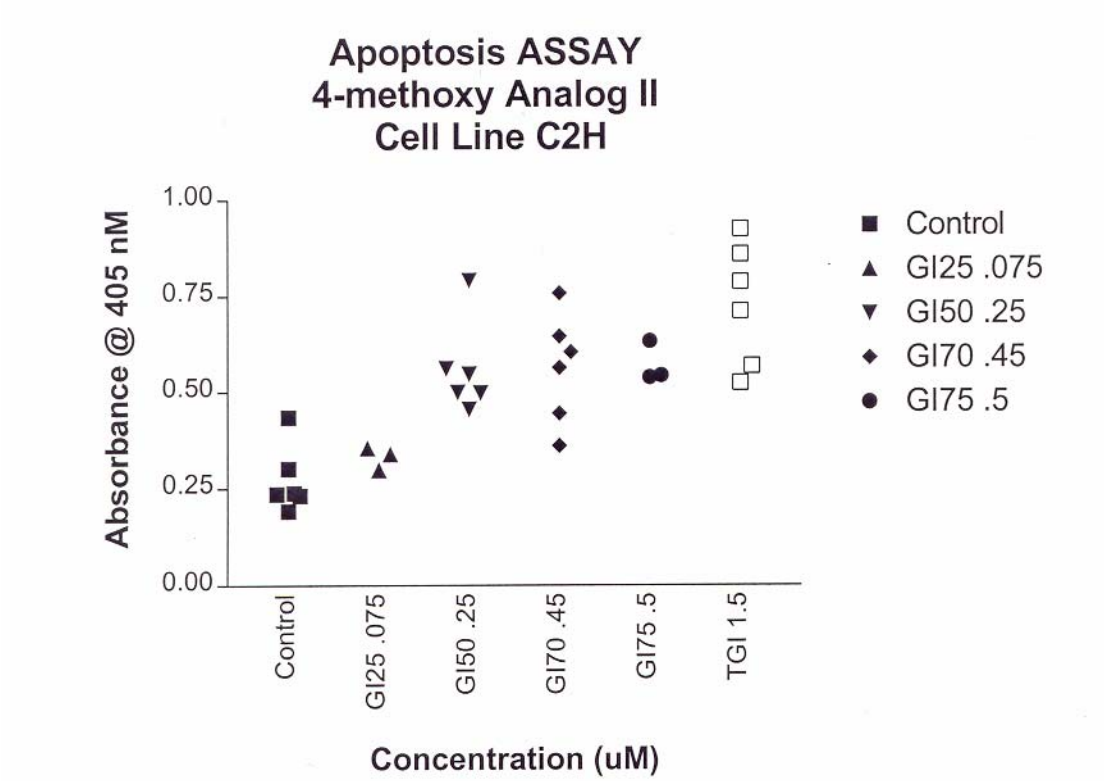
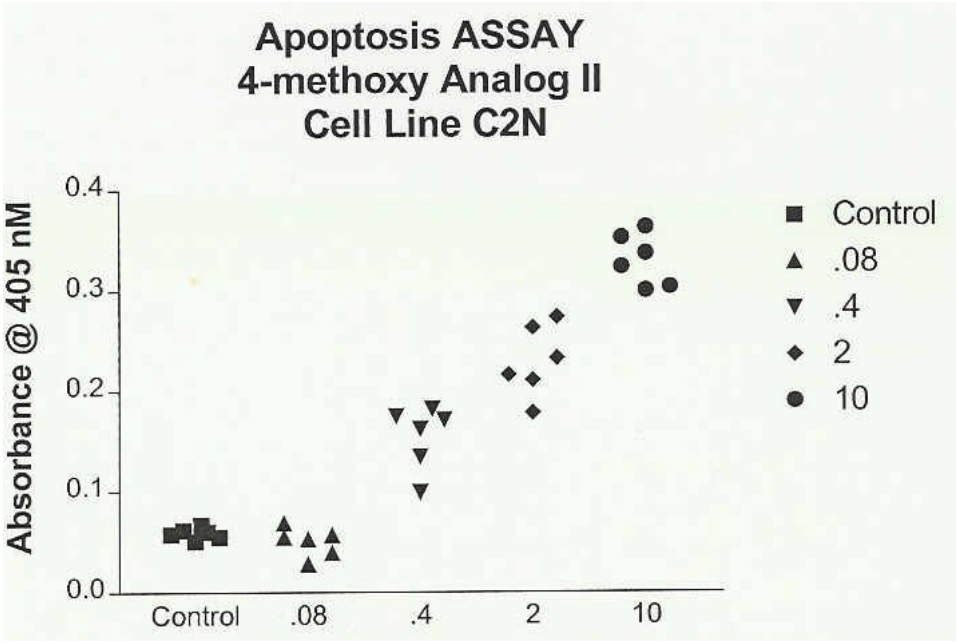


Figure 23. Apoptosis caused by 4-methoxy Analog II on C2H Cell Line



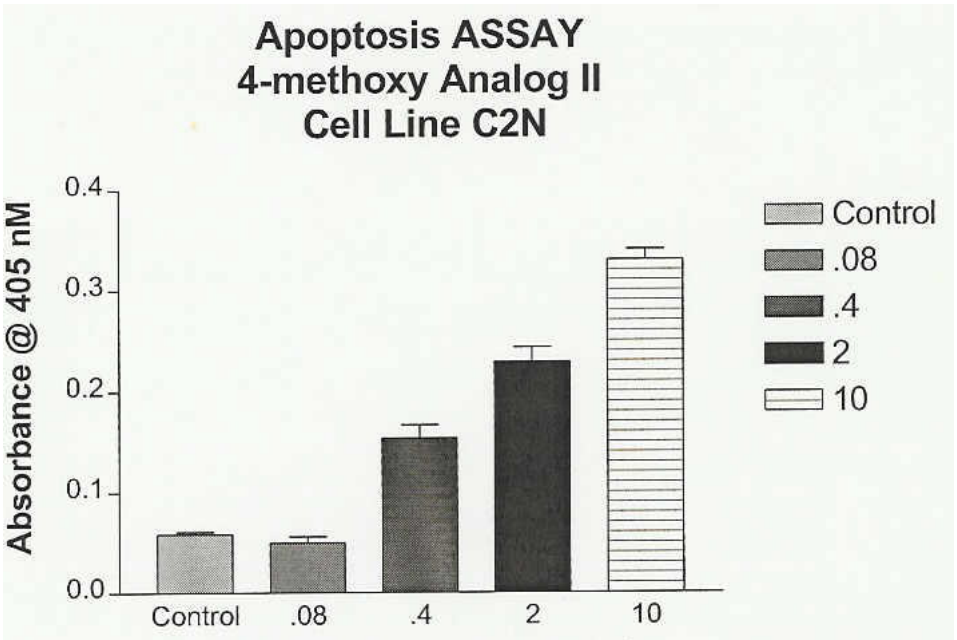


Figure 24. Apoptosis caused by 4-methoxy Analog II on C2N Cell Line

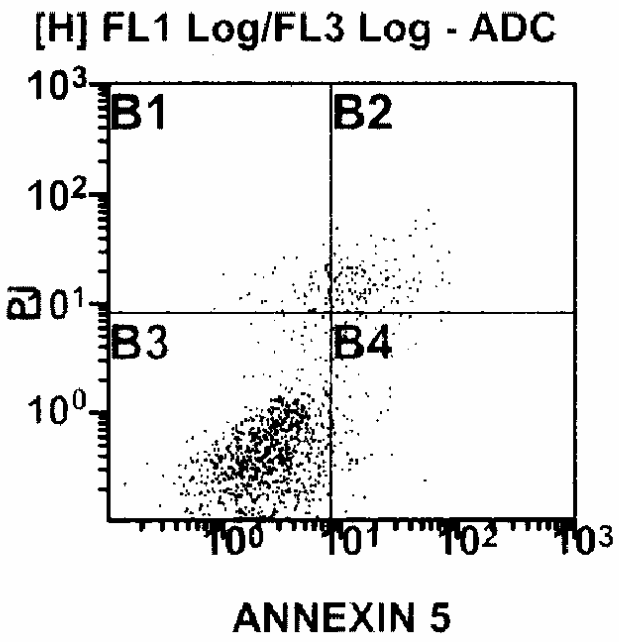
Table 16. Minimum Apoptosis-Inducing Co ncentration of 4-Meth oxy Analog II in Each Cell Line

Cell Line	Minimum Apoptosis-Inducing Concentration (μM)
C1A	0.14 ± 0.05 (μM)
C2H	0.10 ± 0.03 (μM)
C2N	0.12 ± 0.01 (μM)

Figure 25 shows the results of the PARP cleavage assay. The PARP cleavage assay is another test used to verify the occurrence of apoptosis. This is a simple test conducted using Western blot analysis. PARP is a nuclear repair enzyme that is involved in the repair of DNA damage. During apoptosis, certain caspase family members cleave PARP. The full size PARP protein is 116 kDa and when apoptosis is occurs PARP is cleaved into a smaller fragments of 89 kDa and 24 kDa (Satoh and Lindahl, 1992). Due to limitations in resources, the PARP cleavage assay was conducted on only the C1A cells, using a concentration of 1.5 μM (TGI concentration calculated previously) of 4-methoxy Analog II at times of 0, 24, 48 and 72 h. The TGI was chosen because it was expected that apoptosis would be most detectable at this concentration. The analysis showed no detectable PARP cleavage in the control groups (i.e., no band at 89 kDa), whereas the 48 hour treatment group showed PARP cleavage band at 89 kDa. Also note that in the treated C1A cells at 72 hours, the sample was underloaded. This can be seen in the Western blot analysis of the actin.

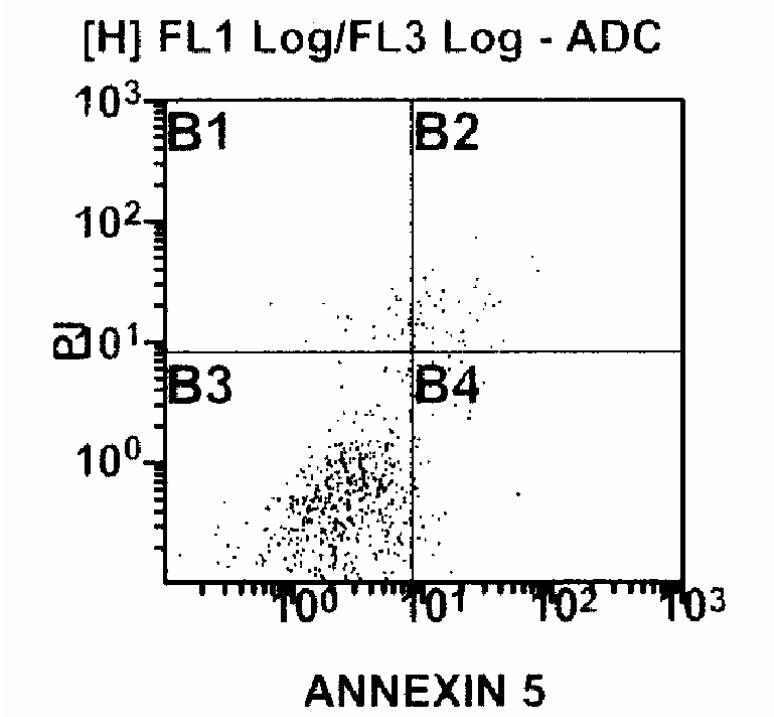
Figure 25. PARP Cleavage Western Blots of C1A cells, Untreated (Control) and Treated with 1.5mM 4-methoxy Analog II on 10% Resolving Gel at Times of 0, 24, 48 and 72 Hours. There were 3 independent experiments performed.

Next, flow cytometry was used to quantify the apoptosis resulting from treatment of C1A cells with 4-methoxy Analog II. C1A cells were again treated with of 1.5 μM (TGI previously calculated) 4-methoxy Analog II for 0, 48, and 72 h. Untreated cells and treated cells were sequentially stained with annexin V (green fluorescence) and propidium iodide (PI) (red fluorescence), then analyzed by flow cytometry. Figure 26 shows the dot plots for annexin V and propidium iodide staining at time 0 (untreated). Apoptotic cells stained with annexin V but not PI appear in the lower right quadrant of the dot plots. Necrotic cells appear in the upper right quadrant stained with both PI and annexin V. At 48 h after treatment, 16.12% of the cells treated with 4-methoxy Analog II were in the apoptosis quadrant as compared to 8.71% of the control cells in the apoptosis quadrant. In addition, at 72 h of treatment, 29.64% of the cells treated with 4-methoxy Analog II had demonstrated apoptosis, as compared to 18.13% apoptosis, in the control culture. This is approximately a 50% increase in apoptotic cells when comparing the cell culture treated with 4-methoxy Analog II to the control group.



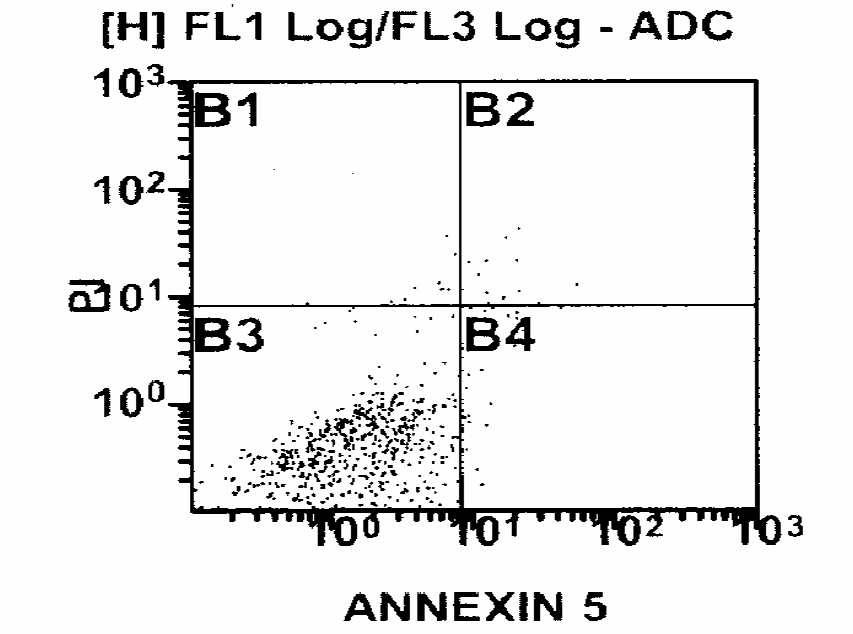
[H]FL1 Log/FL3 Log		
Region	**Number**	**Percent Gated**
B1	112	3.50
B2	225	7.04
B3	2426	75.91
B4	433	13.55

Figure 26. Two Color Flow Cytometric Evaluation of Cells for Apoptosis. Control Time 0.



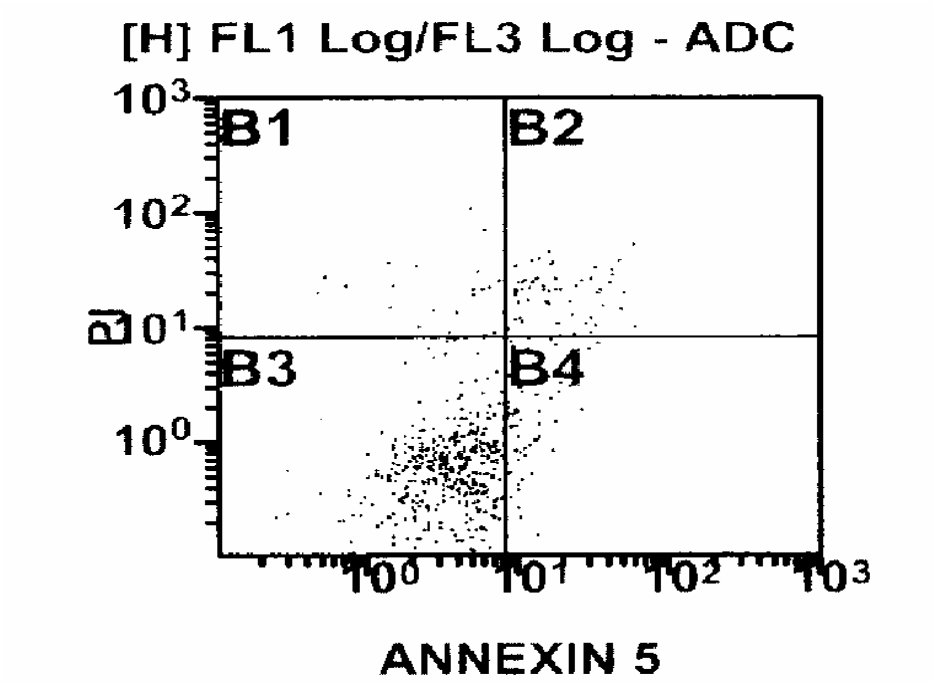
[H]FL1 Log/FL3 Log		
Region	**Number**	**Percent Gated**
B1	61	3.66
B2	89	5.34
B3	1310	78.58
B4	207	12.42

Figure 27. Two Color Flow Cytometric Evaluation of Cells for Apoptosis. Treatment at Time 0.



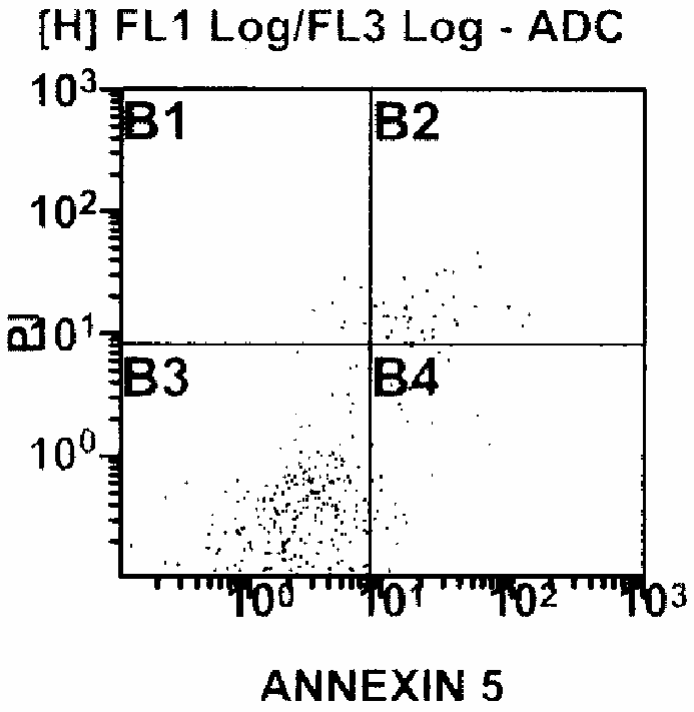
[H]FL1 Log/FL3 Log		
Region	**Number**	**Percent Gated**
B1	21	1.19
B2	32	1.81
B3	1561	88.29
B4	154	8.71

Figure 28. Two Color Flow Cytometric Evaluation of Cells for Apoptosis. Control at Time 48 hours.



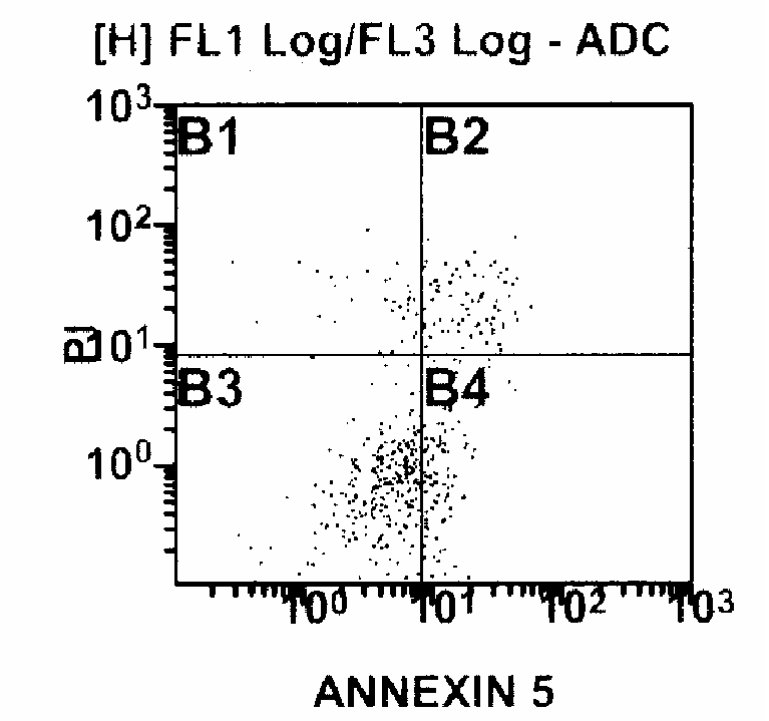
[H]FL1 Log/FL3 Log		
Region	**Number**	**Percent Gated**
B1	51	4.09
B2	114	9.14
B3	881	70.65
B4	201	16.12

Figure 29. Two Color Flow Cytometric Evaluation of Cells for Apoptosis. Treatment at Time 48 hours.



[H]FL1 Log/FL3 Log		
Region	**Number**	**Percent Gated**
B1	16	1.39
B2	84	7.32
B3	839	73.15
B4	208	18.13

Figure 30. Two Color Flow Cytometric Evaluation of Cells for Apoptosis. Control at Time 72 hours.



[H]FL1 Log/FL3 Log		
Region	**Number**	**Percent Gated**
B1	78	6.35
B2	131	10.67
B3	655	53.34
B4	364	29.64

Figure 31. Two Color Flow Cytometric Evaluation of Cells for Apoptosis. Treatment at Time 72 hours.

Finally, a microarray analysis was conducted on C1A cells treated with 1.5 μM 4-methoxy Analog II in comparison to a control population of untreated C1A cells. The objective of the microarray analysis was to determine whether apoptosis inhibitor genes and activator genes were experiencing altered expression due to 4-methoxy Analog II. The raw data obtained from the microarray analysis was reduced using a two-tailed p value of 0.005 and 0.995 as per the manufacturer's guidelines for significant change in gene expression between the control group and the treatment group. The complete file of the probe IDs were then entered into Onto-Express to identify the genes associated with apoptosis. From the probe IDs, Onto-Express identified 10 distinct genes associated with apoptosis. Onto-Express also categorized these genes as apoptosis inhibitors or activators. Table 10 lists the apoptosis related genes identified by Onto-Express, along with the change in expression levels of these genes due to treatment with 4-methoxy Analog II. The apoptosis activator genes Bok and Siva-pending were upregulated due to treatment of C1A cells with 4-methoxy Analog II. In addition, the apoptosis inhibitor genes Birc 4, Dad1, and Atf5 were downregulated due to treatment of C1A cells with 4-methoxy Analog II.

Table 17. Results of microarray analysis-change in expression of apoptosis related genes in the treatment versus the control

Gene	Gene Title	apoptosis activator or inhibitor	Change in Gene Expression (treatment compared to control)	Gene Signal (control)	Gene Signal (treatment)	Change p-value (two tailed)
Bok	Bcl-2 related ovarian killer protein	activator	increase	173.6	314.6	0.00002
Siva-pending	CD27 binding protein	activator	increase	441.2	534.1	0.00003
Siva-pending	CD27 binding protein	activator	increase	125.8	168.1	0.00097
sh3glb1	SH3-domain GRB2-like B1 (endophilin)	activator	decrease	266.7	165.5	0.99951
PTEN	phosphatase & tensin homolog	activator	decrease	875.1	615.8	0.99976
trim35	tripartite motif-containing 35	activator	decrease	458.8	312.9	0.99987
PTEN	phosphatase & tensin homolog	activator	decrease	318.7	194.3	0.99996
sh3glb1	SH3-domain GRB2-like B1 (endophilin)	activator	decrease	215.6	147.2	0.99998
Hspa1b	heat shock protein 1B	inhibitor activity	increase	251.4	652.7	0.00002
Hspa1b	heat shock protein 1B	inhibitor activity	increase	214.2	557.4	0.00002
Hspa1b	heat shock protein 1B	inhibitor activity	increase	252.5	973.9	0.00002
Api 5	Apoptosis inhibitor 5	inhibitor activity	increase	448.4	633.7	0.00249
Birc 4	baculoviral IAP repeat containing 4	inhibitor activity	increase	10.2	23.5	0.00336
Birc 4	baculoviral IAP repeat containing 4	inhibitor activity	decrease	30.9	20.7	0.99696
Dad1	defender against cell death 1	inhibitor activity	decrease	483.9	321.7	0.99880
Birc 4	baculoviral IAP repeat containing 4	inhibitor activity	decrease	25.4	17.3	0.99997
Dad1	defender against cell death 1	inhibitor activity	decrease	784.3	620.9	0.99998
Atf5	activating transcription factor 5	inhibitor activity	decrease	594.3	186.7	0.99998

4.4 TRAMP DISCUSSION

The first step in this study was to determine the most desirable of the four promising antiproliferative agents Analog II, 4-methoxy Analog II, JR oxime 1 and TDH 169, using the MTT assay, in terms of antiproliferative activity against TRAMP cell lines. The agents Analog II and 4-methoxy Analog II have previously been tested in other prostate cancer cell lines (PC-3 and LNCaP), but never against the prostate TRAMP clonal cell lines C1A, C2H and C2N (Balachandran et al, 2000). The screening revealed that 4-methoxy Analog II showed the most potency and reproducibility.

4-methoxy Analog II has previously been shown to inhibit the prostate specific antigen production of and to induce apoptosis in both LNCaP and PC-3 cell lines (Balachandran et al, 2000). In the present study, 4-methoxy Analog II was found induce apoptosis in the C1A, C2H and C2N cell lines. The C1A TRAMP cell line displayed the most severe reaction to 4-methoxy Analog II. Three methods were used to detect apoptosis induction by the agent. The ELISA assay was conducted on all three cell lines to quantify apoptosis and determine the minimum apoptosis inducing concentrations of 4-methoxy Analog II. Apoptosis was verified with the PARP cleavage assay on the C1A cell line, giving a minimum apoptosis-inducing concentration of 1.5 μM for 4-methoxy Analog II (Darmon et al, 1995; Li et al, 1998). Two-color flow cytometric evaluation was conducted to further verify the induction of apoptosis. Collectively, these results clearly suggest that 4-methoxy Analog II induces apoptosis in the C1A cell line.

Microarray analysis was used as a tool to determine whether apoptosis-regulating genes were coordinately upregulated or downregulated in the C1A cell line due to treatment with 4-methoxy Analog II. The apoptosis activator genes Bok and Siva-pending were found to be

upregulated, and the apoptosis inhibitor genes Birc 4, Dad1, and Atf5 were found to be downregulated. Table 17 summarizes the changes in regulation as determined by the microarray.

Table 18. Apoptotic Gene Changes in Regulation and Function

Bok ↑	-Bok is tissue mediator of cell death & promotes apoptosis by interacting with selective anti-apoptotic protein (Hsu et al, 2000; Yakovlev et al, 2004).
Siva-pending ↑	-Siva binds to CD27 -Overexpression of Siva in various cell lines induces apoptosis this suggests siva increases CD27 transduced apoptotic pathway (Yoon et al, 1999).
Sh3glb1 ↓	-Interacts with known pro-apoptotic Bax -Overexpression of sh3glb1 has no major effect on mammalian cell death -Increase in apoptosis due to binding with pro-apoptotic Bax (Pierrat et al, 2001).
PTEN ↓	-Known to play a role in progression of prostate cancer -Thought to be tumor suppressor gene because it down regulates phosphoinositide 3-kinase/Akt pathway -Phosphoinositide 3-kinase/Akt pathway induces cellular transformation -Mouse studies have shown that lack of PTEN is a mechanism of progression of prostate cancer (Di Vizio et al, 2005).
Trim35 ↓	-No apoptosis studies on this gene -Considered an anti-apoptotic protein
Hspa1b ↑	-Also known as Hsp70 -Hspa1b inhibits the nuclear import of apoptosis-inducing factor (Ito et al, 1998).
Api5 ↑	-No apoptosis studies on this gene -Considered an anti-apoptotic protein
Dad1 ↓	-Loss of Dad1 protein triggers apoptosis -Shown to bind with Bok and prevent Bok from generating apoptosis (Ayala et al, 2004).
Atf5 ↓	-Few studies have been performed to characterize Atf5 and its biological function -Has been shown to inhibit apoptosis (Angelastro et al, 2003).

The mechanism by which this occurs is currently unknown but could be due to mitotic block induced by 4-methoxy Analog II. Based on these experimental results, additional work would include further investigation of the specific mechanisms at different stages of the cell cycle by which the genes identified by microarray actually produce the apoptotic results on the C1A cell line.

In relating the findings of this research to humans it should be noted that the homology of the mouse proteins produced by these genes is similar to that of humans. Table 19 summarizes the homology of these genes between mice and humans. Based on these similarities it is reasonable to expect the effects of 4-methoxy Analog II on the human prostate cancer cell lines to be similar to the effects found in the TRAMP model.

Table 19. Similarities Between Genes in Human and Mouse

Gene	Mouse chromosome	Human chromosome	% similarity to humans over the aa sequence
Bok	chromosome 1 syntenic	2(q37.3)	100% / 213 aa
Siva-pending	12(F2)	14q32.33	70% / 174 aa
Sh3glb1	chromosome 3 syntenic	1(p22)	95% / 365 aa
PTEN	19(24.5cM)	10(q23.3)	99.7% / 403 aa
Trim35	14(D1)	8(p21.1)	28% / 475 aa
Hspa1b	17(18.9cM)	6(p21.3)	91.59% / 225 aa
Api5	2 syntenic	11(p12-q12)	98.2% / 504 aa
Dad1	14(24.0cM)	14q11-q12	100% / 113 aa
Atf5	7 syntenic	19(q13.3)	87% / 282 aa

Additional research would include in vivo studies using the TRAMP C57BL/6 mice treated with various doses of 4-methoxy Analog II to yield plasma concentrations centered around the LC_{50}. The TRAMP C57BL/6 line is genetically engineered to develop prostatic intraepithelial neoplasia by 8-12 weeks of age that progress to adenocarcinoma with distant

metastases by 24-30 weeks of age. This in vivo assay with TRAMP mice would provide additional insight into the effectiveness of 4-methoxy Analog II as a potential therapeutic agent for human prostate cancer. Specifically, the in vivo testing would provide insight into the biological transport and interactions of 4-methoxy Analog II, as well as its ability to target the prostate cancer cells within a living mouse.

5 SUMMARY AND DISCUSSION

This investigation outlines the development of a chemical-animal model utilizing a toxicological and therapeutic approach. The results are developed from the latest techniques being implored in the chemical and biomedical field. This research outlines a model building approach which progresses from a preliminary agent screening technique (QSAR/SAR) based on literature studies through in vivo studies using assays such as the Chernoff-Kavlock Assay (CK Assay) to high throughput mechanistic targeted assays, such as the TRAMP model.

Drug discovery and development is not new to humans. Many ancient populations reported the medicinal properties of various plant extracts and elixirs, all the result of a necessary trial and error search for remedies of specific ailments (Sneader, 1985). In early civilization, man used elements from his natural surroundings to treat specific diseases. In ancient civilizations and today, the single most important source of drugs or drug precursors has been nature (Verpoorte, 1998). Up until the 19th century the development of therapeutic agents was a trial and error process that was developed and based on experience, and passed on from generation to generation. In the 19th century scientists developed techniques for analyzing and determining the specific components of these ancient extracts and were thus able to determine the individual components of these natural therapies with therapeutic activity.

True drug discovery as we know it today began to develop when the first structures of receptors were found. The first rational drug discovery effort can be traced back to John Langley and Paul Ehrlich, the discoverers of the receptor concept. By the early 20th century the fields of biology, chemistry and pharmacology increased rapidly, and theories about drug action and mechanism expanded.

In traditional modern medicine, drug discovery is a process that begins with a known disease or ailment in an organism and a therapeutic theory is developed to combat the disease or ailment. With the existing knowledge of biological processes and interactions, scientists develop a list of potential therapeutic treatments and begin a screening process to determine the viability of these agents at combating the ailment. Typical screening techniques include in vitro and in vivo testing of each specific agent. This traditional method of drug screening and drug development, while used to develop many of the treatments of today, is extremely time consuming and may or may not lead to an effective treatment.

Today, scientific advancement has created opportunities for researchers to become more proficient at drug screening and development, as compared to the accepted development process of just a few years ago. Today scientists are able to identify thousands of new targets through the ability to sequence a genome and identify every expressed gene. This provides scientists with the ability to rapidly identify specific gene expression relevant to the onset and persistence of a disease. With this genetic information researchers may know the role, function, structure, gene location, biochemical pathway, molecular interactions, and expression levels of each and every protein coded by a particular genome. The impact of these developments in genomic sciences on drug discovery will change the course of this field remarkably. In fact, at present in most major pharmaceutical companies, 10% to 25% of new discovery projects are based on genomics (Caron et al, 2001).

There are several ways to use genomic analysis to identify specific molecular targets (Jones et al, 1999). One powerful method to rapidly analyze the expression of thousands of genes is the use of microarrays. In microarrays, oligonucleotides are attached to glass slides to form arrays and then hybridized with cDNAs from some particular tissue or cell type. A fluorescent

detection system allows for the quantification of interaction of the cloned gene with the cDNA. Using microarrays, gene expression patterns for many different animal tissues can quickly be obtained under different experimental conditions. Microarrays can be utilized in research to determine the effect of a treatment on gene expression associated with a specific disease. For example, microarrays can be used to analyze groups of cells that are diseased versus not diseased. By comparing the gene expression between diseased and normal cells, scientists can designate specific proteins encoded by those genes more highly expressed in the diseased state to be a potential target for therapy (Neamati and Barchi, 2002). In addition, microarrays can be utilized on diseased cells that are either treated or untreated. By determining the changes in disease associated gene expression between the control and the treatment group, researchers can compare and correlate the effects on the disease due to a specific toxin or inhibitor.

With the development of genomic analysis there are many new targets to be evaluated in drug discovery. New techniques have been developed to expedite the preliminary drug screenings. These techniques combine elements of biology and chemistry with mathematics, statistics and computer sciences. These techniques are referred to as bioinformatics and chemoinformatics, and focus on large datasets such as macromolecular structures, genome sequences, 3D chemical databases and compound libraries. Informatic methodologies rely on a variety of computational techniques (Manly et al, 2001; Luscombe et al, 2001) including sequence and structural alignment, database design and data mining, macromolecular geometry, phylogenetic tree construction, prediction of protein structure and function, gene searching and expression data clustering, chemical-similarity clustering, diversity analysis, library design, virtual screening and QSAR (Luscombe et al, 2001).

The informatic technology utilized in this research is the QSAR/SAR model (Quantitative Structure Activity Relationship/Structure Activity Relationship). The QSAR/SAR model was developed through (1) conformational analysis; (2) generation of physico-chemical properties; (3) preliminary model development; (4) linear discriminant analysis; and (5) choice of the final candidate model. Conformational analysis is utilized to determine the final three-dimensional structure that represents the lowest energy (i.e., in terms of steric strain) arrangement of atoms comprising a molecular structure (i.e., the global minimum energy conformation). Computational analysis is then used to determine the chemicals properties. The chemicals properties describe the size or shape of the compound and provide metrics of the molecule. These metrics include electronic aspects of repulsion and attraction within and with other molecules that might affect potential binding sites, and transport properties of how compounds cross membranes in the body and how they are transported through the body.

Next the preliminary model is developed using statistical analysis. Histograms are generated to examine the normality assumption necessary for discriminant analysis. A correlation matrix is made for all possible independent variables to determine information redundancy. All-possible regression is used as a tool to explore all of the potential models and the r^2 value is utilized to determine the best models from the group of candidate models. Next, linear discriminant analysis is used to generate the difference between two or more groups with respect to several variables simultaneously. Linear discriminant analysis is used to generate functions for the classification of chemicals into actives versus inactives. For the choice of the final candidate model, a complete leave one out cross validation procedure is employed. The complete leave one out cross validation is a technique where each compound is removed once from the total data set and the remaining compounds are used to train and build a new model.

The new model based on the reduced training set is then applied to the single compound that was removed during the generation of the current model. This procedure repeats until models with each of the individual observations removed are generated. Using this method, the sensitivity, specificity and squared distance (deviation) is calculated.

Once a model is developed, the results need to be confirmed. Depending on the specifics of the application and treatment, researchers typically employ an animal assay or clinical trials. For this research, a QSAR/SAR model predicted a chemical compound was positive for an adverse outcome on the developing fetus. Due to the nature of the resulting effect, an animal study was the only acceptable in vivo validation technique. The animal assay utilized in this study was a modified version of the Chernoff-Kavlock (CK) assay. The CK assay utilized pregnant female mice that were orally gavaged with 13-cis retinoic acid. The dosing of this compound was during the period of major organogenesis. The mother is examined during the entire pregnancy for signs of toxicity and the dams are allowed to deliver the pups. The endpoints evaluated in this modified version of the CK assay were fetal death and growth retardation. To determine growth retardation, pups were tagged and weighed on postnatal day one and three. To determine fetal death the number and status of all pups born was monitored. After postnatal day three the dams were killed and determination of implantation sites were conducted to determine the number of absorptions.

This investigation introduced multiple disease endpoints and multiple treatments during the process of summarizing a method for the development of a chemical animal model. The specific adverse health conditions this research was conducted on are developmental toxicity and prostate cancer. Both of this health conditions play a predominant role in the health status of society today.

Developmental toxicology studies exposures to agents that cause abnormal development, and it is estimated that 1 in 3 children born have developmental defects. In addition, these defects are the leading causes of infant mortality and disability in the world. Many birth defects are the result of chemical interactions resulting from exposure during pregnancy, however the effects of many chemical compounds on the outcome of human pregnancies are unknown. Researchers currently utilize clinical studies and animal testing to determine the effects of chemical exposure on the outcome of pregnancies, however both of these methods have shortcomings. In clinical research, it is difficult to determine all of the chemicals a mother has been exposed to, and/ or if a combination of these chemicals lead to the defect. Animal testing is expensive, time consuming and requires each chemical compound to be tested individually. In addition, results from animal tests are also difficult to apply to humans, and the results obtained may or may not apply to the developing human fetus. Therefore, a quicker screening process is needed to predict the outcome of these chemicals on the human fetus. A potential screening process that can be applied to the exposure of chemicals on the developing fetus is the use of QSAR/SAR model development. Utilizing QSAR/SAR models, preliminary data can be developed for chemicals with little to no information on their effects on the developing fetus. Thus chemicals can quickly be segregated into active versus inactive for adverse effects on the developing fetus and additional studies can be concentrated on those chemicals that are predicted to have an adverse effect.

The other health endpoint utilized in this research is adenocarcinoma of the prostate. Prostate cancer treatment and awareness have drastically improved over the last decade, however prostate cancer remains the most common cancer in American men. (Gingrich and Greenberg, 1996). Public health awareness, improvements in early detection of prostate cancer and new

treatments of prostate cancer have increased the survival rate, however prostate cancer remains the second leading cause of cancer deaths in American men (Parker et al, 1997). In fact, an estimated 29,900 American men lose their lives to prostate cancer each year, with one death occurring every twenty minutes (National Prostate Cancer Coalition, 2003). There are many available treatment options for prostate cancer, including prostatectomy, radiation, brachytherapy (radioactive seeds), hormone deprivation therapy, chemotherapy, and many other alternative therapies. However, all of these treatments techniques have drawbacks and side effects. Ideally, researchers would like to understand the specific genes associated with the onset of prostate cancer and develop drugs to prevent the expression of these genes, and therefore eliminate the onset of the disease.

There are currently many accepted models for prostate cancer research. However, one of the most promising models is the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model. The TRAMP model was genetically engineered so that each mouse will develop its own prostate cancer. The TRAMP model has advantages over other models, including the use of a pure developing prostate cancer that mimics the development of prostate cancer in humans, and the ability to conduct research in a mouse that has an intact immune system. In addition, since the TRAMP mouse develops prostate cancer on it's own it's use presents the ability to conduct preventative studies and observe the effects of environment, diet and other factors on prostate cancer development. Therefore, while the majority of existing knowledge and treatment is based on correcting or treating the prostate cancer after it has developed the TRAMP model allows research into factors and treatments that may lead to actual prevention of the onset of prostate cancer altogether.

QSAR are developed to predict the effect of chemicals on biological systems, including the reproductive system. In this research, Molecular Modeling Pro™ was used to calculate the physicochemical properties for a series of dopamine mimetics. The study was conducted on a twelve compound database consisting of a series of dopamine mimetics to generate a model to predict actives versus inactives.

The modeling in this study was based on research conducted by SmithKline Beecham Pharmaceuticals. Their work consisted of a series of dopamine mimetics developed as potential therapeutic agents. These dopamine mimetics were for therapeutic use in a population that include fertile female humans. Therefore, the reproductive toxicity of the dopamine mimetics was a concern. In the original study, the dopamine mimetics were tested in rats, who were treated orally by gavage from days 6 to 15 post coitum. A qualitative assessment of developmental followed by the application of QSAR techniques (Ridings et al, 1992). The results of this study was a 22 parameter model predicting biological activity of these dopamine mimetics.

In the present study, the original rat study data of these dopamine mimetics was used to construct an equally accurate, but mathematically simpler biological activity predictive model, using molecular modeling, physico-chemical properties, and statistical techniques. The outcome of this re-evaluation was a two parameter model described by the molecules' HLB (hydrophilic lipophilic balance) and density.

The two final predictive models were:

Biological Activity of Dopamine Mimetics = 4.45 - 0.528 HLB

and

Biological Activity of Dopamine Mimetics = -7.30 + 7.14 density

Through statistical methods these models were validated and the results of the analysis show that, individually, the parameters HLB and density effectively predicted biological activity of all compounds except for compound 6. Compound 6 is, however, not truly a dopamine mimetic. Compound 6 is without any monoamine potentiating properties. Therefore, these two parameters were 100% effective at predicting the biological activity of the true dopamine mimetic compounds analyzed, and 91.7% effective at predicting the biological activity of all twelve compounds. The squared distance was 5.48, which showed that the means of the two groups were 5.48 standard deviations apart. This showed there was very little overlap between the groups when using ± 3 standard deviations per group. This also showed that the predictions were divided into two distinct groups (biologically active or inactive).

In addition these results display that the categorization of compound 8 as biologically active is a questionable call based on the limited experimental data obtained, and that based on the model development techniques compound 8 is likely inactive. Additionally, analysis was conducted using specific endpoints of fetal death, fetal weight and malformations. Accurate predictive models were generated categorizing compounds 1,2,7,11 as positive for the endpoints of fetal weight and malformations and accurate predictive models were generated categorizing

compounds 1,2,7 as positive for the endpoint of fetal death. In all models generated density was a major physicochemical property associated with activity in this series of dopamine mimetics. The parameters HLB, Hansen dispersion, hydrogen bond acceptor and dipole moment are also all associated with the biological activity of these dopamine mimetics.

From the application of the generated models to determine values of the specific parameters in the models that would indicate biological activity of unknown compounds with similar structure to those used to develop the models, a density value of 1.16 or greater is indicative of a compound that is teratogenic, a density of approximately 1.22 or greater or a dipole moment greater than 4.86 indicates the compound is active for fetal death, and a density value of 1.16 or greater is indicative of a compound that is active for growth retardation and/or malformations. While the specific values determined from the models are not exact limits for activity versus inactivity, any compounds that are similar in structure to the compounds used to develop these models and have parameters approximately equal to those derived should be suspect for the specific activity listed. As with any QSAR/SAR model, these determinations are not exact, however researchers synthesizing similar compounds in the future can utilize these parameter values for early initial predictions of the newly synthesized compounds biological effects.

The results of the QSAR/SAR model show that a mathematically simpler model is effective at predicting the biological activity of the dopamine mimetics. This illustrates that when developing a QSAR/SAR model a large complex model is not always necessary, and a simpler model may be just as effective at predicting biological activity as the complex model. The usefulness of generating a QSAR/SAR model is to quickly and accurately screen related

compounds for biological activity. The simpler the model and the more readily available the variables are, the more effective and useful a model is in application.

The next segment of research was validating a reproductive QSAR/SAR model through in vivo animal testing. A series of chemicals not used for the QSAR/SAR model development was submitted for prediction and based on the physico chemical properties the model predicted retinoic acid was positive for developmental toxicity. The in vivo validation utilized a timed pregnant mouse study using Retinoic Acid as the chemical agent and the CK assay. The CK assay quantitatively measures effects on fetal viability and growth. The specific endpoints analyzed in this study were maternal toxicity, fetal growth and pup death.

In this study, maternal toxicity was slightly evident but not significant, and no dose dependent reduction in fetal growth was evident. The endpoint that was unambiguously associated with influenced cis RA treatment was fetal death. In all instances, when the dose increased the number of surviving pups decreased. In all categories of fetal death that were evaluated, the relationship between increased dose and increased death was statistically significant, giving p values of 0.05 or less. Statistical analysis revealed these effects were dose-dependent. These results demonstrated, in a quantitative manner, the developmental toxicity of retinoic acid in the mouse, as was predicted by our SAR model and as was expected from developmental literature.

The final segment of research was the in vitro experimentation conducted on a series of four microtubule perturbing agents that have been classified as promising antiproliferative cancer agents (Analog II, 4-methoxy Analog II, JR oxime I and TDH 169). Research was conducted with these four agents to determine their effects on cell viability, cell death and gene expression of three TRAMP cell lines, C1A, C2H and C2N.

The MTT assay revealed that 4-methoxy Analog II treatment displayed the most promising antiproliferative effects. Therefore, additional research was conducted with 4 -methoxy Analog II alone. An ELISA assay was conducted on all TRAMP cell lines and revealed the minimum apoptosis-inducing levels of 4-methoxy Analog II on each cell line. Due to limitations in resources additional experiments were limited to the C1A TRAMP cell line, because the C1A cell line showed the most promising results. The ELISA assay was followed by PARP cleavage and flow cytometry assays to confirm and quantify apoptosis attributable to 4 -methoxy Analog II on the C1A cell line. Finally, a microarray was conducted to determine alterations in gene regulation of the C1A cell line due to treatment with 4 -methoxy Analog II. Changes in gene regulation due to treatment were found in apoptosis activator genes Bok and Siva-pending (found to be upregulated) and the apoptosis inhibitor genes Birc 4, Dad1 and Atf5 (found to be downregulated). Further research would include in vivo testing of the agents identified by the TRAMP cell lines experiments that produce the most therapeutic results. The potential benefits from this research would be the development of a drug treatment that would combat prostate cancer in the human.

In summary, this research provides an outline of a chemical animal model building approach utilizing the latest techniques being implored in the chemical and biomedical field. The process outlined in this research begins with a QSAR/SAR model development by means of computational chemistry on a series of dopamine mimetics that are expected developmental toxicants. The research progresses through in vivo model validation using a QSAR/SAR model predicted positive developmental toxicant, retinoic acid. The final portion of this research includes in vitro testing on a novel cell lines, TRAMP, to evaluate four promising antiproliferative cancer agents Analog II, 4-methoxy Analog II, JR oxime 1 and TDH 169.

The overall results display a systematic approach for use in drug research to evaluate a series compounds by developing a chemical animal model. This systematic approach provides a guideline for future efforts in developing therapeutic treatments for various diseases. In addition, the results also provide insight into the toxic effects of several compounds (dopamine mimetics and retinoic acid) in the emergent field of developmental toxicology. The results also present information regarding the effect of the promising antiproliferative cancer agents 4-methoxy Analog II on the C1A TRAMP cell line. The microarray analysis provides data of the alteration of specific genes expression due to treatment. Through additional in vivo research, using TRAMP mice, may lead to further understanding of the genes associated with prostate cancer and insight into therapeutic agents that may control the expression of these prostate cancer genes.

APPENDIX A: MICROARRAY EXPRESSION DATA

Separate file attached

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