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## Cellular Proliferation, Death and Histology in *Gambusia Affinis* Livers after Exposure to 2-Aminofluorene and Benzidine

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CELLULAR PROLIFERATION, DEATH AND HISTOLOGY IN  
GAMBUSIA AFFINIS LIVERS AFTER EXPOSURE TO  
2-AMINOFLUORENE AND BENZIDINE

by

Susan J. Lentz, Ph.D.

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Faculty of The Graduate College  
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CELLULAR PROLIFERATION, DEATH, AND HISTOLOGY IN  
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Susan J. Lentz, Ph.D.

Western Michigan University, 2003

A fundamental need exists for aquatic animals in the biomonitoring of aquatic ecosystems for environmental carcinogens. *G. affinis*, a small fish species, is selected for this study due to its hardiness, non-migratory nature, geographic diversity and ability to develop tumors. Molecular tools for cellular proliferation and death are examined for use with *G. affinis*. Additionally, these tools are used to investigate the early carcinogenic effects of 2-aminofluorene and benzidine, both individually and combined, on *G. affinis* livers.

Antibodies to proliferating cell nuclear antigen (PCNA), p53, cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) were examined for use in *G. affinis*. While p53 and cleaved caspase-3 and PARP results were inconclusive, PCNA was successful in detecting cellular proliferation in *G. affinis*. Additionally, the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method was examined and found to be a successful indicator of cell death.

Therefore, cellular proliferation, death, and their difference in *G. affinis* livers were assayed after dietary exposure to 0.069 mM and 6.9 mM doses of 2AF, BZ and 2AF/BZ for 4, 8 and 12 weeks. Hematoxylin and eosin (H&E) staining was used for

histological examination. All treatment groups had similar responses at differing degrees. These results from *G. affinis* exposure to 2AF, BZ and 2AF/BA indicate:

- A transient increase in cellular death at 4 weeks that decreased at 8 and 12 weeks, some below control levels.
- A substantial increase in cellular proliferation throughout the 4, 8 and 12 exposures above control levels.
- A net increase in cellular growth above control levels.
- Increases in oval cell proliferation, altered foci and tumor development.



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Susan J. Lentz

## TABLE OF CONTENTS

ACKNOWLEDGMENTS .....	ii
LIST OF TABLES .....	iv
LIST OF FIGURES.....	v
CHAPTER	
I. INTRODUCTION .....	1
Cancer and Environmental Carcinogens .....	1
Polycyclic Arylamines.....	3
Small Fish in Carcinogenic Studies.....	4
Scope of Dissertation.....	6
II. LITERATURE REVIEW .....	7
Carcinogenesis .....	7
A Historical Overview.....	7
Cellular Origins of Cancer .....	10
From Totipotent Stem Cells to Differentiated Tissue Cells.....	11
Embryonal Theory of Cancer.....	12
Dedifferentiation of Mature Cells in the Origin of Cancer .....	13
Maturation Arrest of Stem Cell Differentiation in the Origin of Cancer.....	14
Stages of Carcinogenesis.....	16

## Table of Contents—continued

Initiation .....	17
Promotion.....	17
Progression.....	18
Contributing Factors to the Genesis of Cancer.....	19
Oncogenes .....	20
Tumor Suppressor Genes.....	22
Environmental Contributors and Biotransformation .	23
Progress and Prevention.....	24
The Cell Cycle.....	26
Proliferation Cell Nuclear Antigen.....	28
p53 Tumor Suppressor Gene.....	30
Caspase-3 .....	33
Poly (ADP-Ribose) Polymerase.....	35
Detection of DNA Fragmentation During Cell Death.....	38
Benzidine .....	43
2-Aminofluorene.....	47
Gambusia Affinis.....	50
Aquatic Species in Carcinogenic Studies.....	50
Gambusia Affinis in Environmental Carcinogenesis Studies.....	51
III. MOLECULAR TOOLS.....	54

## Table of Contents—continued

Purpose of Investigating Molecular Tools.....	54
Experimental Design .....	55
Methods and Materials .....	56
Fish and Housing.....	56
Flow Cytometry, Fluorescence Microscopy and Differential Interference Contrast Microscopy.....	59
Cell Staining.....	60
Flow Cytometry .....	61
Fluorescence Microscopy and DIC Microscopy.....	61
Western Blots for Immunodetection of PCNA, p53, Cleaved Caspase-3 and PARP Proteins .....	62
Total Protein Extraction and Quantification.....	63
Western Blotting .....	63
Results .....	65
Flow Cytometric Analysis of AML21 Hepatocytes .....	65
Fluorescence Microscopy and DIC Microscopy of AML12 Hepatocytes .....	66
Western Blots of PCNA, p53, and Cleaved Caspase-3 and PARP .....	69
Discussion .....	74
IV. DETECTION OF CELLULAR PROLIFERATION AND DEATH IN G. AFFINIS EXPOSED TO 2-AMINOFLUORENE AND BENZIDINE .....	79

## Table of Contents—continued

Purpose of Investigation .....	79
Experimental Design.....	80
Methods and Materials .....	82
Fish and Housing.....	82
Tissue Sectioning of Livers.....	83
PCNA Antibody and TUNEL Labeling of Tissue Sections.....	83
PCNA Antibody Labeling of Tissue Sections .....	84
TUNEL Labeling of Tissue Sections.....	85
Determination of PI Index and TUNEL Index.....	85
Results .....	87
PCNA Index.....	87
TUNEL Index .....	89
PCNA Index Minus TUNEL Index.....	101
Discussion.....	103
V. HISTOPATHOLOGY OF 2-AMINOFLUORENE AND BENZIDINE TREATED G. AFFINIS LIVERS .....	111
Purpose of Study.....	111
Experimental Design.....	111
Methods and Materials .....	112
Results .....	113
Discussion.....	116

Table of Contents—continued

VI. CONCLUSIONS .....	121
REFERENCES .....	126
APPENDICES .....	174
A. Institutional Animal Care and Use Committee Approval form .....	175
B. Individual TUNEL Index, PCNA Index and (PI – TI) Counts .....	176



## LIST OF TABLES

1. Experimental Design .....	81
2. Means for TUNEL Index, PCNA Index and Their Differences .....	90
3. PCNA – Tests of Time and Treatment Effects and Interaction .....	95
4. PCNA – Fisher’s Paired Least Significant Differences With Statistical Significance.....	96
5. TUNEL – Comparison of Tests of Treatment and Time Effects and Interactions.....	99
6. TUNEL – Adjusted Comparison of Treatment and Time Effects and Interactions.....	100
7. TUNEL – Fisher’s Paired Least Significant Differences With Statistical Significance.....	100
8. PCNA and TUNEL Differences – Test of Treatment and Time Effects and Interaction.....	106
9. PCNA and TUNEL Differences – Fisher’s Paired Least Significant Differences With Statistical Significance.....	106
10. Histopathological Examination of G. Affinis Livers After Exposure to 2AF and BZ.....	114

## LIST OF FIGURES

1. Schematic of the Cell Cycle.....	27
2. Dual Filtration System.....	57
3. Circulating Air and Water Through CPVC Piping .....	58
4. Bulkhead Attachment for Continual Water Drainage .....	58
5. Filtration of Drainage Water.....	59
6. Flow Cytometry Histogram of Propidium Iodide (PI) Stained Freeze/Thawed AML12 Hepatocytes .....	67
7. Flow Cytometry Histogram of Propidium Iodide (PI) Stained Camptothecin Treated AML12 Hepatocytes.....	67
8. Flow Cytometry Histogram of Propidium Iodide (PI) Stained Non- Treated AML12 Hepatocytes.....	68
9. Flow Cytometry Histogram of Hoechst 33342 Stained Camptothecin Treated AML12 Hepatocytes.....	68
10. Flow Cytometry Histogram of Hoechst 33342 Stained Non-Treated AML12 Hepatocytes.....	69
11. Western Blot of Anti-PCNA Antibody Labeling of Total Protein Extracts .....	72
12. Western Blot of Anti-p53 Antibody Labeling of Total Protein Extracts .....	72
13. Western Blot of Anti-Cleaved Caspase-3 Antibody Labeling of Total Protein Extracts .....	73
14. Western Blot of Anti-Cleaved PARP Antibody Labeling of Total Protein Extracts .....	73
15. Histogram of PCNA Index Means and Standard Error for Treatment Groups and Controls at 4 Weeks.....	91

# List of Figures—continued

16. Histogram of PCNA Index Means and Standard Error for Treatment Groups and Controls at 8 Weeks.....	91
17. Histogram of PCNA Index Means and Standard Error for Treatment Groups and Controls at 12 Weeks.....	91
18. Histogram of TUNEL Index Means and Standard Error for Treatment Groups and Controls at 4 Weeks.....	92
19. Histogram of TUNEL Index Means and Standard Error for Treatment Groups and Controls at 8 Weeks.....	92
20. Histogram of TUNEL Index Means and Standard Error for Treatment Groups and Controls at 12 Weeks.....	92
21. Histogram of PI - TI Index Means and Standard Error for Treatment Groups and Controls at 4 Weeks.....	93
22. Histogram of PI - TI Index Means and Standard Error for Treatment Groups and Controls at 8 Weeks.....	93
23. Histogram of PI - TI Index Means and Standard Error for Treatment Groups and Controls at 12 Weeks.....	93
24. PCNA Positives from a G. Affinis Liver Tissue Section.....	94
25. PCNA Boxplot of Wilcoxon Studentized Residuals.....	94
26. PCNA Q-Q Plot of Studentized Residuals.....	95
27. TUNEL Positives from a G. Affinis Liver Tissue Section .....	98
28. TUNEL Boxplot of Wilcoxon Studentized Residuals .....	98
29. TUNEL Q-Q Plot of Studentized Residuals.....	99
30. PCNA and TUNEL Differences Boxplot of Wilcoxon Studentized Residuals .....	105
31. PCNA and TUNEL Differences Q-Q Plot of Studentized Residuals.....	105
32. Altered Foci, Granuloma and PMA, Respectively, in G. Affinis Liver .....	117

List of Figures—continued

33. Basophilic Foci of Spindle Cell Proliferation in G. Affinis Liver.....	117
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## CHAPTER I

### INTRODUCTION

#### Cancer and Environmental Carcinogens

Cancer has been around longer than civilization itself. It has been reported that neoplastic lesions were observed by paleopathologists in the bones of dinosaurs [1]. Efforts to understand the genesis of cancer date back to the 4<sup>th</sup> century when Hippocrates' put forth that excess black bile was the cause of cancer. However, it wasn't until 1838 that Johannes Müller observed that cancer tissue was made up of cells [2]. Epidemiologic research into cancer brought about a significant increase in knowledge of human cancer causes after World War II including the relationship between human cancer and environmental carcinogens [3]. Another significant increase in cancer research was in the early 1970's with the advent of recombinant DNA technology. That technology led the advancement from animal models to molecular models [4]. Although our understanding of the processes involved in the onset and progression of cancer has grown tremendously over the past 30 years, there is still no comprehensive picture of carcinogenesis available today.

In broad terms, cancer is a complex, dynamic, and often, fatal disease in which cells undergo molecular changes that lead to their uncontrolled proliferation. This uncontrolled cellular proliferation lends itself to tumor formation that may or may not become cancerous and metastasize. The underlying causes of cancer are attributed to either or both genetic or epigenetic alterations [4-9]. Genetic origins are often due to mutations in cancer related genes. These mutations are either germline (inherited) or

somatic mutations (environmental) or a combination of both. Viruses are also capable of inducing cancer by integration of their genome into the host cell [10]. While only a small number of cancers are due to germline mutations, environmental components account for about 80% to 90% of all cancers [3, 11, 12].

With environmental carcinogenesis being a major factor in human cancers, great effort has been made in elucidating the role these environmental carcinogens play in the development and progression of cancer. However, it is important to note that this is a difficult task with more than 100 different cancers in more than 35 different organs and tens of thousands of chemicals in the environment [5]. The environmental components associated with carcinogenesis can either be localized, in cases such as improper disposal of industrial waste and run-off, or widely distributed, as with automobile exhaust products and agriculture. World Health Organization data indicates that death rates due to lung, breast, prostate, colon, rectal, stomach, and uterine cancers, are consistently lowest in Thailand for both males and females. However, the highest death rates for these same cancers are widely distributed across geographic regions according to sex and cancer type indicating the geographic variation of environmental carcinogens that target specific organs. In addition to the geographic distribution of environmental carcinogens, variation exists in their concentration at different sites and their partitioning properties. Additional culprits in environmental carcinogenesis are cultural, lifestyle, age, species, and sex differences.

The study of environmental carcinogens is essential in understanding the underlying events leading to the formation of cancer. While a great deal of knowledge has been attained through scientific research regarding carcinogenesis, its relationship

with environmental compounds is limited [13]. With over 50,000 chemicals registered with the EPA and FDA, and more being continuously introduced, it is imperative to understand the biological cellular mechanisms involved in environmental carcinogenesis. With that understanding will come the ability to interfere with those mechanisms [14].

The National Institute of Cancer (NIC) cites a special concern for products originating from the combustion of fossil fuels in diesel engines and automobile exhaust, industrial emissions, residential and commercial space heating, and oil and coal fired power plants. The byproducts of these processes include polycyclic aromatic hydrocarbons (PAH), polycyclic aromatic amines, and numerous other cancer related compounds. While many of these chemicals themselves may not directly initiate carcinogenesis, their metabolites can initiate and promote the carcinogenic process. Thus it is necessary to test these compounds in biological systems to determine their effects, along with that of their metabolites, on living organisms.

### Polycyclic Arylamines

As previously stated, several of the environmental pollutants listed for special concern by the NIC contain PAH. Different processes (e.g. industrial, automotive) emit different concentrations and types of PAH, of which many are considered potent carcinogens [15]. . The detrimental effects of a wide range of PAHs are well documented. These effects include formation of DNA-adducts, sister chromatid exchanges, lung, bladder and skin cancer, transplacental transfer, premature ovarian failure, and activation of the aromatic hydrocarbon receptors (AhR) [16-22]. Due to the

numerous compounds that fall under the classification of PAH, it is only feasible to use representative compounds for investigative inquiry.

There are two broad classes of PAHs, the biaryls and the condensed benzenoid hydrocarbons. These classes differ in whether their hydrocarbon ring structures are fused (sharing two carbons) or linked (a single carbon bond). The representative class selected here is the biaryls because they undergo electrophilic aromatic substitution more readily than the condensed benzenoid hydrocarbons. This substitution occurs when one electrophile (typically a proton) is replaced by another electron-deficient species [23]. The electrophilic amine groups of polycyclic arylamines (PAA) are metabolized in the liver to highly reactive nitrinium ions that bind DNA, forming DNA adducts [24].

The individual polycyclic arylamines chosen for this study are benzidine and 2-aminofluorene. Both of these compounds and their metabolites are well studied and are involved in carcinogenesis. Their effects on liver cells are of particular interest because of the metabolic enzymes present in livers. These enzymes are capable of either metabolizing the compound to a less toxic form or a more toxic form. This paper specifically examines the exposure of benzidine (BZ) and 2-aminofluorene (2AF) on cellular proliferation, death, and growth in the livers of the western mosquitofish, *G. affinis*.

### Small Fish in Carcinogenic Studies

Interest in the use of fish models for environmental carcinogenesis studies has grown as the need for more efficient animal models for statistical significance in cancer research has also grown. An Interagency Agreement between the U.S. Army and the



U.S. Environmental Protection Agency was initiated in the early 1980's for the purpose of developing more efficient animal models for evaluating the health effects of environmental pollutants. It found development of fish models to be of special interest in carcinogenic studies [25]. The use of fish in carcinogenic studies is growing due to the similarities that fish have in their metabolic pathways with that of mammals for disposition of certain carcinogens [26]. Fish have also been demonstrated to form neoplasms in short periods of time, are relatively easy to house and care for and due to the small size of numerous species, statistical significance can be more readily achieved in an efficient manner [27-29].

Several fish models have been and are currently utilized in studies to demonstrate the carcinogenic effects of chemical toxins [28-34]. These studies, along with numerous others, indicate the feasibility of small fish models in environmental carcinogenesis studies. This study uses the western mosquitofish, *Gambusia affinis*. This fish is ubiquitous in the United States, is non-migratory and is distributed throughout the globe for mosquito control [35-37]. Thus, it can be monitored at numerous sites and is representative of each site it resides. Additionally, they have been demonstrated to develop neoplasms in response to chemical carcinogens [27]. Many of the other fish species used in research are either exotic and reside in restricted waters, have low tolerance of poor quality water, are migratory, or are large and not easily maintained. Therefore, *G. affinis* has a promising future as both a sentinel species for biomonitoring of aquatic ecosystems and carcinogenesis studies.

## Scope of Dissertation

Environmental carcinogenesis is responsible for the majority of human and animal cancers. Understanding the impact these environmental compounds have at the cellular and molecular levels is imperative in determining the mechanistic pathways involved in the early onset of neoplastic development. Once these pathways have been identified, they can ultimately be targeted for interference in efforts to eliminate or control the process of carcinogenesis. This paper examines the individual and combined effects of two polycyclic arylamines, BZ and 2AF, on cellular proliferation and death, and net cellular growth in the liver tissue of *G. affinis*.

*G. affinis* is not a traditional research organism. Therefore, in order to investigate the effects of PAAs on cellular proliferation and death, five potential biomarkers are tested for compatibility with *G. affinis*. The first biomarker is an antibody to proliferating cell nuclear antigen (PCNA). It is used for the detection of cellular proliferation. The next three biomarkers are antibodies to p53 and cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP) proteins. These antibodies detect proteins associated with programmed cell death. The last biomarker tested uses the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. TUNEL detects DNA strand breaks associated with cell death.

Additionally, formation of foci, tumors and neoplasms are examined using hemotoxylin and eosin (H&E) staining of *G. affinis* liver tissue sections. HE staining, along with the use of biomarkers for cell proliferation and cell death are examined to reveal the extent of aberrant growths and overall changes in cellular proliferation, cell death and net cellular growth in *G. affinis* livers after exposure to 2AF and BZ.

## CHAPTER II

### LITERATURE REVIEW

#### Carcinogenesis

##### A Historical Overview

Cancer has plagued humans and animals for millions of years and continues to do so to this day. Neoplasms have been documented to occur as far back as the Mesozoic era when dinosaurs ruled [1, 2]. The bones of petrified, prehistoric, human remains, a million and a half years old, have been found to have tumors [10]. Evidence of squamous papillomas, nasopharyngeal carcinomas and several suspected malignant melanomas have been reported in Egyptian and Peruvian mummies dating back five to six thousand years [38, 39]. An interesting note is that nasopharyngeal carcinomas are still prevalent in the Egyptian population today [10]. Attempts to rid our selves of the scourges of cancer date back to ancient times.

Ancient Egyptian physicians were the first to recognize different type of tumors. They treated soft tumors by incision and drainage and hard tumors by excision. It was “Galen the Pergamite”, (130-200 A.D.), who first categorized tumors, he considered swellings, as 1) according to nature (swelling of breasts and uterus during pregnancy), 2) exceeding nature (bone fracture calluses) and 3) contrary to nature (neoplasms). He also characterized tumors as benign or malignant and was the first to recognize metastasis of tumors [10]. However, it wasn’t until the 1700’s that significant strides were made in understanding the relationship of increased cancer incidences with that of environmental

influences.

Beginning in 1713, with Ramazzini's observations that the high incidence of breast cancer in nuns may be attributable to their childless lifestyle, is when rudiments for the groundwork on environmental influences in carcinogenesis was set forth. The late 1700's continued on this path when J. Hill published, "Cautions against the immoderate use of snuff", and P. Pott published, "Chirurgical observations relative to the cancer of the scrotum". These publications documented the statistically significant relationship of snuff to nasal polyps and chimney soot exposure to cancer of the scrotum. Further case studies in the 1800's continued to find associations with environmental agents, such as arsenic, sunlight and aniline dyes, and increased cancer risk [40]. The advent of the microscope and achromatic lenses in the early 1800's brought about significant advancements in understanding cancer, including diagnostic techniques.

Three pathologists, Johannes Müller, Rudolf Virchow and Karl von Rokitanski, were able, with the use of microscopic techniques, to establish the cellular basis of life and more specifically the cellular basis of cancer. Papanicolaou (1883-1962), a physician and cytologist used cells shed from malignant neoplasms. He stained and observed them on glass slides under the microscope and was able to morphologically define them as cancerous. This method is routinely used today in screening tests for cancer: it is the "Pap-smear" [2, 10, 41]. Evidence continued to accumulate in relating environmental agents to cancer and these were especially evident in epidemiological studies.

Epidemiological research identified geographic, ethnic, lifestyle, occupational, dietary, age, and gender differences, along with individual susceptibility, in occurrence of tumors as well as tumor types. One of the earliest epidemiologic publications on lung

cancer, by Doll and Hill (1950), hypothesized that increases in lung cancer were related to atmospherically polluted areas and smoking of tobacco[42]. Additional studies by Doll and Hill using 1465 lung cancer cases and 1465 controls supported their earlier suspicions that smoking tobacco was a significant contributor to lung cancer. However, they also emphasized that not all smokers would develop lung cancer, nor were all lung cancers due to smoking indicating individual variation [43, 44]. Another well known epidemiological study in the 1960's demonstrated that when people migrated from Japan to the United States, their children did not exhibit the cancer patterns of their parent's homeland, but instead, they acquired the characteristic patterns of the new country indicating geographic/cultural influence [45]. As epidemiologic studies identified statistically significant environmental components in the etiology of cancer, molecular techniques were developed that catapulted a revolution in the biological sciences including environmental carcinogenesis.

The past 20 years has brought about the power to isolate, manipulate and propagate genes and other genetic elements outside the host, then reintroduce them into the same or different host. This has lead to extraordinary advancements in understanding the complexities of carcinogenesis. These advancements include the use of restriction enzymes, bacterial and viral cloning vectors, DNA ligases, DNA and RNA polymerases, oligonucleotide probes, DNA, RNA and protein isolation and separation, immunoassays and transfection just to name a few.

In the mid 1970's, Sanger developed a technique for sequencing the nucleotide sequence of genes and fragments of DNA [46]. This technique allowed investigators to examine the differences in mutant and wildtype genes, especially those related to the

onset of cancers. The 1993 Nobel Prize in Chemistry was presented to Kary Mullis for his work in 1986 in developing the polymerase chain reaction (PCR). This technique amplified segments of DNA  $10^6$  to  $10^9$  times in a matter of hours, reducing the need for week's worth of cloning. Molecular techniques have also shed light on gene function. For example, in 1975, Kohler and Milstein reported the production of antibodies of a predefined specificity [47]. The production of antibodies has been essential in immunoblotting and immunohistochemistry. This allows the investigator to identify the expression of particular genes in cells, such as those related to carcinogenesis. The 1980's brought about the use of gene transfer experiments, wherein the genes of one organism can be transferred and expressed into genome of another organism. This transfer of genes gives insight into the function of particular genes, including oncogenes and tumor suppressor genes, and possibly, the impact of carcinogens on the regulation of homeostasis [2, 48-50].

With the ever-growing body of data and techniques now available to investigate the etiology of cancer, its complexities still elude us. While all cancers may fall into a very broad definition, historical perspectives and current understandings have indicated a disease process that does not fit into any comprehensive explanation. Debates over the cellular origins of cancer, while fine-tuned, occur in scientific journals today [51-54].

### Cellular Origins of Cancer

It is common knowledge that cancers are composed of cells that proliferate outside the normal boundaries of cell cycle control mechanisms. How this proliferative capacity is brought about by viral infection, chemicals, radiation or inherited disorders

has been of great interest and study over the years. That cancers are cellular in origin begs the question: From which cells does the cancer originate? Is it the mature tissue cells dedifferentiating? Is it unregulated pluripotent stem cells? Different possible cellular origins have been suggested such as: the embryonal theory of cancer, dedifferentiation of mature cells capable of dividing and the maturation arrest of stem cell differentiation [53-58]. In a review of current literature on the topic, it appears that the maturation arrest of stem cells is a likely candidate for the origin of cancerous cells, although dedifferentiation of mature cells may play a role outside of teratocarcinomas [52, 53, 56, 58-62]. To understand the different possible cellular origins of cancer, a short review of the different stages and progression of various cell types follows.

#### From Totipotent Stem Cells to Differentiated Tissue Cells

The journey from a totipotent stem cell to a terminally differentiated tissue cell is well documented in developmental biology. Totipotent stem cells have the capacity to become any cell type of the organism [53, 63-66]. The fertilized egg, a zygote, is an excellent example of a totipotent cell. In the correct environment, the zygote has the potential to divide, differentiate and organize into a complex organism [67]. In all vertebrates, these totipotent zygotes produce three populations of pluripotent stem cells, the ectoderm, endoderm and mesoderm. These cells are described as pluripotent because while they are stem cells and still have the capacity to differentiate into variety of cell types, this differentiation potential is limited [68]. For example, the cells comprising the ectoderm produce skin and nerve cells, but cannot produce the digestive and respiratory

cells produced by cells of the endoderm and vice versa [53]. These pluripotent stem cells have lost totipotency and are now restricted in their differentiation potential.

Research with hematopoietic stem cells have shown that these pluripotent, determined stem cells produce committed stem cells. These stem cells have further restrictions on their potential for differentiation and are committed to produce only predetermined cell types [52]. Once a cell is terminally differentiated, it no longer has the ability to differentiate into another cell type. For example, a granulocyte precursor cell may terminally differentiate into a basophil or eosinophil [69]. These basophils and eosinophils do not further differentiate nor can they revert back to a granulocyte precursor cell. These terminally differentiated cells are now fixed. Other terminally differentiated cells, such as hepatocytes have the same restrictions in that they can only produce more hepatocytes.

In summary, as totipotent stem cells produce pluripotent stem cells, and these pluripotent stem cells produce more committed progeny cells, the potential for differentiation becomes sequentially more restrictive. This continues until the progeny cells become terminally differentiated. These sequential events of differentiation have been extrapolated to models of carcinogenesis due to the morphological and biochemical similarities observed in cancerous cells in different tissues [52, 53, 58, 60, 70-72].

### Embryonal Theory of Cancer

The embryonal theory of cancer states that embryonal stem cells somehow become isolated in tissue while still maintaining the potential to proliferate. Because the stem cells are isolated in the developing organ, they are unable to differentiate with the



surrounding cells. Then at some later time those isolated stem cells express their potential for persistent growth. This proliferation occurs later in adult life and forms tumors. That theory was widely accepted throughout the 19<sup>th</sup> century and was contrived as early as 1829, when Recamier hypothesized that tumors originated from embryonal cells that proliferated into adulthood [73].

While this embryonal theory of cancer has been laid to rest due to the fact that the resting embryonic cells have never been observed, it isn't that far off from the currently held theories on the origin of cancerous cells. One of these current theories is the dedifferentiation of terminally differentiated cells.

#### Dedifferentiation of Mature Cells in the Origin of Cancer

Dedifferentiation is defined as the loss of phenotype and the gain of potential for differentiation. While dedifferentiation occurs during development, it has also been attributed to the cellular origin of cancer. However, this has been debated because the malignant cells in teratocarcinomas originate from undifferentiated germ cells and result in an undifferentiated, not dedifferentiated, appearance in the tumor [74]. Thus some have dismissed dedifferentiation in carcinogenesis and attribute its conception as a lack of understanding of the normal molecular and cellular mechanisms involved in the development and renewal of tissue in organs, especially determined stem cells [53].

While this may be true for teratocarcinomas, it is argued that the majority of cancers in any one tissue show a broad spectrum of degrees of differentiation such as basal cells to keratinocytes in squamous cell carcinomas and the biochemical properties of mature differentiated hepatocytes in hepatocarcinomas [56]. Also, prostatic cancer

cells are so well differentiated that it's difficult to distinguish them from normal prostatic acinar cells [75].

#### Maturation Arrest of Stem Cell Differentiation in the Origin of Cancer

Maturation arrest of stem cell differentiation is another currently held hypothesis for the origin of cancerous cells in scientific literature. The first direct evidence for a stem cell origin of cancer was with the use of transplantable teratocarcinomas of mice [76]. In 1970, Roy Stevens injected teratocarcinomas from the genital ridge of F1 mice into the parental mice testes. These transplanted teratocarcinomas have since given rise to teratocarcinomas from the normal genital ridge cells for over 200 generations in inbred parental mice [58]. It is the morphological and developmental characteristics between embryonic cells and cancer that has kept the stem cell origin of cancer in the spotlight.

In 1994, Stewart Sell and G Barry Pierce published a paper reviewing the cellular origin of cancers for the following tissues: teratocarcinomas, epidermal/transitional cell carcinomas, adenocarcinomas, hepatocellular carcinomas, pancreatic carcinomas, breast cancers, prostate cancers and lung cancers. In each incidence they were able to identify a determined stem cell that was required for tissue renewal in the carcinomas. The tumors that resulted were not dedifferentiated. They contained many stem cells and imperfect differentiation. In the late 1960's these undifferentiated tumors were described as caricatures of the normal process of tissue renewal [74].

It was once argued that malignant tumors were able to originate from benign tumors. This was supported by erroneous assumptions that adenocarcinomas arose from adenomatous polyps in the colon [77, 78]. However, it wasn't understood at that time

that adenomatous polyps are not benign tumors, but actually focal areas of hyperplasia that have not progressed to cancer. Benign tumors have a narrow range of differentiated cells, whereas a focal area of hyperplasia contains a wide range of undifferentiated stem cells along with their differentiated progeny cells [58]. It is the focal hyperplasias that are the target for secondary or tertiary mutations due the numerous cycling cells that may act as targets. This is seen in classical gastrointestinal carcinomas as well as hematopoietic malignancies.

Both gastrointestinal and hematopoietic cells have a high turnover rates such that early initiation events in differentiating cells would be gone before application of promotional events several months later. However, if the initiation event takes place in renewable stem cells, then the application of a promoter months or years later would still be able to induce a malignancy [79, 80]. This is especially evident in hematopoietic stem cell research where a great deal of research has been conducted on the regulation of self-renewal since both the normal stem cells and cancer cells from this tissue are well characterized and documented [52].

Several morphogenic and biochemical similarities have been observed between stem cells and cancer cells. Pathways involved in the regulation of normal self-renewal such as inhibition of apoptosis by expression of *bcl-2*, and promotion of self-renewal by the Notch, Shh and Wnt signaling pathways have also been shown to be involved in leukemias, colon carcinomas, epidermal tumors, medulloblastomas, basal cell carcinomas and mammary tumors [52, 81-85]. An article in Science recently reported the discovery of a gene product that is expressed in the early, multipotential state of embryonic stem cells that virtually disappears at the beginning of differentiation. In efforts to elucidate its

function, over-expression and gene silencing of this protein in both embryonic stem cells and human cancer cells resulted in hindered cell proliferation, suggesting a role in regulation of proliferation [61].

Further evidence has accumulated in the tumor stem cell hypothesis including the escape of tumor cells from immune surveillance. G. Manzo has published a paper suggesting the role of MHC-null/HSP70-very high phenotypes as a sort of 'primordial self' tumor cell that cannot be recognized by either the MHC-restricted or the MHC-non-restricted immunosurveillance systems, thus eluding detection and allowing for proliferation as with undifferentiated embryonal cells [57].

As research progresses and evidence continues to mount, the stem cell hypothesis for the origin of cancer cells has held steady, but not without dissenters. They argue that in hepatocarcinogenesis, the precursor lesions are clearly the mature hepatocytes [56]. This was observed in preneoplastic foci of rat livers 11 days after initiation with diethylnitrosamine and promotion with 2-acetylaminofluorene in the three separate experiments [86]. Therefore, both dedifferentiation and stem cell arrest theories have neither been proved nor disproved. Moreover, they appear to demonstrate the variance observed in different neoplasm classes.

### Stages of Carcinogenesis

Carcinogenesis is the multistep progression of cells sequentially undergoing a selective growth advantage, clonal expansion, and neoplastic formation. This multistep progression can be categorized into two main themes, the stages of neoplastic formation and the components contributing to the process [2]. The three major stages of neoplastic

development are often described as initiation, promotion, and progression. Several other substages occur during each major stage [87]. Although there is no uniform definition for initiation, promotion, or progression due to their links with individual experimental procedures and interpretations, they can be generally defined by their molecular, cellular, and tissue level characteristics [2].

### Initiation

Initiation is generally referred to as the earliest stage of cancer where DNA damage has occurred in a cancer related gene. This damage may originate from spontaneous mutations, UV radiation, chemical mutagens, hypomethylation, inherited mutations, DNA adduct formation, replication errors and a host of other factors [12, 41, 88-93]. Initiation typically is associated with a rare change in cellular phenotype [56]. This new phenotype is a critical component to initiation. It gives the rare cell a selective growth advantage and an altered response to its environment when compared to the neighboring normal cells [41, 45]. In a study of hepatocarcinogenesis, four major factors were identified in initiation: a) DNA interaction, b) dependence on a round of cellular proliferation, c) cells with resistance to some of the inhibitory effects of the carcinogen, and d) selection of new resistant cells [94, 95]. In a minireview of environmental carcinogenesis and biotechnology, initiation is described as damages to DNA associated with the early changes in cancer development such as base pair mutations [2]. Initiators, with rare exceptions, usually act as both initiators and promoters in the progression of neoplasms.

### Promotion

Although the general characteristics of initiation are virtually identical to promotion, promotion is considered a subsequent step to initiation [96]. The promotion stage is thought to last much longer than the initiation stage. Promotion is carried out by proliferation and expansion of the rare initiated cell to form focal proliferations that appear similar to benign neoplasms [97]. These focal proliferations either redifferentiate to normal appearing tissue or slowly develop into cancer [98]. During promotion, initiated cells survive to a greater extent than non-initiated cells and the odds of additional genetic damage accumulating in the expanding and proliferating population of initiated cells is enhanced [41]. While the mechanism of clonal expansion is not evident in most systems, differential inhibition has been identified as a dominant mechanistic pattern in the liver of rats [45].

To differentiate initiation from promotion a mathematical model has been postulated where an initiator is any agent that increases the transition of a normal cell to a once-hit intermediate cell and that a promoter is any agent that acts on these intermediate cells to increase their proliferation or decrease the rate of differentiation or death giving rise to intermediate lesions [72]. It is these intermediate cells, not normal cells, which may on rare occurrence progress to malignant cells.

### Progression

Progression, the last stage of neoplastic development, is self-promoting and can be interfered with by diet, drugs, or xenobiotics. It is characterized by increased changes to chromosomes/karyotypes and the progressive nature of the cell to elude normal growth

and differentiation controls [2]. It has been demonstrated in hepatocytes, that preneoplastic nodules are phenotypically homogenous in a given tissue even with the use of different initiators and promoting environments, however, the resulting cancers are quite diverse and heterogeneous [97]. While the acquisition of heterogeneity and diversity has not been worked out yet, it appears to be acquired with the malignant state.

Agents that promote the transition of a cell from a promotion to a progression have the characteristic of inducing chromosomal aberrations [99]. The mechanisms involved in karyotypic instability are numerous. Contributing mechanisms may be altered telomere function, gene amplification, topoisomerase function, DNA hypomethylation, mismatch repair genes, oncogene and tumor suppressor gene function, spontaneous instability and sister chromatid exchanges [19, 45, 93, 100-105]. One or more these mechanisms are exhibited in neoplasms and are highly likely to be involved in the progression of carcinogenesis [99].

#### Contributing Factors to the Genesis of Cancer

As research continues to clarify our basic understanding of the development of cancer, the factors contributing to the process have grown. While numerous contributing factors have been identified, they are classified into two major groups, heritable and environmental factors. These major classes may act individually or in combination with one another. The heritable forms of cancers are either indirect contributors or direct contributors. The indirect heritable forms are more common and are associated with predispositions to certain cancers depending on additional factors such as environmental

factors, whereas direct heritable forms of cancer act independently and are rare by comparison.

Directly heritable cancers include familial polyposis coli, Gardner's syndrome, Paget's disease, and retinoblastoma to name a few from a short list. All of these heritable cancers are inherited as autosomal dominant genes [106]. As the environment comes into play in the development of cancer, the list grows exponentially regarding indirect heritable predispositions to certain cancers. Numerous genes have been implicated in the development of cancers. These genes range from oncogenes and tumor suppressor genes to metabolically active genes [41].

### Oncogenes

Oncogenes originate from proto-oncogenes and certain viruses. Proto-oncogenes are genes involved in the normal growth and differentiation processes. However, when genetic changes occur to proto-oncogenes, such as an assault by a chemical carcinogen, they can be activated to oncogenes [107, 108]. Additionally, viral sources can integrate oncogenes into the genome of a host cell [10, 48]. An example is the human papilloma virus (HPV) that is associated with the majority of uterine and cervical cancers [109].

Oncogenes perform various functions such as growth factors, protein kinases and nuclear transcription factors. Growth factors act as signals for the stimulation of normal growth and development. However, when regulatory mechanisms or the structural architecture of these signals is disrupted, these same growth factors can cause uncontrolled growth of the effected cells [110]. In addition to the influence of growth factors on cellular growth and differentiation, protein kinases also play a significant role.



Disruptions in the normal functioning of protein kinases are evident in human tumors [111]. For examples, the *ras* family of genes, act as protein kinases and they specifically code for guanosine triphosphatases. These protein kinases function in association with membrane-associated G proteins in signal transduction pathways for normal growth and differentiation[112]. During normal functioning, *ras* gene products participate in the phosphorylation of GDP to GTP upon signaling from an external mitogenic signal. When the *ras* gene is mutated, GTP cannot be hydrolyzed to GDP [113]. The result is a continuous mitogenic signal and accounts for large number of human tumors [111]. Ultimately, the signaling cascade for normal growth and differentiation leads to highly regulated gene expression through transcriptional regulation.

Transcriptional regulation is mediated by downstream transcription factors. Transcription factors have two functional domains, one that binds DNA and another for protein-protein interactions. These binding domains are critical to transcription, since RNA polymerase II cannot directly bind DNA promoter sites and requires interaction with transcription factors to bind DNA and initiate transcription [114]. An example is the well studied nuclear transcription factor, c-myc. It has been shown to regulate cell proliferation, differentiation and transformation. Mitogenic stimuli such as chemical carcinogens can induce c-myc expression in quiescent cells and those cells can enter the cell cycle [115]. Genetic aberrations and expression amplifications of c-myc have been associated with bladder cancer, breast cancer, ovarian cancer and non-small cell lung cancer [116-119].

In summary, oncogenes, resulting from inappropriately activated proto-oncogenes augment the potential of neoplastic formation [41]. Chemical carcinogens can induce inappropriate activation of proto-oncogenes [120]. This includes base pair substitutions and frameshift mutations that arise from DNA adduct formation after exposure to chemical carcinogens such as polycyclic arylamines [121, 122]

### Tumor Suppressor Genes

Contrasting oncogenes are tumor suppressor genes that when inappropriately inhibited are also involved in neoplastic formation [41]. Tumor suppressor genes are multi-functional and their expression is required for negative regulation of cell growth. If both copies of the gene are absent or inactive, uncontrolled cellular proliferation will result [123]. Examples of tumor suppressor genes are p53, RB-1, BRCA1, BRCA2, NF1 and hMLH1 [124].

The p53 protein, for example, is usually found in low concentrations in the cell and can be activated by DNA strand breaks for cell cycle arrest and/or apoptosis [125]. Mutations in the p53 tumor suppressor gene appear in approximately 50-55% of all human cancers [126]. Environmental carcinogens are involved in these mutational events and can lead to the inhibition of p53's tumor suppression functions either by inactivation of p53 or point mutations [127]

As evidenced, both proto-oncogenes and tumor suppressor genes are critical for the normal growth, development and division of cells. However, alterations to these genes, their expression and/or function play a significant role in neoplastic formations [45, 128-131]. In addition to genetic predispositions to cancer, environmental

carcinogens also act as highly significant predictors of increased cancer incidence through their interaction with proto-oncogenes and tumor suppressor genes.

### Environmental Contributors and Biotransformation

It has been unequivocally determined that numerous components of the environment contribute the onset of cancers. These include x-rays, irradiation, infections, pollution, diet, tobacco, combustion products, occupation, chemicals, and reproduction just to name a few [2, 88, 89, 132, 133]. These environmental components act either directly or indirectly in altering the normal functioning of cellular growth and division [44, 101, 134]. For example, tobacco smoke contains polycyclic aromatic hydrocarbons (PAH) that form DNA adducts. In a study, samples from 40 heavy smokers who recently quit were assayed for PAH-DNA and 4-aminobiphenyl-hemoglobin adducts. A significant reduction in adduct formation was observed in those ex-smokers negative for cotinine, a component of tobacco smoke indicating that tobacco smoke was responsible for adduct formation [135]. Even the sun's ultraviolet rays have been shown to induce a variety of skin cancers [136].

Acting in concert with environmental agents are biologically active proteins that function in the biotransformation of xenobiotics and metabolism of endogenous chemicals [24, 137, 138]. Several chemicals identified as carcinogens are metabolically activated to their carcinogenic state and are referred to as promutagens and procarcinogens [139, 140]. The metabolism of xenobiotics in the liver is largely due to the cytochrome P450 family of biotransformation enzymes [141, 142].

Cytochrome P450 is the most catalytically versatile enzyme and functions in detoxifying or activating a large number of environmental compounds to reactive intermediates [143]. The standard reaction catalyzed by cytochrome P450 is monooxygenation, the transfer of one oxygen atom to a substrate and the other to water via NADPH [24]. Different compounds can interact with different cytochrome P450s acting as either substrates for biotransformation, P450 inhibitors or P450 inducers [24]. Polymorphisms, heritable or chemically induced, in the alleles of these metabolically active enzymes can alter their function and result in increased susceptibility to neoplastic formation upon exposure to environmental carcinogens [144, 145]. There are more than 15 different forms of P450 enzymes in human liver microsomes such as CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9/18/19, CYP2D6, CYP2E1 and CYP3A3/4/5/7 [146, 147]. Subjects with two active CYP2D6 alleles, rapid metabolizers, are at an increased risk of developing hepatocellular carcinomas [148]. The interaction between environmental carcinogens and metabolic variation has fueled increased efforts to measure biomarkers in exposure studies.

### Progress and Prevention

The pathogenesis of cancer is more often than not, associated with environmental factors [6, 13, 89, 132, 149, 150]. Higginson addresses two major control strategies for cancer. The first being increased regulation limiting or prohibiting as little as minute quantities of pollutants into the surrounding environment. While this has reduced some incidences of cancers, due to regulatory and legislative hurdles, along with resistance to lifestyle changes this is not likely to result in a substantial decrease in future mortality

rates associated with cancer [13]. The second control strategy is long term research into the cellular biological mechanisms underlying carcinogenesis and interfering with those mechanisms. Although long term, the second control strategy is much more likely to have a substantial impact on future mortality rates associated with cancer.

While much of risk assessment and management 20 years ago was attributed to mathematical extrapolation, a substantial increase in our understanding of molecular mechanisms has been driven by new biotechnologies [41]. For example, DNA adducts can now be detected by gas chromatography coupled to mass spectrometry at levels as low as 10 fmol/g. This allows for the direct measurement of a specific chemical and/or its metabolite's interaction with DNA. It can also indicate exposure of individuals to specific carcinogens when adducts are detected in tissue samples [4]. Once a chemical has been identified to interact with DNA, protein and DNA sequencing along with utilization of biomarkers can determine what effects at the molecular level are occurring due to this interaction, this is especially relevant in environmental carcinogenesis studies.

Treatment in the prevention of cancer mortality rates has increased as our knowledge of the etiological processes involved has grown. Typically, the more that is known about a particular cancer, the better the rate of success in its treatment [2]. The current hypothesis into tissue-specific stem cells and neoplastic development has the potential to change the manner in which we approach cancer therapy. Existing therapies target the greater mass of tumor cells. However, normal stem cells from various tissues are more resistant to these chemotherapies than their mature cells from the same tissue [52]. The use of human stem cells for cancer therapy has opened possibilities for vaccination approaches in several solid tumors and hematologic malignancies [151].

## The Cell Cycle

In order for cells to maintain a homeostatic environment in complex organisms, an equilibrium is achieved in tissues where some cells are dividing, some are growing, some are differentiating, some are quiescent and others are dying [152]. This equilibrium is essential for the normal development and maintenance of tissue. Cells are able to sustain this homeostatic environment through a process referred to as the cell cycle. The cell cycle is a highly regulated process that is dependent on an orderly sequence of signals received from its internal and surrounding environment [153]. Cells that are not in the process of growth and replication are said to be in the quiescent G<sub>0</sub>-phase. In replicating cells, there are 4 major phases involved in the cell cycle; G<sub>1</sub>-, S-, G<sub>2</sub>- and M-phase. A schematic of the cell cycle is illustrated in Figure 1 [154].

During G<sub>1</sub>-phase, DNA uncoils from its condensed state and RNA and protein synthesis occur in preparation for DNA synthesis. DNA is synthesized in the S-phase for replication of the genome in preparation for cell division. The G<sub>2</sub>-phase prepares the cell for division by condensing the DNA, and further RNA and protein synthesis occurs. The M-phase is when the cell undergoes active cytoplasmic and nuclear division either through mitosis or meiosis. Checkpoints occur before the transition from one phase of the cell cycle onto the next [154-156].

There are 3 checkpoints that ensure the proper completion of one phase of the cell cycle before progression to the next. During normal cellular cycling, the G<sub>1</sub>/S checkpoint acts in G<sub>1</sub> and assures the cell is competent (i.e. DNA is not damaged) for entry into the S-phase. The G<sub>2</sub>/M checkpoint oversees the completion of S-phase and passage into M-phase. The M checkpoint regulates spindle formation and exit from M-

phase [157]. There are also well-established divergences from this cycling, for example when DNA damage occurs due to environmental assaults such as exposure to chemicals, UV light, viruses, etc. When DNA damage occurs in the cell, a cascade of regulatory proteins such as RB, p53, cyclins C, D and E and cytokines function to keep the cell in G1-phase until the damage has been corrected before allowing entry to the S-phase [12, 101, 152, 158-160]. This allows the cell to maintain its genomic integrity by repairing DNA damage before it is passed on to daughter cells.

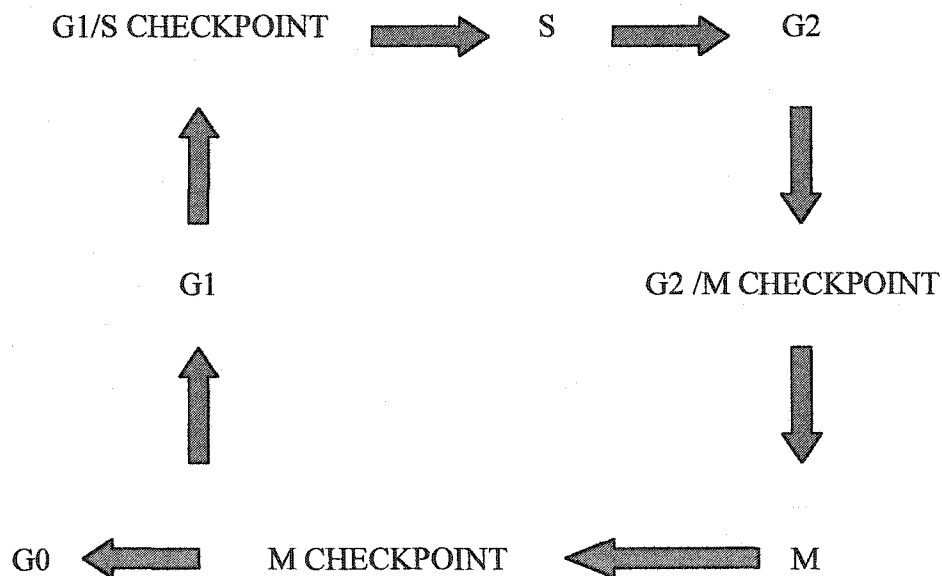


Figure 1. Schematic of the Cell Cycle.

In circumstances where the DNA damage is too extensive for the cellular DNA repair machinery, the cell will undergo an internally programmed cell death [161]. This pathway to cellular death is called apoptosis and is also highly regulated through a cascade of signaling events [162-164]. The pathway from the cell cycle to apoptosis was

proposed as: p53 → WAF1/CIP1 → Cyclin E/CDK4 → G1/S arrest → apoptosis [158, 165-167].

Once the cell is committed to apoptosis another sequential cascade of signaling events occur. These signals involve the family of caspases and poly(ADP-ribose) polymerase, among a number of other signaling molecules that lead to cellular condensation and the eventual fragmentation of the cell's genome. The specific cell cycle control and apoptotic components addressed in this paper are the proliferating cellular nuclear antigen (PCNA), (PARP), caspase-3 and p53 tumor suppressor protein. .

### Proliferating Cell Nuclear Antigen

PCNA is a highly conserved protein, present in higher plants, yeast and mammals, that was originally isolated from patients with systemic lupus erythematosus [168, 169]. It is typically a 36 kDa cyclin that functions with DNA polymerases  $\delta$  and  $\epsilon$  and is involved in DNA replication and repair, transcription and cell cycle control [170-176]. PCNA appears in the nucleus in late G1-phase, increases during the S-phase, and decreases in G2- and M-phases [177, 178]. Its synthesis directly correlates with cellular proliferation [175, 179]. Several studies have demonstrated that normal non-cycling cells and tissues synthesize very little or no PCNA, while normal cycling cultured cells (limited life span) synthesize PCNA during their low number passages, and in transformed cells (immortal) PCNA synthesis is significantly elevated in varying amounts [180]. PCNA has been observed as a prominent component of many cancers including ovarian, gastric, oral, pharyngeal, bladder, hepatic and leukemia among others and tumor cell lines [181-186]. Upon mitogen-induced blast transformation, peripheral



blood lymphocytes tested positive for PCNA, whereas normal peripheral blood lymphocytes did not exhibit detectable amounts, indicative of PCNA's involvement in DNA replication [187]. PCNA has been shown to interact with several other biologically active constituents, such as p21, cyclins, MyD118, Gadd45, mismatch repair proteins, human DNA ligase and Fen1 [172, 188-191].

Owing to PCNA's presence in replicating cells, it has been utilized as a biomarker for cellular proliferation. This has led to numerous studies investigating the expression of PCNA, especially in neoplasms for prognostic significance. For example, a positive correlation was observed between the malignancy index and PCNA index (PI) in glottic squamous cell carcinomas [192]. Significant correlations were found between PI and grade, cervical metastasis, lymphovascular invasion, tumor margins, local-regional recurrence and survival in laryngeal cancer patients [193]. In another study, a higher percentage of intense PCNA staining was observed in laryngeal carcinomas that progressed into invasive cancers [194]. These results indicate the possible utility of PI in treating and assessing prognosis in laryngeal carcinomas. In breast cancers, PCNA scores were associated with 'shorter' disease-free and overall survival times than tumors with low PCNA scores [195]. A strong correlation was also found to exist between the PI and prognosis in squamous cell carcinoma of the cervix [196]. In addition to human cancer studies, PCNA has also been used as a biomarker in aquatic species for proliferation and carcinogenesis studies.

Teleost fish undergo continual growth and their eyes expand in concert with this growth. In studies with these fish, PCNA was used to determine the spatial distribution of cellular proliferation in these teleost retinas [174, 197]. Immunochemical localization

of PCNA in mussel digestive gland tissue revealed its capacity to proliferate in all cell types, opposing the suggested existence of one stem cell in the epithelium [198]. PCNA was also used in an investigation into the regulation of cell death and proliferation of chemically induced neoplasias in *Fundulus grandis* after exposure to 6.7  $\mu$ M N-methyl-N'-nitro-N-nitrosoguanidine and 6.9 mM 2-aminofluorene. The authors noted the use of PCNA as a biomarker significantly augmented the assessment of these chemicals on liver cells [199]. PCNA's preferential expression in dividing cells makes it a useful molecular tool in the studies of cellular proliferation. And, when used in conjunction with other biomarkers, can be used to determine the ratio of cellular proliferation to cell death.

#### p53 Tumor Suppressor Gene

The p53 tumor suppressor protein is one of the most highly characterized cancer related proteins. Its major functions are in maintaining genomic integrity by pausing the cell cycle to allow for DNA damage repair before replication, or with extensive DNA damage, inducing programmed cell death (apoptosis) to prohibit passing on of damaged DNA to daughter cells [161]. This is accomplished by the accumulation of biologically active p53 upon DNA damage and is highly regulated [159]. p53's expression is also associated with increased levels in transformed and tumor cell lines [200]. Mutations in the p53 gene are one of the major genetic aberrations associated with human cancers [93]. Therefore the status of p53 expression, along with other indicators, is useful in investigating cell cycle disruption including apoptosis [201, 202].

A major role of the p53 gene is its ability to induce the transcription of genes that contain a p53 response element. The p53 protein's DNA-binding domain is located in

the central region (amino acids 90-290) and its protein-binding domain is in the amino-terminal end (amino acids 1-42), while the carboxy-terminal negatively regulates its DNA-binding activity [203-208]. The DNA sequence of p53 has been characterized in over 20 different species and while the genes are highly conserved, their homology is not uniformly distributed. Five blocks were identified as highly conserved, and four of those five blocks are located in the central DNA-binding region and the other is located in the amino-terminal protein-binding region [209]. These conserved regions are indicative of the critical importance of p53's DNA-binding function and mutations in this central region have been implicated in neoplastic development [93, 126, 210-216].

The tumor suppressor function of p53 is accomplished by its regulation of the cell cycle in response to DNA damage. The absence of p53 does not inhibit mitotic or meiotic cell cycles, but it does significantly correlate with high incidences of tumor formation in p53-null mice [217]. In UV-irradiated normal human skin cells, p53 protein levels are significantly increased [218]. This presence of high p53 protein levels during DNA damage correlates with the time involved in repairing the DNA damage [219]. Repression of the cell cycle by p53 in response to DNA damage is suggested to occur through transcriptional suppression and transcriptional activation of specific target genes [220]. For example, the p53 protein inhibits the expression of PCNA and *c-fos* whose products are required for cell proliferation and cell cycle progression [221, 222]. When extensive DNA damage occurs, such as that with radiation, normal mice thymocytes undergo apoptosis, however, mice that lack functional p53 develop cancer and are resistant to radiation-induced programmed cell death [223].

Further evidence of the tumor suppressor role of p53 was examined in hypoxia-induced apoptosis. Hypoxia exposed p53-expressing mouse embryonic fibroblasts (MEFs) accumulate wild-type p53 and undergo significantly increased apoptosis while their p53- null counterparts were resistant to hypoxia-induced apoptosis [224]. Additional studies also revealed an association with p53 and apoptosis. In a study of apoptosis in cardiac myocytes, chronic rapid ventricular pacing for 3 weeks induced congestive heart failure, increased levels of p53 and apoptosis, whereas control cells did not [225]. MN/CA IX (MN) is a carbonic anhydrase that has a strong association with cervical, bladder, oesophagus, and colon cancers [226, 227]. In Ma Tu cells, an MN promoter was repressed by wildtype p53, but stimulated by (143<sup>Val→Ala</sup>) mutant p53 in a dose-dependent manner [227]. This is indicative of p53's tumor suppressor function and the opposing effect mutations can induce in the p53 protein.

More than 5000 mutations have been described in the p53 gene and it is estimated that ≈50% of all human cancers contain mutated p53 genes [126, 228]. These mutations are typically point mutations and can have a profound effect on the resulting mutant p53 protein. The overwhelming majority of p53 mutations detected in human cancers are localized in the central region of the gene in exons 5-8 [229]. The occurrence of p53 mutations in exon 5 of occupational bladder tumors was around 70%, with C to T transitions being the most common mutation while codons 151 and 152 acted as 'hot spots' [212]. In oesophageal carcinomas, 38% had p53 gene mutations in exons 5-9) [213]. This suggests that exposure to environmental carcinogens may be the predominant etiological factor in oesophageal cancers. Thyroid neoplasms vary from slow growing differentiated adenocarcinomas to rapid proliferation of undifferentiated carcinomas.

When investigating the p53 mutations in carcinomas of the thyroid gland, 6 of 7 undifferentiated carcinomas had base pair substitution mutations in exons 5-8, while no mutations were detected in differentiated papillary adenocarcinomas [230]. Thus, in addition to the use of biomarkers for wildtype p53 levels in apoptotic studies, mutations in p53 can also be used as biomarkers in carcinogenesis studies.

### Caspase-3

Caspase-3 is a member of a family of intracellular aspartate-specific cystein proteases, called caspases. Caspases play a significant role in apoptosis through a downstream cascade of activation and self-activation [231-234]. This cascade ultimately results in cytoskeletal changes, chromatin condensation and DNA fragmentation [162, 235]. Caspases are divided into 2 subclasses, the initiators (apoptosis signaling events) and the executioners (cellular dismantling). The initiator caspases (i.e. caspases-8 and -9) activate the executioner caspases (i.e. caspases-3 and -7) [235, 236]. Caspase-3 is one of the major proteases responsible for the cleavage of PARP in programmed cell death. Activation of caspase-3 from its proform, called apopain, to its active form, named CPP32, is essential to its function as an initiator signal for apoptosis [237, 238].

Activation of caspase-3, to CPP32, is mediated by a number of upstream proteases: caspase-6, -8, -9 and -10, interleukin-1 $\beta$  converting enzyme (ICE), granzyme B and synthetic peptides containing the arginine-glycine-aspartate (RGD) motif [237, 239-241]. These proteolytic proteins activate caspase-3 by cleaving the full length proform, at critical Asp residues in the interdomain linker loop, into large and small subunits, p17 and p12. These two subunits are closely associated in forming two

heterodimers that form a tetramer, resulting in the enzymatically active form of CPP32 [242-244]. Once activated, caspase-3/CPP32 is involved in the cleavage of several substrates involved in DNA repair, pre-mRNA splicing, sterol biosynthesis, sustaining Rho-GTPases, DNA fragmentation, and pyrimidine nucleotide synthesis [244, 245].

The cleavage products of these substrates are all associated with processes involved in programmed cell death. Several studies using caspase-3 inhibitors (Z-VAD-fmk, Z-DEVD-fmk or Ac-DEVD-CHO) allow for the examination of caspase-3 activity [245-247, 248: Park, 1998 #840, 249]. These studies have helped elucidate some of the functional characteristics of caspase-3. In the early stages of apoptosis in SK-HEP-1 hepatoma cells induced by ginsenoside Rh2 or staurosporine, caspase-3 activity increased and cleaved p21, an inhibitor of cyclin kinases, in a dose-dependent manner [250]. Caspase-3 protease is implicated in genistein-induced apoptosis of prostatic carcinomas cell lines. Genistein was shown to activate caspase-3 and inhibit growth and proliferation of prostatic carcinoma cell lines via apoptosis. When caspase-3 activation was inhibited so was genistein-induced apoptosis [247]. Activation of DFF-45/ICAD, a subunit of the DNA fragmentation factor (DFF), mediates genomic DNA fragmentation during apoptosis. Tang and Kidd reported that DFF-45/ICAD has two cleavage sites for activation and that caspase-3 is required for cleavage of its carboxyl-terminal caspase cleavage site [235]. Caspase-3 deficient mice gave the first strong evidence for a major role in neuronal apoptosis by caspase-3 [251]. Additionally, caspase-3 is also hypothesized to be involved in c-Myc-induced apoptosis [252]. Recent studies have identified another caspase-3 substrate, hPMCA4b. hPMCA4b is a subunit of plasma

membrane  $\text{Ca}^{2+}$  pump (PMCA) that maintains low intracellular  $\text{Ca}^{2+}$  levels. Changes in intracellular  $\text{Ca}^{2+}$  homeostasis are associated with apoptosis [253].

Another critical role in caspase-3 activation is its downstream cleavage of poly(ADP-ribose) polymerase (PARP), which also leads to DNA fragmentation. Caspase-3 cleavage of PARP and the resulting DNA fragmentation is a hallmark of apoptosis. Caspase-3 activation of PARP is specific to programmed cell death, whereas, during necrotic cell death an additional cleavage is involved [254]. These studies underscore the advantage of using cleaved and full-length caspase-3 biomarkers in investigations of cellular apoptotic events.

#### Poly(ADP-Ribose) Polymerase

Poly(ADP-ribose) polymerase is a 113-116 kDa protein involved in DNA replication, DNA repair, genomic stability, cellular differentiation, cell death (both apoptotic and necrotic) and chromatin functions depending on whether it is in its full length or cleaved form [255-257]. PARP is divided into three major functional domains: 1) a DNA-binding domain in the N-terminus, 2) automodification sites in the central fragment, and 3) catalytic and nucleotide-binding domains in the c-terminus. The DNA-binding domain exhibits two zinc fingers and two helix-turn-helix motifs that recognize altered DNA structures and bind to them. It also contains a DEVD sequence that is cleaved by caspases (i.e. caspase-3) during apoptotic cell death resulting in 89- and 24-kDa fragments. The automodification domain has 15 glutamate residues that may act as potential ADP-ribose polymer acceptors. This catalytic domain is involved in nicotinamide adenine dinucleotide (NAD) catabolism. The ADP-ribose moiety of NAD

is utilized in the formation of chains of poly-ADP-ribose onto acceptor proteins, resulting in the acceptor protein's inactivation [257, 258].

PARP itself is the main target protein of poly(ADP-ribosyl)ation both *in vitro* and *in vivo* [259]. When DNA damage resulting in DNA strand breaks occur, PARP binds to DNA at the site of the DNA strand break and concomitantly synthesizes oligo- or poly(ADP-ribose) (PAR) chains that covalently bind itself (or various acceptor proteins). Binding of PARP to DNA strand breaks is suggested to function in the suppression of chromosomal recombination. This is observed in PARP-1 knockout cells, where spontaneous and DNA damage-induced sister chromatid exchanges occur at high frequencies [260]. When PAR binds PARP at PARP's NAD binding-domain it results in a negative charge that causes PARP to dissociate from the DNA strand breaks. This catalytic activity of PARP is dependent on DNA strand breaks and NAD, and is modulated by automodification [257].

In addition to DNA repair, numerous studies have implicated PARP in the apoptotic process [238, 255, 261-265]. Investigators have shown that caspase-3 cleavage of PARP occurs at the onset of apoptosis and separates the two DNA-binding zinc fingers from the automodification domain thus precluding the catalytic domain of PARP from being recruited to DNA strand breaks [238]. It was also demonstrated that during the early stages of Fas-mediated apoptosis osteosarcoma cells, HL-60 cells, 3T3-L1 cells and PARP<sup>+/+</sup> fibroblasts all underwent a transient burst of poly(ADP-ribosyl)ation 4 days after exposure to anti-Fas and cyclohexamide for apoptosis induction. This transient burst did not occur with PARP-depleted 3T3-L1 antisense cells or PARP<sup>-/-</sup> fibroblasts. Following the transient burst, poly(ADP-ribosyl)ation declined significantly, and at the



same time, caspase-3 cleaved PARP, DNA was cleaved and PAR moieties bound to proteins were degraded [255].

Further investigation into the transient burst of poly(ADP-ribosyl)ation prior to caspase-3 cleavage of PARP suggests it may be implicated in histone H1 correlation with internucleosomal DNA fragmentation. PARP inhibitors have demonstrated an inhibitory effect on the generation of DNA fragmentation during apoptosis. It is hypothesized that a change in polarity of the basic portions of histone H1 by PAR reduces its affinity for the internucleosomal DNA region, thus relaxing the solenoid structure of chromatin resulting in an increased susceptibility of chromatin to cellular endonucleases [265]. This is further supported by the restoration of the chromatin solenoid structure through the degradation of PAR units by poly(ADP-ribose) glycohydrolase on histone H1 [266].

In addition to caspase-3 cleavage of PARP, granzyme B and perforin have also been shown to cleave PARP. However, granzyme B and perforin's cleavage of PARP, in a cell free extract of purified bovine PARP, produces four fragments, 64, 61, 54 and 42 kDa, that differs from the major 89 kDa fragment produced by caspase-3 cleavage. The 64 and 54 kDa fragments contained the amino-terminal and carboxy-terminal end, respectively, of which only the 54 kDa fragment was active since it contained the catalytic domain. Secondary cleavage of the 64 and 54 kDa fragments produced 61 and 42 kDa fragments, respectively. Of these fragment, the 42 kDa fragment was also shown to be active. When Jurkat cells were incubated with granzyme B and perforin both 89 and 64 kDa PARP fragments were observed at different time points [267]. While this demonstrates the different enzymes capable of cleaving PARP, it also indicates the variable cleavage patterns of PARP that may be detected.

Different fragmentation patterns of DNA by PARP have also been documented during necrotic and apoptotic cell death in HL-60 cells. Using etoposide to induce apoptosis and cytochalasin B to induce necrosis, agarose gel electrophoresis of DNA revealed significant DNA degradation in the apoptotic cells and only a slight increase over the controls in necrotic cells. Cleavage of PARP itself also differed between apoptotic and necrotic cells. Control cells revealed a major band at 113 kDa and a minor band at 89 kDa and developed another minor band of 55 kDa after 24 hrs. The apoptotic cells began with a minor 113 kDa band and major 89 kDa band at 3 hrs and by 10 hrs the 113 kDa band had disappeared and the 89 kDa band stayed strong for the full 48 hrs. The necrotic cells maintained a strong 113 kDa band and an 89 kDa band that produced more fragments (50, 40 and 35 kDa) after 24 hrs. This stable presence of additional fragments of PARP from necrotic cells suggests that this is not a random degradation. Additionally, this could be utilized as an assay to differentiate apoptotic and necrotic cell death [254]. Biomarkers for the detection of PARP in both its full-length and cleaved forms are useful in environmental carcinogen studies. A decrease in the full-length form of PARP with a concomitant increase in its cleaved form is indicative of cell death.

#### Detection of DNA Fragmentation During Cell Death

In maintaining genomic integrity, cells are amazingly resilient. They have several mechanisms that give them the ability to repair moderate amounts of DNA damage and when DNA damage is too extensive for repair, programmed cell death ensues. This ensures that aberrant DNA is not passed on to daughter cells, thus maintaining the genomic integrity of future cell populations [268, 269]. An aspect of cell cycle disruption

that occurs with exposure to toxic chemicals is whether or not the chemical directly causes injury to the cell resulting in necrotic cell death, or whether the chemical affects the genetic machinery inducing an apoptic cell death [202].

Morphological and biochemical distinctions differentiate necrotic and apoptic cell death [270-272]. In contrast to necrotic cells, apoptic cells exhibit cellular shrinkage, DNA fragmentation, plasma membrane blebbing, protein production and condensation of the cytoplasm [164, 273]. Necrotic cells have cellular swelling, a loss of cell membrane integrity, and a release of lysosomal enzymes. Recently, evidence has been presented that programmed cell death occurs in pathways other than apoptosis. Another pathway is autophagic-programmed cell death (PCD) and it is a biochemically and morphologically distinct pathway from apoptotic [273]. These forms of PCD serve a protective function by removing damaged or initiated cells, thus ensuring they do not proliferate [274]. Interference with these protective functions by toxic chemicals may enhance or prohibit PCD [161, 275].

The most highly characterized type of programmed cell death is apoptotic cell death. Morphological characteristics of apoptosis are condensation and fragmentation of the cell and of nuclear chromatin, along with phagocytosis by neighboring cells [161, 164, 274, 276]. The fragmentation of chromatin by endonucleases during apoptosis produces a signature DNA ladder, ranging from about 50-300 base pair multiples depending on the cell type [277]. To compare and contrast this pattern of DNA fragmentation in apoptotic and non-apoptotic cells, DNA from separate nuclear and cytoplasmic extracts were examined. Agarose gel electrophoresis of isolated DNA from the fractions of apoptotic cells resulted in two classes of oligonucleosome chains. One of

a low molecular weight DNA that freely dissociates from the nucleus, and one of a higher molecular weight DNA, that remains bound. These apoptotic fractions contrast with the very high molecular weight DNA from both fractions observed in normal cells that do not even migrate on the gel. The contrasting results suggest that DNA fragmentation is associated with apoptotic cell death [276].

Although, there have been studies where the signature DNA ladder was not observed during apoptosis, these observations may be partially due to the method used in detecting DNA fragmentation [278, 279]. Cell types that had previously not exhibited DNA fragmentation during apoptosis were examined using a technique that selectively amplifies blunt, 5' ends by a modified ligation-mediated polymerase chain reaction. This method resulted in DNA laddering of all cell types tested after induction of apoptosis by dexamethasone or UV irradiation. The cell types tested include normal thymic DNA, UV irradiated P19 cells, genomic DNA from normal BALB/c mouse thymus, spleen, liver, kidney, intestine, ovary, testes and brain, genomic DNA from *D. melanogaster* embryos and *M. Sexta* intersegmental muscles, and the P5 cerebellum and P7 cortex from adult and embryonic mouse. Although all tissues tested were positive for DNA fragmentation, the adult brain had very low levels. This coincides with the lack of evidence for apoptotic cell death in that tissue [280]. Thus, the DNA ladder may not be detected with gel electrophoresis without prior amplification due to a lack of sensitivity, not because DNA fragmentation did not occur.

Although DNA fragmentation is often referred to as a hallmark of apoptosis, it has also been observed in necrotic cell death. In a study of DNA degradation in apoptotic and necrotic human leukemia cell deaths, the nuclear and mitochondrial DNA fractions

were examined separately. Both of the necrotic fractions but only the nuclear fraction of apoptotic cells resulted in DNA fragmentation. The mitochondrial DNA fraction from the apoptotic cells appeared resistant to degradation [281]. These results indicate that necrotic cell death also results in DNA fragmentation (although not in the organized manner observed with apoptosis) and that the resistance of the apoptotic mitochondrial DNA fraction may be a way to differentiate apoptotic and necrotic cell death. Further evidence of DNA fragmentation in both apoptotic and necrotic cells was observed in a study of intracellular adenosine triphosphate's effect on apoptosis and necrosis. Both the apoptotic and necrotic cells exhibited DNA fragmentation during this study, although the necrotic cells resulted in a lower level of DNA fragmentation [282].

While there are several distinguishing characteristics between apoptotic and necrotic cell death, both ultimately result in DNA degradation. This common feature of cell death is exploited by using methods to identify DNA strand breaks. Specifically, the terminal deoxynucleotidyl transferase-mediated dUPT nick end labeling (TUNEL) method has been shown to be an excellent tool for observing these DNA strand breaks. This method is based on a protocol first developed in the early 1990's by Frank Rösl due to problems associated with agarose gel electrophoresis. These problems include the insensitivity of ethidium bromide staining and the time consuming labeling with  $^3\text{H}$ -thymidine and  $^{32}\text{P}$  that can also create high background signals in autoradiography when trying to detect DNA fragmentation. The availability of free 3'-OH ends resulting from endonuclease cleavage, were end-labeled with only one nucleotide (to avoid false positives by any nick-translation effect due to 3'-5' exonuclease activity) such as  $^{32}\text{P}$ -dCTP or  $^{32}\text{P}$ -dATP using a Klenow polymerase. The unincorporated nucleotides were

removed and the resulting DNA was electrophoresed on a 1.8% agarose gel, the gel was dried on 3MM Whatman paper and exposed for autoradiography [283]. This method has since undergone several modifications such as the substitution of Klenow polymerase with DNA polymerase or terminal deoxynucleotidyl transferase (TdT), and the substitution of  $^{32}\text{P}$ -nucleotide labeling by fluorescein, digoxigenin, or biotin labeling. These modifications opened the way for *in-situ* labeling of paraffin embedded sections [284-286].

Although the TUNEL method was initially used to distinguish apoptotic cell death, subsequent research has indicated it also detects DNA damage from necrotic and autolytic cell death, cardiomyocytes undergoing active DNA repair and calcification of matrix vesicles in atherosclerotic plaques [287-290]. Well-defined models of apoptotic, necrotic and autolytic liver cell death in rats were used to investigate the TUNEL assay. All three types of cell death resulted in TUNEL-positive staining of nuclei. The autolytic cells were the result of insufficiently fixed livers and could be avoided with optimal fixation. These results indicated that DNA fragmentation is common to different types of cell death [291]. TUNEL-positive staining of apoptotic cells and necrotic cells was also seen in tissue from frontal lobe contusions and verified using morphological criteria [292]. These findings are consistent with numerous other studies demonstrating the detection of DNA fragmentation in various cell types undergoing necrotic cell death [289, 291, 293, 294]. With regard to TUNEL-positive staining of cardiomyocytes undergoing DNA repair, both Laszlo Hegyi and the original authors independently responded to Motoo Kanoh, et al.'s results. They both came to same conclusion that the TUNEL-positive staining of cardiomyocytes, that also stained positive for PCNA

indicating DNA repair, was the result of severe DNA repair and would likely progress to apoptosis [295, 296]. The TUNEL-positive staining of calcified matrix vesicles in atherosclerotic plaques can be avoided by pretreatment with a chelator such as EDTA [297]. They can also be distinguished from apoptotic and necrotic cells by morphological examination. Thus, the TUNEL technique is a sensitive molecular tool for investigating cellular death whether from apoptosis or necrosis.

### Benzidine

Benzidine (4,4'-diaminodiphenyl, 4,4'-dephenylenediamine and Fast Corinth Base B) and its metabolites are widely studied carcinogens [8, 269, 298-302]. It has been classified as a Group A, human carcinogen by the Agency for Toxic Substance and Disease Registry (ATSDR). The Environmental Protection Agency (EPA) lists benzidine (BZ) as a hazardous air pollutant in the "Clean Air Act of 1990." BZ is a polycyclic arylamine and its chemical structure is that of a diphenyl with an amine moiety at both the C4 and C4' positions [303]. It is a white, grayish-yellow, or reddish crystalline solid or powder and usually present as benzidine hydrochloride or benzidine sulfate [304]. In the environment it is either in its free form as an organic base or in a salt form such as benzidine sulfate. In the air it is usually found attached to suspended particles [305]. BZ is primarily used in the production of azo dyes for the leather, textile, and paper industries [304]. Exposure may occur from contaminated soils and groundwater. Health effects occur with both chronic and acute exposure, and range from dermal sensitivity to liver, bladder and other cancers [20, 304].

With regard to the environmental fate of and exposure to BZ, the EPA's National Priorities List (NPL) lists 1,408 hazardous waste sites as the most serious in the nation. BZ has been identified in at least 27 of these NPL sites [305]. Human exposure to BZ may occur at hazardous waste sites containing benzidine or in facilities that manufacture or use benzidine. However, the general population's potential risk of exposure, based on available data, is almost nil [305].

Human exposure to benzidine has been researched through occupational exposures and the use of cultured cell lines. Bladder cancer have been reported in workers with an occupational exposure to BZ, aniline, betanaphthylamine, and BZ-dyes [44, 302, 306]. Studies of kimono painters exposed to BZ-based dyes by licking their brushes revealed an increased risk of bladder tumors and bladder cancers [307]. A 22-year study of a cohort of 3,322 Japanese employees from plants manufacturing benzidine and beta-naphthylamine a statistically significant risk for liver, gallbladder, and bile duct cancers were found. From this study, 244 who had died of cancer of the genitourinary organs, 11 males also had primary liver, gallbladder, bile duct, large intestine, and lung cancers [308]. Dermal sensitization to BZ was reported in 5% of 4,600 patients tested, and of that 5%, 88.5% were diagnosed with occupational allergic contact dermatitis [309].

Experiments investigating the effects of BZ have been conducted on various animal models including hamsters, mice, rats, rabbits, grass frogs and dogs. The effects from BZ varied depending on the organism tested. Neoplastic development due to BZ exposure has been reported in rats, mice, hamsters, grass frogs, dogs and humans (occupational studies) [305, 310, 311]. Liver neoplasms were observed in rats, mice,



hamsters and humans upon exposure to BZ [308, 312-315]. Intermediate intragastric exposure in female Sprague-Dawley rats (3.4 mg/kg/day x 3 days) to BZ was investigated 9 months after exposure and resulted in mammary carcinomas [316]. An inhalation study using 28 rats exposed to 10-20 mg/m<sup>3</sup> of BZ dust, 4-hrs/day x 5 days/wk for 20 months, 5 developed myeloid leukemia, 4 had fibroadenoma, adenocarcinoma or squamous carcinoma of the mammary gland and 1 with a hepatoma [304, 317]. Chronic exposure studies in male BALB/c and female C57BL/6 mice revealed that doses of 1.8 to 2.5 mg/kg/day over their lifetime resulted in spongiform leukoencephalopathy, Harderian gland adenomas and liver carcinomas in a number of the exposed mice [305, 318]. Hamsters fed 0.1% BZ base or BZ dihydrochloride in a lifetime study reported occurrence of malignant and benign multiple cholangiomas, hepatomas, and/or liver carcinomas in >50% of those surviving [313]. In dogs orally administered 17.6 mg/kg/day x 5 days of BZ for 5 years recurrent cystitis was observed, and at doses of 24.2 mg/kg/day x 6 day for the same duration, bladder carcinomas had developed [319].

Tumor induction at various sites by BZ is mostly species-specific and has been attributed to species differences in metabolism of benzidine. The pathways for BZ metabolism in various species have been correlated with acetylation at the nitrogen position [301, 320, 321]. Species with efficient *N*-acetylators are susceptible to liver tumors and those that are deficient are susceptible to bladder cancer. This is seen with humans polymorphic for *N*-acetylation, the slow acetylators who appear to have an increased risk for bladder cancer [8, 322]. A comparison of human and rat hepatoma cell lines with Chinese hamster lung-derived fibroblast cell lines, that have little or no metabolic activity, resulted in sister chromatid exchanges in the hepatoma cell lines, but

not in the fibroblast cell lines [8]. These results reflect a metabolic requirement for induction of sister chromatid exchanges during exposure to BZ.

An *in vivo* study using high-pressure liquid chromatography (HPLC) identified 17 urinary and/or biliary metabolites of BZ in rats. The most potent mutagen of these metabolites was *N*-hydroxy-diacetylbenzidine (*N*-OH-DABZ) with 10X greater potential than *N*-acetylbenzidine (ABZ) and *N*-*N'*-diacetylbenzidine (DAB) and was also the major metabolite at higher doses (50 mg/kg vs. 0.5-5 mg/kg) [323]. A study in rats identified the metabolism of BZ to its reactive metabolite *N*-OH-DABZ to include the *N*-oxidation of ABZ to *N'*-OH-ABZ. The major pathway proposed for the formation of benzidine's reactive metabolites is:  $BZ \rightarrow ABZ \rightarrow N'-OH-ABZ \leftrightarrow N-OH-DABZ$ . The authors also confirmed the formation of the DNA adduct, *N'*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine, from *N*-OH-DABZ in the presence of *N,O*-acetyltransferase. They additionally revealed the formation of the same DNA adduct via a direct interaction with *N'*-OH-ABZ under acidic conditions [324].

Several studies have found correlations in the formation of DNA adducts with the development of tumors and neoplasms in carcinogenesis studies [325-329]. Formation of DNA adducts have been associated with the induction of chromosomal aberrations in the livers of mice after exposure to BZ. A linear dose response was demonstrated for BZ-DNA adducts with a correlation of 0.91 between adduct levels and aberrations when analyzed by dose groups [105]. Rat livers exposed to BZ also resulted in BZ-DNA adduct formation, specifically the *N'*-(3'-monophosphodeoxyguanosine-8-yl)-ABZ-DNA adducts [330]. This formation of BZ DNA adducts and the resulting neoplastic development in organisms exposed to BZ and its metabolites, indicate its potential for the

early stages of polycyclic arylamine carcinogenesis studies in the western mosquitofish, *G. affinis*.

### 2-Aminofluorene

The arylamine, 2-aminofluorene (2-fluorenamine, 2-fluoreneamine and 9H-fluoren-2-amine)), has also been widely studied in laboratories, although not as widely studied as benzidine with regard to human exposures. 2-aminofluorene (2AF) is a white to tan, solid, synthetic arylamine, not known to occur in nature, with a molecular weight of 181.23, and is used in chemical research as an experimental carcinogen [331]. In 1940, 2-aminofluorene (2AF) was patented as an insecticide against the tobacco hornworm and that use discontinued a year later due to discovery of its carcinogenicity [332]. Numerous publications have since shown that the metabolic products of 2-AF are mutagenic [269, 299, 333-336]. 2-AF was the first chemical carcinogen to demonstrate a direct metabolic conversion of a chemical to a more active form, 2-acetylaminofluorene (2-AAF) [337].

No data on long-term effects of 2-AF exposure in humans was found in searches of scientific journals or books. Genotoxic potency of 2-AF and several of its metabolites was measured using the *Drosophila* DNA repair test with *Drosophila melanogaster* and resulted in positives for DNA damage for 2-AF and all its metabolites tested [338]. Sister-chromatid exchanges were observed in a dose dependent manner in human leukocytes exposed to 2-AF [103]. DNA-strands breaks were demonstrated in primary mouse hepatocytes after administration of 2-AF and its metabolites [339]. A study on rats administered 1.62 mM/kg of 2-AF and 2-AAF in their diets for 8 months resulted in

a significant number of liver tumors, mammary gland tumors, ear duct tumors and small intestine tumors in both treated groups [340]. In another study using mice, doses of 0.25% 2-AF in their diet for 326 days resulted in liver adenomas, hepatic hyperplasia, bladder carcinomas and kidney carcinomas [341]. These and numerous other studies have reported cancers resulting from dermal and oral administration of 2-AF in both rats and mice [342]. It is important to note that different species respond differently to administration of 2-AF. Both monkeys and guinea pigs have not exhibited tumor formation (the former may be due to experimental design). While liver and bladder cancers are seen in dogs, mice and rats administered 2-AF, only liver tumors are seen in chickens, fish, cats and hamsters, and only bladder tumors in rabbits. Differences in the metabolism of 2-AF are reported within a species such as mice and rats, along with differences reported between the sexes [334, 343]. These studies intimate the importance of individual metabolisms in the conversion of 2-AF to its reactive derivatives.

The metabolism of 2-AF in organisms varies between and within species and more specifically tissues [334]. Intra-species variation was also reported between male and female rats, where male rats were more susceptible to 2-AAF toxicity, than were the females [344]. The metabolism of 2-AF has been extensively studied, along with the formation of DNA adducts from its DNA binding derivatives. The metabolites and derivatives of 2-AF that have been detected are 2-acetylaminofluorene (2-AAF), *N*-hydroxy-2-aminofluorene (*N*-OH-2-AF), *N*-hydroxy-2-acetylaminofluorene (*N*-OH-2-AAF), *N*-Acetoxy-2-acetylaminofluorene (*N*-acetoxy-2-AAF), *N*-sulfonoxy-2-acetylaminofluorene (*N*-SO<sub>4</sub>-2-AAF), 1-hydroxy-2-acetylaminofluorene (1-hydroxy-2-AAF) and (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-gluconsid)uronate (*N*-GIO-2-AAF)

[333]. Both of the *N*-hydroxylated metabolites can be metabolized to the reactive *N*-sulfonyl-2-AF and -2-AFF by sulfotransferases. Noteworthy evidence supports the role of higher levels of sulfotransferase activity in males for the higher hepatocarcinogenic response reported in male rats [345]. *N*-hydroxy-2-AF and -2-AAF are metabolized to the reactive *N*-Acetoxy-2-AF by *O*-acetylase and *N,O*-acyltransferase, respectively. The reactive metabolites *N*-SO<sub>4</sub>-2-AF and *N*-acetoxy-2-AF interact with DNA form dG-C8-AF adducts, while the *N*-SO<sub>4</sub>-2-AAF reactive metabolite forms *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF) and 3-(deoxyguanosin-*N*<sup>2</sup>-yl)-2-acetylaminofluorene (dG-*N*<sup>2</sup>-AAF) DNA adducts [333, 342].

The formation of DNA adducts in hepatic and urinary tract tissue after exposure to 2-AF and its derivatives have been reported in dogs [334]. 2-AF DNA adducts are also reported in several cell lines: rat glial tumor cells, human malignant astrocytoma and brain glioblastoma multiform cells, human colon tumor cells, human bladder tumor cells, human leukemia cells, and human lung tumor cells [346-353]. Syrian hamsters consistently produced higher 2-AF DNA adduct levels in the liver than in the bladder [9]. Studies that have compared DNA adduct levels from 2-AF and its metabolites in various tissues usually found the highest incidence to occur in livers, however, route of administration and sex of the animals appear to have an influence DNA binding levels in different tissues [333]. As with BZ, 2-AF DNA adduct formation and neoplastic development after exposure to 2-AF and its derivatives are excellent candidates for early carcinogenesis studies. Its use as a potential molecular tool, for the investigation of the effects of polycyclic arylamine carcinogenic studies in *G. affinis*, is examined in this study.

## Gambusia affinis

### Aquatic Species in Carcinogenic Studies

Neoplasms have been reported in fish since as early as 1793 when multiple osteomas were found in the butterflyfish (*Platax pinnatus*) [30, 354]. Numerous studies have since reported neoplasms in bony and cartilaginous fish in both fresh- and salt-water species [355-358]. However, it wasn't until the late 1970's and early 1980's that the use of aquatic species in environmental carcinogenic studies became of national interest. In August of 1978, the EPA and NCI collaborated on a program to study and develop aquatic organisms for carcinogenicity studies. An additional interagency agreement was initiated between the U.S. Army Medical Research and Development Command and the EPA to develop more sensitive, shorter term, and less expensive monitors for evaluating environmental pollutants and their health effects using two fish species [359].

These collaborative efforts have revealed that fish have metabolic pathways similar to mammals, develop neoplasms in laboratory settings that have been reported to develop in relatively short time periods, exhibit hardiness under experimental conditions and are easily maintained [26, 360, 361]. In addition to these benefits of using fish in carcinogenic studies, is the often overlooked, yet profound importance of the usefulness of aquatic species in monitoring the overall health of an environmental setting.

For example, an investigation of environmental carcinogenesis in fishes of the Great Lakes was conducted from 1973-76 and occurrences of neoplasms were found to be associated with polluted versus non-polluted areas [362]. Epizootiologic studies in Burlington Harbour (a heavily polluted bay due to industrial and domestic effluents) on

Lake Ontario revealed increased tumor frequency in Burlington Harbour and Oakville Creek white suckers that decreased significantly in collections made at varying distances in both directions from this region [363, 364]. Also, a high prevalence of hepatic neoplasms was reported in mummichog from a PAH-contaminated site in the Elizabeth River [365]. Contaminated waters not only affect the organisms residing in them, they also impact the organisms surrounding them. An example is the statistically significant associations that were found between cancer mortality rates in white males in Louisiana and drinking water obtained from the Mississippi River [366]. Therefore, these studies, along with numerous others studies indicate the considerable utility of aquatic organisms as sentinel animals for biomonitoring in the early detection of environmental carcinogens.

While histologic investigations have shown the utility of aquatic organisms in tumor formation and cancer development, molecular studies have further advanced their utility in carcinogenic studies. This is demonstrated in DNA adduct analysis investigations. These DNA adduct studies have been conducted on small fish models in carcinogenic studies and have demonstrated the formation of DNA adducts with exposure to carcinogens [17, 28, 32, 367]. Although molecular and immunologic applications with fish lag behind mammals, it has been found that most reagents and procedures used in mammalian research can also be used with fish [368].

#### *Gambusia Affinis* in Environmental Carcinogenesis Studies

A variety of fish species are used in environmental carcinogenesis studies including rainbow trout, medaka, guppy, sheepshead minnow, zebrafish, and the western mosquitofish just to name a few of the different fish species used in research [29, 34, 365,

368-376]. While the rainbow trout, medaka, and zebrafish are probably the most highly characterized in research, it is the geographic ubiquity of the western mosquitofish (*Gambusia affinis*) that makes it stand-out as an aquatic sentinel species for environmental biomonitoring. The limited geographic distribution of several small fish species studied imposes restrictions for direct biomonitoring for these species in diverse environmental settings. Additionally, *G. affinis* are not a migratory fish and can be used to biomonitor specific ranges [27]

The widespread geographic locations of *G. affinis* are attributed to its hardiness in hostile environments. They are able to tolerate a variety of waters from that of high quality to harsh conditions. A recent study of its survival in a hostile environment, revealed a salinity tolerance above that of full sea water, >10 ppm ammonia, >100 ppm nitrates and <1 ppm O<sub>2</sub> [377]. *G. affinis* has been used as an aquatic model of choice for toxicity studies of pollutants to fish in several investigations [378-380]. In a study examining the histopathological effects of Thiodan® on *G. affinis* livers, numerous morphological changes were observed ranging from vacuolization of cell cytoplasm and infiltration of lymphocytes to pycnotic state of nuclei and degeneration of cells [380]. DNA strand breaks have also been observed in *G. affinis* inhabiting retention ponds heavily contaminated with radionuclides [381].

It has also been demonstrated that they develop hepatocarcinomas after relatively short exposures to methylazoxymethanol acetate, [27]. The development of neoplasms in the liver of *G. affinis* is important to its usefulness as a sentinel species for environmental biomonitoring. Correlational evidence of a relationship between contaminant exposure and hepatic neoplasms and related lesions are useful histopathological biomarkers of



contamination exposure effects [382]. While hepatic neoplasms have been observed in *G. affinis* after exposure to methylazoxymethanol acetate, there exists a need for examining the effects of other compounds and their mixtures on these fish to ascertain their responses to other carcinogens of different mechanistic classes [27]. Thus, the polycyclic arylamines, BZ and 2-AF, which have conclusively shown DNA adduct formation and neoplastic development in the livers of several species tested and are examined here for their effects on cellular proliferation and death, along with histopathological observations in the liver's of *G. affinis*.

## CHAPTER III

### MOLECULAR TOOLS

#### Purpose of Investigating Molecular Tools

Molecular tools (biomarkers) have made an extraordinary contribution to biological research by allowing investigators to visualize microscopic phenomena taking place at cellular and molecular levels. These tools can range from enzyme assays of molecular events to immunoassays of protein levels. The molecular tool of interest here is immunological assays of proteins. Antibodies are used in research to 1) measure minute amounts of a material, usually proteins, in a complex mixture, 2) to detect specific proteins in gels, tissues and cell cultures 3) or to isolate a specific protein in a complex mixture [383]. While the use of antibodies in research has lead to great advancements in our understanding of biological processes, they are usually developed against a particular species and therefore restrictive in their use. Typically, the species is a highly characterized organism in scientific literature. In less investigated animal models, this pool of molecular tools is even more limited. Human, mouse and rat antibodies are by far the more abundant antibodies commercially available.

Since *G. affinis* is our animal model of choice, for the reasons stated in the literature review, and is not a popular animal model in research, there are no species-specific antibodies for cellular proliferation or death commercially available. However, all is not lost. Fortunately, evolutionarily highly conserved proteins in one organism often contain the same antibody recognition site as the related protein in another

organism [191]. This permits the use of an antibody developed for one animal model to be used in another unrelated animal model.

With that in mind, this experiment tests the specificity of antibodies to conserved proteins in unrelated species (rat or human) against four of the same type of proteins from *G. affinis*. The antibodies being tested here are against PCNA, p53 and cleaved caspase-3 and PARP proteins. PCNA protein is involved in cellular proliferation while p53 and cleaved caspase-3 and PARP proteins are both involved in cellular death (see literature review). The conserved nature of these proteins was verified using protein sequences obtained from the Swiss-Protein/TrEMBL web site. The sequences were compared between all sequence-available non-related species using the Blast sequence alignment tool. All four proteins had a high degree of sequence homology with non-related species, indicating the conserved nature of these proteins. To determine whether these antibodies bind specifically to the protein of interest, and only to that protein, western blots were conducted for each antibody.

### Experimental Design

This experiment is designed to demonstrate the antibody specificity of rat specific anti-PCNA, mouse specific anti-p53, and human specific anti-cleaved caspase-3 and PARP antibodies on *G. affinis* PCNA, p53, caspase-3 and PARP proteins. Therefore, 10 male *G. affinis* were randomly selected and half were placed in a glass bowl containing a liter of filtered water for 2 days, while the other half were placed in a glass bowl containing a liter of filtered water and 50 ppm carbon tetrachloride, to induce liver injury, for 4 hrs, then transferred to a glass bowl containing a liter of filtered water for 2 days.

Once treatment was complete, fish were immediately processed for total protein extraction and quantification using the micro Lowery method, and western blot analysis of antibody specificity.

To ensure the accuracy of carrying out the experimental procedures and the integrity of the reagents, internal controls were used. For internal controls, AML12 proliferating mouse hepatocytes (ATCC, Manassas, VA) were cultured in Corning tissue culture dishes (VWR Scientific, South Plainfield, NJ) in Vitacell F-12K medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum and 0.1 % penicillin-streptomycin solution (Sigma Aldrich, St. Louis, MO) and incubated in 5% CO<sub>2</sub> at 37° C. Culture media was replaced every 2 days until confluency was attained. Half of the cells were incubated at 37° C for 8 hrs while the other half were treated with 20 µM camptothecin (Genotech, St. Louis, Mo) in medium, to induce apoptosis, and incubated at 37° C for 8 hrs. When treatment was complete, cells were detached from the culture plates using 2 ml 0.25% trypsin (ATCC, Manassa, VA) and suspended in culture medium. Samples from the cell suspensions were processed to test for live cells, necrotic cells and apoptotic cells by flow cytometry and bright field and phase contrast microscopy. This was followed by total protein extraction of the suspended cells, protein quantification, and use as internal controls in western blot analysis.

## Methods and Materials

### Fish and Housing

*G. affinis* were purchased from Aquatic Research Organisms, Hampton, NH, and treated in accordance with an animal use protocol (99-06-02) approved by the

Institutional Animal Care and Use Committee of Western Michigan University. They were housed in 10-gallon aquariums in a flow-through dual filtered water aquaria system. The dual filtration system shown in Figure 2 consisted of an iron filter for iron removal before an activated carbon filter for chlorine removal. Filtered water and air were circulated through CPVC piping and delivered to the appropriate aquaria as shown in Figure 3. Bulkheads were attached to each aquarium for water drainage in order to maintain water levels while water was continually fed to the aquarium as in Figure 4. Drainage water filtered through a tub of activated carbon, as seen in Figure 5, before entering floor drain. Aquarium water temperature was maintained at 76-78° C and light cycled at 12 hrs on and 12 hrs off. General health was monitored daily, while alkalinity, ammonia, pH, dissolved O<sub>2</sub>, CO<sub>2</sub>, nitrites, chloride, chlorine and hardness of the aquaria water was monitored on a weekly basis. Fish were fed freshwater aquarium flake food, approximately 1% of their averaged weight, twice daily from automated fish feeders.

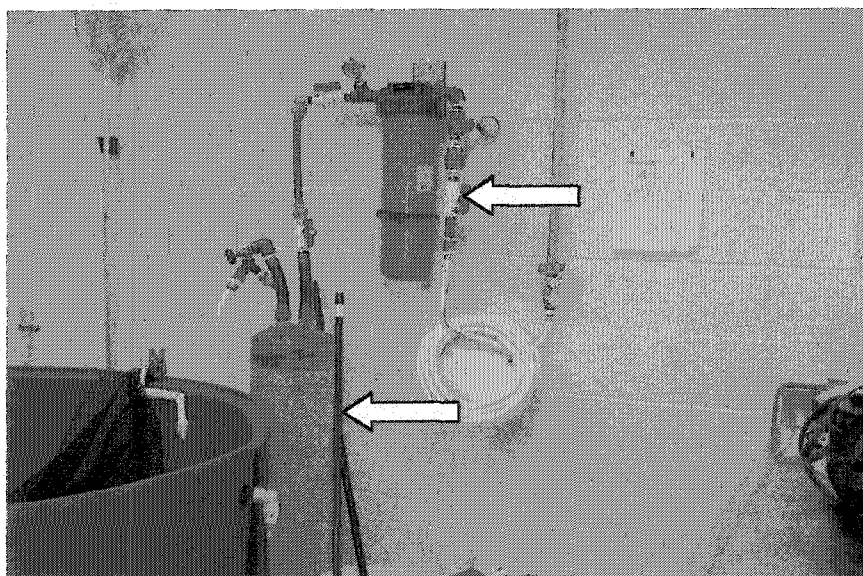


Figure 2. Dual Filtration System.

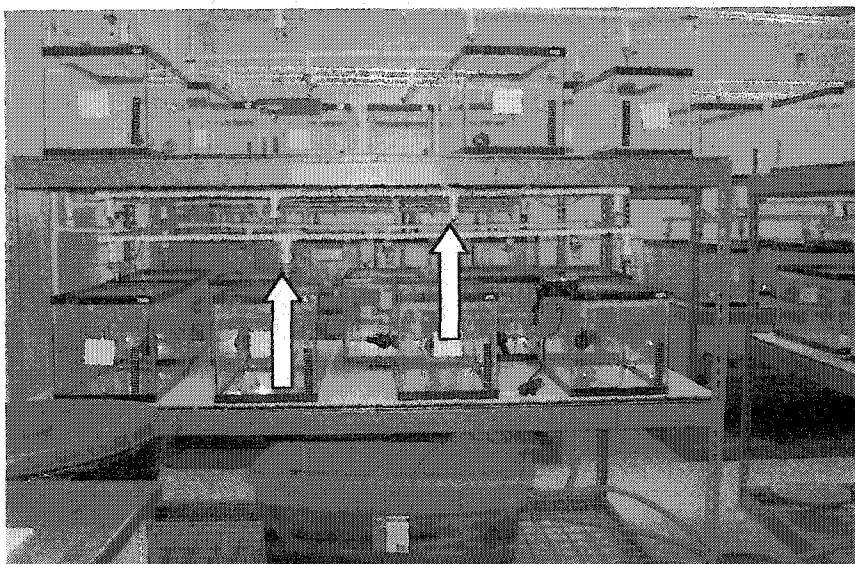


Figure 3. Circulating Air and Water Through CPVC Piping.

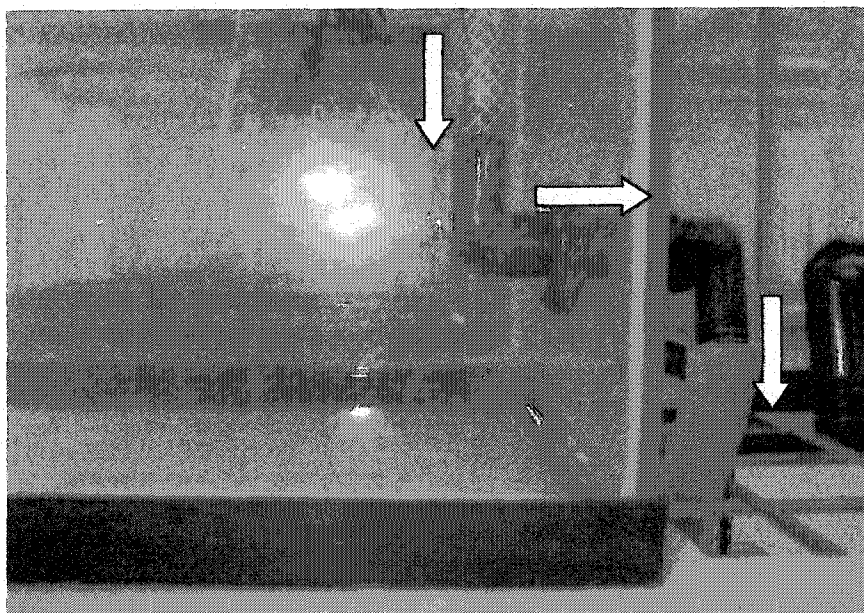


Figure 4. Bulkhead Attachment for Continual Water Drainage.

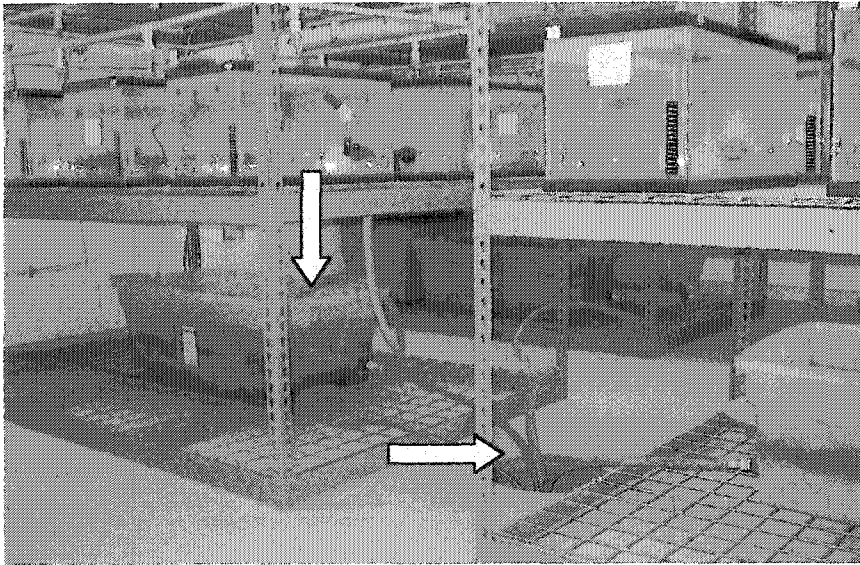


Figure 5. Filtration of Drainage Water.

When treatments were completed, fish were sacrificed by placing on ice for 45 min and decapitation before tissue was removed.

#### Flow Cytometry, Fluorescence Microscopy and Differential Interference Contrast Microscopy

Flow cytometry and microscopy are frequently used to differentiate live, necrotic and apoptotic cells, based their biochemical and/or morphological characteristics [384-390]. Fluorescent dyes specific for the detection of live, necrotic and apoptotic cells, were used here on samples from treated and non-treated AML hepatocytes, and examined on a BD LSR flow cytometer (BD Biosciences, San Jose, CA) and a Nikon Microphot-FXA upright microscope (Nikon Inc., Tokyo, Japan). Flow cytometry measures the intensity of fluorescence emitted by the stained cells and translates these fluorescent signals into electronic signals that interface with a computer for data transfer. The resulting data is represented in histograms created in the Cell Quest software package

(BD Biosciences, San Jose, CA). These results will be utilized to characterize AML12 hepatocytes as internal controls in the western blotting analysis.

Morphological verification of apoptosis is always recommended due to the possibility of non-specific binding of the fluorochromes [387, 391-394]. Therefore, fluorescence microscopy and differential interference contrast (DIC) microscopy were used to verify apoptosis. This allows for the visualization of individual cells in the sample. Fluorescence microscopy was used to identify fluorescing cells and DIC microscopy to visualize the apoptotic morphology of the fluorescing cell.

#### Cell Staining

Three fractions of the AML12 hepatocytes were prepared for fluorochrome staining, one fraction was live untreated cells, the second fraction subjected to 3 freeze/thaw cycles to induce necrotic cell death and the third fraction contained camptothecin-induced apoptotic cells. These cell suspensions were all labeled with 1  $\mu$ l/ml propidium iodide staining solution for 10 min (Biosure, Grass Valley, Ca). Propidium iodide binds DNA, but does not cross the plasma membrane efficiently and is used to differentiate live and apoptotic cells from dead cells because the dead cells have fragmented membranes [388]. To differentiate the live cells from the apoptotic cell suspensions, the live and apoptotic samples were stained with 30  $\mu$ l/ml Hoechst 33324 (Molecular Probes, Eugene, OR) for 30 min. Hoechst 33342 also binds DNA, however it is rapidly taken up by the increased membrane permeability of apoptotic cells for that dye [395, 396].



### Flow Cytometry

The flow cytometer was calibrated using SPHERO™ rainbow blank and fluorescent calibration particles (Pharmingen, San Diego, CA) and Align Flow™ 633 nm and UV beads (Molecular Probes, Eugene, OR). Once the AML12 hepatocytes were stained, 1 ml from each of the live, necrotic, and apoptotic cell suspensions were analyzed by flow cytometry. The detection of propidium iodide fluorescence was measured on the flow cytometer using a 488 nm argon laser, while Hoechst 33342 fluorescence was detected with the 325 nm Helium/Cadmium UV laser. The detector settings for forward and side scatter were set at liner amp gain, and all other detectors set at log amp gain. Ten thousand events were collected on the flow cytometer for each of the cell samples.

### Fluorescence Microscopy and DIC Microscopy

Immediately after flow cytometry, a drop of each of the stained cells was placed on glass slides and observed using the Nikon-FXA epi-fluorescent research microscope with a 100-watt mercury light source (Nikon Inc., Tokyo, Japan). The red (propidium iodide) fluorescence was observed using a G-2A filter cassette to identify dead cells and the blue (Hoechst 33342) fluorescence was observed using a UV-1A filter cassette to differentiate apoptotic cells from live cells. Immediately following fluorescence detection of red (dead), dim blue (live), or bright blue (apoptotic) cell samples with the epi-fluorescent microscope, the cells were observed using DIC microscopy. DIC microscopy was used to identify the morphological characteristics of live, necrotic and apoptotic cells. Once it was verified that the three fractions of cells were live, necrotic,

and apoptotic cells, the AML12 hepatocytes were processed for western blotting as internal controls.

#### Western Blots for Immunodetection of PCNA, p53 and Cleaved Caspase-3 and PARP Proteins

Western blotting of protein extracts is an established method for immunodetection of specific proteins contained in a complex protein extract [397, 398]. This technique allows an investigator to determine whether a particular protein of is expressed in the cells or tissue of interest. Western blotting exploits the differential electrophoretic mobility of proteins. Proteins are isolated from cells or tissues and loaded into wells on a polyacrylimide gel. The gel is the electrophoresed and the proteins are separated by size. Smaller proteins will migrate further through the porous gel than the larger proteins. Once separated, proteins on the gel are transferred to a membrane using an electrical current. The membrane is then incubated with a primary antibody against the protein of interest. After incubation with the primary antibody, it is labeled with a horseradish peroxidase (HRP)-conjugated secondary antibody against the primary antibody. These antibody complexes are then incubated with a light emitting chemiluminescent reagent. The membrane is then exposed to x-ray film for chemiluminescent detection using ELC™ reagents (Amersham Biotech, Arlington Heights, IL). This detection is the result of photons emitted from the antibody complexes bound to the membrane and exposed to an x-ray film where these photon emissions are captured. The resulting x-ray film is developed and any dark dots or bands that are recorded on the film are used for analysis of protein/antibody complexes.

### Total Protein Extraction and Quantification

The livers of non-treated and carbon tetrachloride treated *G. affinis* were immediately removed upon sacrifice, homogenized in a 7 ml Dounce tissue grinder (VWR International, South Plainfield, NJ), rinsed 3x in PBS and suspended in 5-10 ml PBS. Non-treated and camptothecin treated AML12 hepatocyte suspensions were also rinsed 3x in PBS and re-suspended in 5-10 ml PBS. Both *G. affinis* and AML12 hepatocyte cell suspensions were centrifuged at 10K rpm for 5 min and the resulting pellet was collected, by pouring off supernatant, and resuspended in 5-10 ml PBS (this was repeated 2x). Total protein was extracted from the *G. affinis* and AML12 hepatocyte cell suspensions using Chaps cell extract buffer (Cell Signaling, Beverly, MA) in accordance with manufacturers protocol. Total protein extract was then quantified using the Micro Protein Determination kit (Sigma Aldrich, St. Louis, MO), based on a modified Lowery technique, in accordance with manufacturers protocol.

### Western Blotting

Protein separation was carried out by gel electrophoresis with Novex's NuPage gel system (Invitrogen, Carlsbad, CA), in their precast 4-12% gels with MES buffer. 30 µg total protein from non-treated and carbon tetrachloride treated *G. affinis* liver extracts and non-treated and camptothecin treated AML12 hepatocyte extracts, along with 5 µls SeeBlue® pre-stained protein standard (Invitrogen, Carlsbad, CA) were added to sample buffer (15 µls total volume), and loaded into the wells, then ran in an X-Cell II mini cell (Invitrogen, Carlsbad, CA) at 100 V for 1.5 hrs. The gel was removed from the cassette for protein transfer to a nitrocellulose membrane (0.45 µm pore size) using membrane

filter sandwich packs with Novex's NuPAGE transfer buffer (Invitrogen, Carlsbad, CA) in an X-cell II blot module transfer apparatus (Invitrogen, Carlsbad, CA) and ran at 30 V for 1 hr. Membranes were removed and non-specific sites blocked by incubation in 5% BSA/TBS-T overnight at 4° C. Membranes were then washed 4 times for 15, 10, 10 and 5 min each in TBS-T on a rocking platform. Individual membranes were each incubated in one of the 4 primary antibodies; 1) mouse anti-PCNA antibody against rat PCNA, PC10 clone (Dako, Carpinteria, CA), 2) mouse anti-p53 antibody against human p53, Pab 240 clone (BD Immunocytometry, San Jose, CA), rabbit anti-cleaved caspase-3 (Asp175) polyclonal antibody and mouse anti-cleaved PARP (Asp214) monoclonal antibody against human cleaved caspase-3 and PARP, respectively (Cell Signaling, Beverly, MA) at optimized dilutions in TBS-T for 2 hours at room temperature. Membranes were washed 4 times for 15, 10, 10 and 5 min each in TBS-T on a rocking platform. Incubated membranes in the appropriate mouse or rabbit HRP-secondary antibody supplied in the ECL™ western blotting detection reagents (Amersham Bioscience, Arlington Heights, IL) at a 1:1000 dilution in TBS-T for 1 hr at room temperature. Washed membranes as before, and followed manufacturer's protocol for ECL™ western blotting detection reagents for detection of blots on XAR-5 X-OMAT x-ray film (VWR Scientific, South Plainfield, NJ). X-ray film was developed and fixed using GBX Developer and GBX Fixer (Sigma Aldrich, St. Louis, MO).

## Results

### Flow Cytometric Analysis of AML12 Hepatocytes

To verify use of the AML12 hepatocytes as internal controls for western blotting, AML12 hepatocytes were stained with either propidium iodide or Hoechst 33342 and subjected to flow cytometry. Propidium iodide stain was used to identify cells undergoing necrotic cell death and Hoechst 33342 stain was used to differentiate live cells from apoptotic cells. Flow cytometric analysis of the AML12 hepatocyte fractions stained with propidium iodide is represented in histograms in figures 6-8. Propidium iodide staining was most intense in the histogram of the freeze/thawed hepatocytes, figure 6, with a peak of approximately 120 counts in channel  $10^3$ . The camptothecin treated cells, Figure 7, peaked at around 20 counts and the non-treated hepatocytes, Figure 8, around 40 counts in channel  $10^3$ . Thus, propidium iodide staining was most intense in the freeze/thawed AML12 hepatocytes indicating necrotic cells. The two remaining fractions, the non-treated and camptothecin treated fractions were further examined by flow cytometry for differentiation.

Flow cytometry of the Hoechst 33342 stained camptothecin treated and non-treated AML12 hepatocytes is represented in Figures 9 and 10 respectively. The Hoechst 33342 stained camptothecin treated AML12 hepatocytes revealed fluorescence with a peak of about 160 in channel  $10^1$ , while the non-treated hepatocytes fluoresced to a lesser degree at around 100 in channel  $10^1$ . This is indicative of the increased uptake of Hoechst 33342 in apoptotic cells when compared to live cells. Additional examinations of the three AML12 hepatocyte cell fractions were conducted using fluorescence and DIC

microscopy to confirm the flow cytometric analysis.

#### Fluorescence Microscopy and DIC Microscopy of AML12 Hepatocytes

Further examination of the three AML12 hepatocyte fractions using the Nikon-FXA epi-fluorescence microscope with the G-2A filter cassette for propidium iodide's red fluorescence revealed that the vast majority of cells from the freeze/thawed hepatocytes were fluorescing red. This was in contrast with the significant decrease in red fluorescence from the non-treated and camptothecin treated AML12 hepatocytes. These samples were immediately observed using DIC microscopy. The red fluorescing cells demonstrated a typical necrotic morphology including membrane discontinuities, cellular swelling and loss of cellular contents. These were numerous in the freeze/thawed hepatocytes and rare in the camptothecin treated and non-treated hepatocytes. The two remaining fractions, the non-treated and camptothecin treated fractions were further examined by fluorescence and DIC microscopy for differentiation.

For further differentiation of the non-treated and camptothecin treated AML12 hepatocytes, fluorescence microscopy with the UV-1A filter cassette was used to identify Hoechst 33342's blue fluorescence. The camptothecin treated AML12 hepatocytes revealed a significant number of cells with intense blue fluorescence, while the non-treated AML12 hepatocytes contained a large number of cells fluorescing a dim blue fluorescence. DIC microscopy of these samples further differentiated the bright blue fluorescing hepatocytes from the dimly fluorescing hepatocytes. The major observation differentiating these two fractions of AML12 hepatocytes was the significant membrane blebbing observed in the bright blue fluorescing cells that was not seen in the dim blue

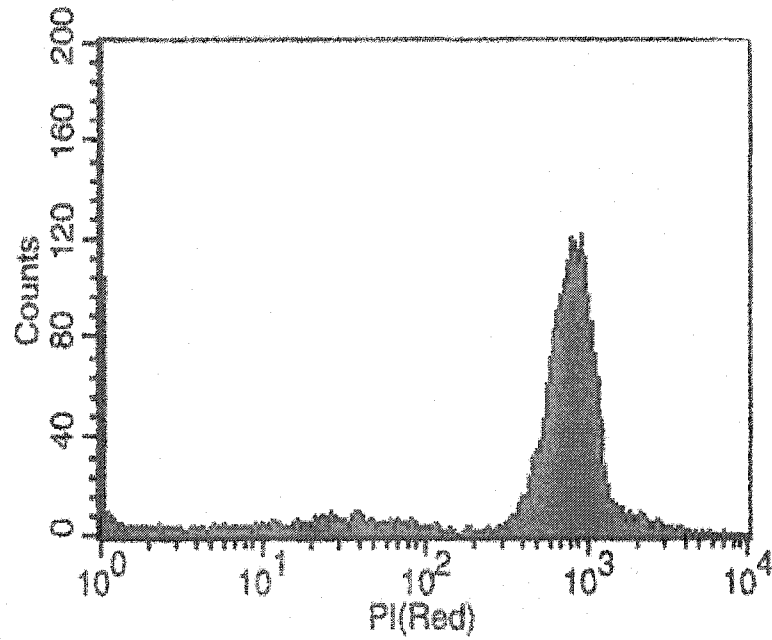


Figure 6. Flow Cytometry Histogram of Propidium Iodide (PI) Stained Freeze/Thawed AML12 Hepatocytes.

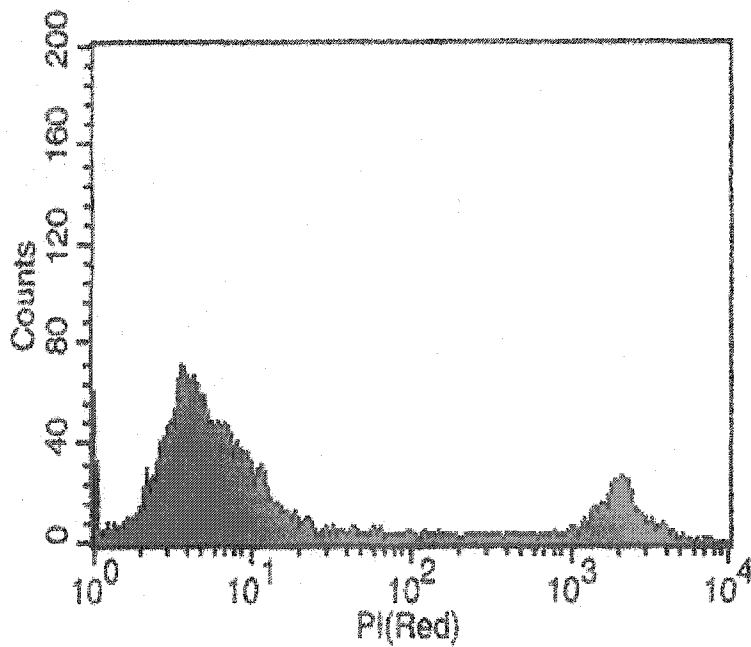


Figure 7. Flow Cytometry Histogram of Propidium Iodide (PI) Stained Camptothecin Treated AML12 Hepatocytes.

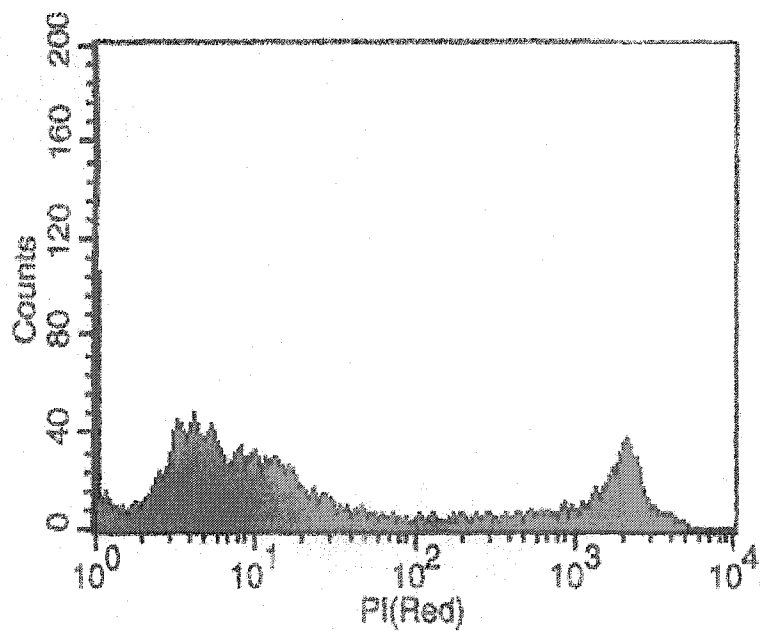


Figure 8. Flow Cytometry Histogram of Propidium Iodide (PI) Stained Non-Treated AML12 Hepatocytes.

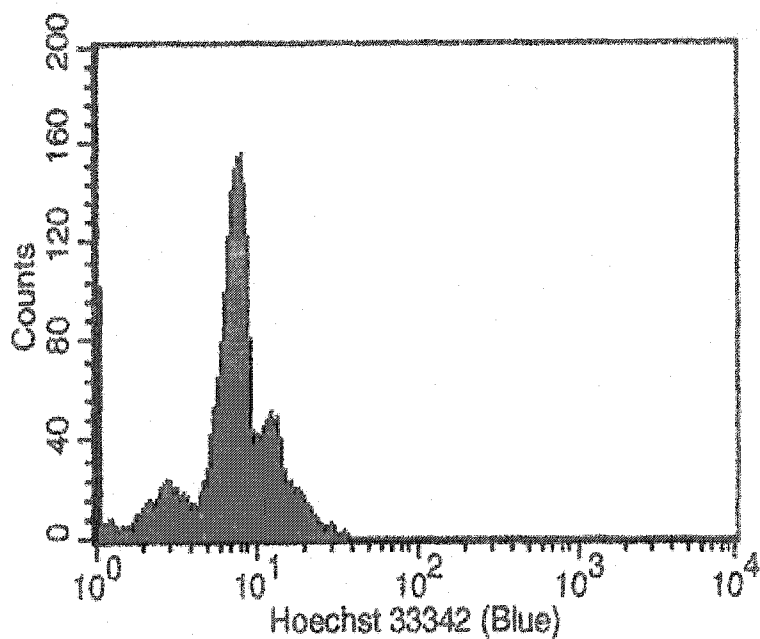


Figure 9. Flow Cytometry Histogram of Hoechst 33342 Stained Camptothecin Treated AML12 Hepatocytes.



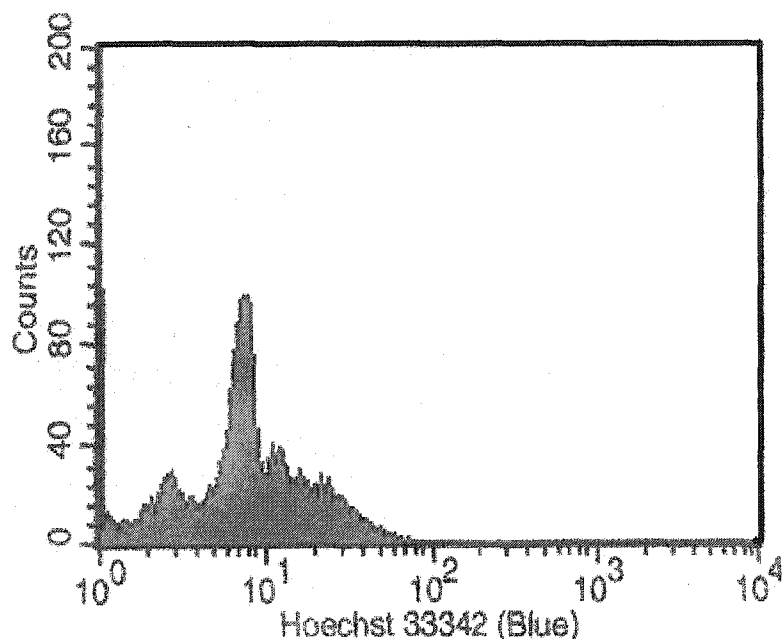


Figure 10. Flow Cytometry Histogram of Hoechst 33342 Stained Non-Treated AML12 Hepatocytes.

fluorescing cells. The dim blue fluorescing cells had intact membranes without observable blebbing. These results from fluorescence microscopy and DIC microscopy

#### Western Blots of PCNA, p53, and Cleaved Caspase-3 and PARP

The western blot technique has been used extensively in scientific research for separating and detecting proteins by antibody recognition [399-408]. Therefore, western blot analysis was employed here for the detection of *G. affinis* PCNA, p53 and cleaved caspase-3 and PARP proteins by respective antibodies. Western blot analysis of PCNA antibody against human PCNA was examined for specificity against *G. affinis* PCNA protein is shown in Figure 11. Lanes are designated as follows: lanes 1) and 2), carbon tetrachloride treated and non-treated *G. affinis* protein extracts, respectively, lanes 3) and

4), camptothecin treated and non-treated AML12 mouse hepatocyte protein extracts, respectively, lane B), blank and the last lane is Magic Mark protein standard (Invitrogen, Carlsbad, CA) with kilo-Daltons indicated on the right. There is some shadowing effect from movement of the membrane on the film. This blot revealed a band at approximately 32-36 kDa in both the carbon tetrachloride treated and non-treated *G. affinis* protein extracts. This 32-36 kDa band corresponds with the same band observed for both the camptothecin treated and non-treated internal controls. These results correlate well with those observed in scientific literature where PCNA protein is demonstrated to be a 28-36 kDa protein depending on the species examined [180, 409].

The anti-p53 antibody against humans was tested for specificity against *G. affinis* p53 protein as shown in figure 12. The lanes are designated as follows: lanes 1) and 2), non-treated and camptothecin treated AML 12 mouse hepatocyte protein extracts, respectively, lanes 3) and 4) non-treated and carbon tetrachloride treated *G. affinis* protein extracts, respectively, and the last lane contains SeeBlue® Plus2 protein standard (Invitrogen, Carlsbad, CA) with kilo-Daltons indicated on the right. Protein standard is cut and pasted from the original membrane. This blot resulted in an approximately 98-104 kDa band for both the carbon tetrachloride treated and non-treated *G. affinis* samples, with a comparatively stronger signal in the treated group. Blots of the internal controls both contained the expected 54-56 kDa band, however, the camptothecin treated group had two additional bands at approximately 85 and 48 kDa's. The results from the *G. affinis* protein samples deviated from the size of the p53 protein reported in literature. The band size expected was around 42 - 53 kDa [216, 410].

Anti-cleaved caspase-3 antibody against human cleaved caspase-3 was used to test specificity against *G. affinis* cleaved caspase-3 protein. These results are represented in Figure 13. Lane designation is as follows: lanes 1) and 2), carbon tetrachloride treated and non-treated *G. affinis* protein extracts, respectively, lanes 3) and 4), non-treated and camptothecin treated AML12 mouse hepatocyte protein extracts, and the last lane is SeeBlue® protein standard (Invitrogen, Carlsbad, CA) with kilo-Daltons indicated on the right. Protein standard is cut and pasted from the original membrane. This western blot revealed a band at about 36 kDa in both the carbon tetrachloride treated and non-treated fish groups. No signal was detected from either of the AML12 hepatocyte internal controls. These results were not aligned with those published in literature. The size expected of the cleaved caspase-3 fragment should be around 17-22 kDa while the full-length caspase-3 is typically around 32-38 kDa [252, 411-414].

The anti-cleaved PARP antibody (against human cleaved PARP) was used to examine its specificity to *G. affinis* PARP protein. The results are shown in Figure 14. Lanes are designated as follows: lanes 1) and 2), carbon tetrachloride treated and non-treated *G. affinis* protein extracts, respectively, lanes 3) and 4), camptothecin treated and non-treated AML12 mouse hepatocyte protein extracts, respectively, and the last lane is SeeBlue® protein standard (Invitrogen, Carlsbad, CA) with kilo-Daltons indicated on the right. Protein standard is cut and pasted from the original membrane. This western blot revealed one band of approximately 40-42 kDa in the carbon tetrachloride treated fish samples with an identical, but weaker band, observed in the non-treated group. The AML12 hepatocytes resulted in an approximately 100-115 kDa band in the non-treated group and an 85-90 kDa band in both the camptothecin treated and non-treated samples.

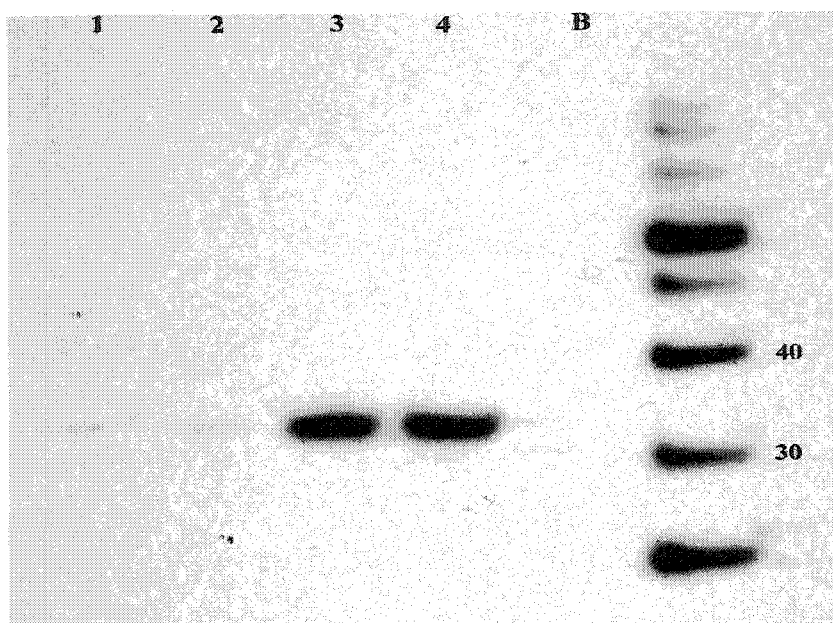


Figure 11. Western Blot of Anti-PCNA Antibody Labeling of Total Protein Extracts.

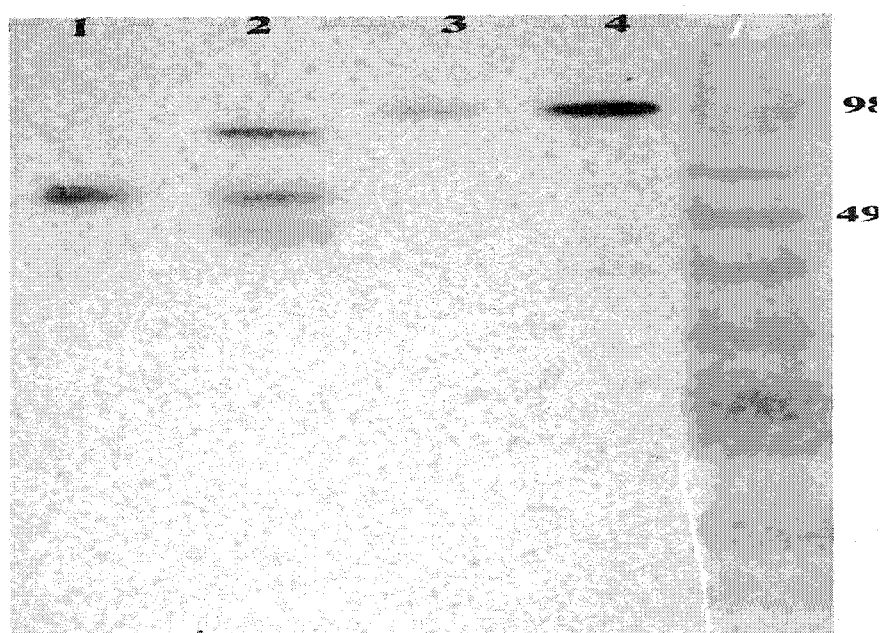


Figure 12. Western Blot of Anti-p53 Antibody Labeling of Total Protein Extracts.

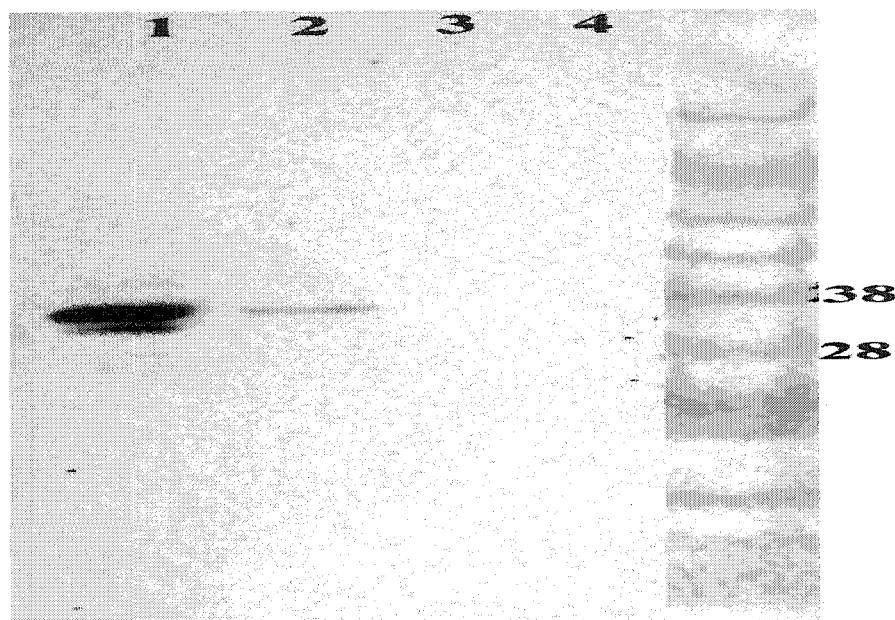


Figure 13. Western Blot of Anti-Cleaved Caspase-3 Antibody Labeling of Total Protein Extracts.

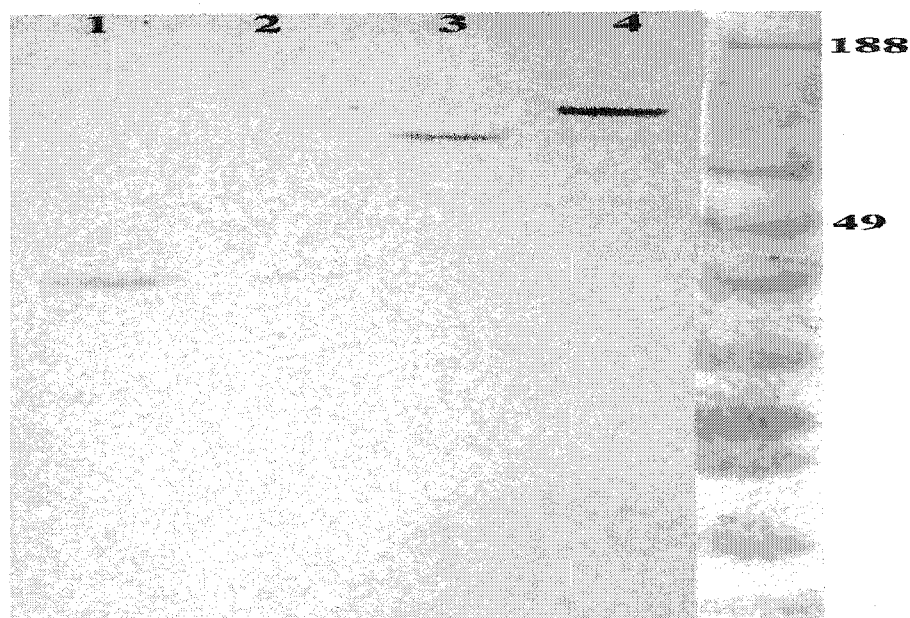


Figure 14. Western Blot of Anti-Cleaved PARP Antibody Labeling of Total Protein Extracts.

Although the results from the *G. affinis* samples do not correspond with that described in literature, the internal controls were as expected with the full length PARP at 113-116 kDa and the large cleaved fragment at 85-89 kDa as demonstrated in numerous publications [128, 216, 252, 255, 267, 412].

## Discussion

The integrity of AML12 proliferating mouse hepatocytes for use as internal controls in western blot analysis was examined here. The internal controls had two major requirements: 1) they had to proliferate, and, 2) they had to be capable of being induced to undergo apoptosis. The reasoning behind these requirements is that the antibodies that will be used in the western blots are against proteins involved in proliferation and apoptosis. AML12 hepatocytes are naturally proliferating cells, however, apoptosis has to be induced. Flow cytometric analysis, along with fluorescence and DIC microscopy demonstrated that the AML12 hepatocytes could be induced to undergo apoptosis when treated with camptothecin and that this could be differentiated from necrotic cells and live cells.

Apoptotic and live cells were differentiated from necrosis by staining the cell fractions representing live (non-treated), necrotic (3 freeze/thaws) and apoptotic (camptothecin treated) cells with propidium iodide and examining with flow cytometry. Flow cytometric analysis resulted in a high intensity of red fluorescence in the freeze/thawed cells, Figure 6. In contrast there was minimal fluorescence of the other two fractions, Figures 7 and 8. Propidium iodide is basically impermeable in cells with intact membranes. Necrotic cells have fragmented plasma and nuclear membranes that

result in greater staining of their nucleic acids by propidium iodide [164, 391]. The non-treated and camptothecin treated cells exhibited a very low degree of propidium iodide staining, indicating that necrosis was minimal in these cells.

These results were further examined with fluorescence and DIC microscopy. Using fluorescence microscopy, the red fluorescence of the freeze/thawed hepatocyte fraction was observed in the majority of cells. Only a few cells from the non-treated and camptothecin treated hepatocytes were fluorescent. When these samples were examined with phase contrast microscopy, they revealed morphological characteristics of necrosis in the fluorescing cells. The morphological characteristics ranged from cellular swelling and membrane fragmentation to complete cellular degradation. These results indicated that the cells induced to undergo necrosis were indeed necrotic cells and that the other cells did not experience any appreciable necrosis.

The non-treated and camptothecin treated AML12 hepatocytes were stained with Hoechst 33342 to verify induction of apoptosis in the camptothecin treated cells. After staining the cells with Hoechst 33342, flow cytometric analysis differentiated the two fractions as live and apoptotic by the intensity of their blue fluorescence. The blue fluorescence from Hoechst 33342, which is readily taken up by apoptotic cells, was much more intense in the camptothecin treated hepatocytes than in the non-treated cells as shown in Figures 9 and 10, respectively. This suggests that the camptothecin treated cells were apoptotic, but not the non-treated cells. Fluorescence microscopy correlated well with the flow cytometric results. A significant proportion of cells from the camptothecin treated AML12 hepatocytes fluoresced bright blue, while the majority of non-treated AML12 hepatocytes fluoresced a dim blue. Examination of these cells by DIC

microscopy revealed morphological characteristics of apoptosis in the brightly stained cells. Cellular condensation was noted, however, most significant, was the membrane blebbing observed in the bright blue fluorescing hepatocytes. These results, from flow cytometric analysis, fluorescence microscopy and DIC microscopy combined, indicate that the AML12 hepatocytes meet the major requirements for use as internal controls in western blot analysis. While their proliferative state is self-evident, this experiment verified induction of apoptosis in the AML12 hepatocytes exposed to camptothecin and differentiated them from necrotic cells and live cells.

These results were imperative for western blot detection of p53, cleaved caspase-3 and PARP, along with PCNA in AML12 hepatocytes for use as internal controls in their respective western blots. Since apoptosis could be induced in the AML12 hepatocytes, it follows that the apoptotic cells would have elevated levels of the apoptosis related proteins, p53 and cleaved caspase3 and PARP. Additionally, the proliferative state of AML12 hepatocytes results in elevated levels of PCNA protein. Thus, these hepatocytes meet the requirements put forth to ensure the integrity of the procedures and reagents used in western blotting.

Western blot analysis was employed for determining antibody specificity of PCNA, p53 and cleaved caspase-3 and PARP antibodies against the respective proteins from *G. affinis*. While results from the p53 and cleaved caspase-3 and PARP western blots were inconclusive, definitive PCNA antibody specificity against *G. affinis* PCNA was revealed. This was demonstrated in the western blot, figure 11, by the appearance of 32-36 kDa bands in both the carbon tetrachloride treated and non-treated *G. affinis* protein extracts. PCNA antibodies have been previously employed in



immunohistochemical detection of *G. affinis* PCNA [27]. The use of PCNA in immunohistochemical detection of proliferating cells in *G. affinis* correlates with the presence of proliferating cells such as intestinal crypt cells, cells in the gill filament and highly mitotic regions of the testis [373]. However, after an extensive search, this author was unable to find any references to western blot analysis of antibody specificity against *G. affinis* PCNA in scientific literature. Therefore, this study validated that the antigenic properties of *G. affinis* PCNA protein is specific for and compatible with the PC10 clone antibody against rat PCNA and that non-specific antibody labeling does not take place.

The antigenic properties of *G. affinis* p53 and cleaved caspase-3 and PARP proteins, however, were not as clear with regard to antibody specificity. The western blot using p53 antibody against human p53 ideally would have produced a band of around 42-53 kDa. This molecular weight range was determined by previously published research in scientific literature and the p53 protein sequences listed in the Swiss-Prot/TrEMBL protein database, accession numbers P79734, Q29537, P02340, P25035 and P04637 [415-417]. The molecular weight of the product observed in the *G. affinis* western blot was around 98-104 kDa, Figure 12, which is double the expected product. Whether or not some complexed form of p53 protein produced this band size is unknown.

The molecular weight of cleaved caspase-3 is typically 17-22 kDa and its pro-form ranges from 32-38 kDa as observed in several studies as reported in the Swiss Prot/TrEMBL database, accession numbers P42574, P70677, P55866 and P04637 [414, 418-420]. While it appears that the 36 kDa band on the *G. affinis* western blot, Figure 13, detected the full-length form the cleaved form was not observed. This may be indicative of low levels of cleaved caspase-3 or a caspase-3 independent apoptotic

pathway in carbon tetrachloride treated *G. affinis* liver cells. Confounding this, the internal controls did not produce any bands on the blots. Several optimization steps for protein concentration, primary antibody and secondary antibody dilutions and incubation times did not have any effect. Therefore, the integrity of the reagents and procedures could not be verified. Although further experimentation is necessary, the recognition of an approximately 36 kDa protein in the *G. affinis* blots may be for the pro-form of caspase-3.

The molecular weight of cleaved and full-length PARP has been established as 85-89 kDa for the large cleaved PARP fragment and 113-116 kDa for the pro-form [257, 420-422]. The PARP western blot of *G. affinis* proteins revealed a 40-42 kDa product, Figure 14. While this product may reflect an additional cleavage of PARP in *G. affinis*, further experimentation is necessary. To examine this discrepancy in the PARP western blot, along with the observed discrepancies in the p53 and cleaved caspase-3 western blot, additional testing such as protein sequencing would be required. Unfortunately, that is beyond the time restraints and scope of this study. Therefore, in conclusion, the PCNA western blots demonstrated that the PC10 clone, PCNA antibody is PCNA specific and can be employed as a useful molecular tool for investigating proliferation in carcinogenic and toxicologic studies with *G. affinis*. While the western blot analysis of *G. affinis* p53 and cleaved caspase-3 and PARP proteins revealed inconclusive results for use of antibodies against these proteins as molecular tools, cell death can be detected using another, more expensive alternative. This is the TUNEL method of labeling DNA strand breaks discussed in the literature review.

## CHAPTER IV

### DETECTION OF CELLULAR PROLIFERATION AND DEATH IN *G. AFFINIS* EXPOSED TO 2-AMINOFLUORENE AND BENZIDINE

#### Purpose of Investigation

The objective of this study is to examine the early carcinogenic effects of the PAAs, 2-AF and BZ both individually and combined on the ratio of cellular proliferation and cell death in *G. affinis* livers at three time intervals. To accomplish this task, PCNA antibodies and the TUNEL technique were used for immunohistochemical (IHC) detection of proliferating cells and labeling of DNA strand breaks to identify cell death, respectively. Both of these techniques are well established as molecular tools in a number of published studies discussed earlier in their literature reviews.

The use of a small fish species as the animal model reflects the ongoing research involved in promoting aquatic species, *G. affinis* in particular, as sentinel species for environmental carcinogenic and toxicology studies [27, 29, 34, 373, 423]. An extensive literature search revealed the only carcinogen class tested on *G. affinis* is the alkylating carcinogen, methylazoxymethanol acetate (MAM-Ac) [27]. Therefore, PAAs were selected as a different metabolic class of environmental carcinogens. Additionally, the combination of 2-AF and BZ exposure to *G. affinis* is examined to determine any variation from their individual effects. The exposure of two different chemicals administered simultaneously can have an additive effect, a synergistic effect, or an antagonistic effect [424-427]. The selection of liver tissue was based on its metabolism of these PAAs as discussed previously in their literature reviews and on its rapid development of neoplasms when exposed to carcinogens [27, 373].

The three time intervals, 4, 8 and 12 weeks were chosen to represent the early stages of carcinogenesis in *G. affinis*. This was based on an earlier study of hepatocarcinogenesis in *G. affinis* where the formation of tumors was reported in 33% of the fish examined at 25 weeks [27]. Liver carcinomas have been reported as early as 6 weeks to 3 months in medaka [428, 429]. The early stages are important as they reflect the cellular events occurring at the onset of neoplastic formation.

Here we will examine cell proliferation and death in serial liver sections to determine any early changes from their normal proliferative/death status due carcinogen exposure. While the proliferation index and TUNEL index will be considered in the analysis, the difference between cell proliferation and cell death will also be used. This difference will control for the uncommon occurrence of cells staining positive for both proliferation and death. As discussed in the literature review this may occur when cells are undergoing extensive DNA repair. Therefore, the difference between the two assays will give a more accurate picture of cellular proliferation by controlling for false positives and subtracting out the dying cells will reveal the net proliferation.

### Experimental Design

To test the carcinogenic effects of 2-AF and BZ on *G. affinis* livers, 21-22 fish are randomly selected for each of 8 treatment groups and placed in 8 different 10-gallon aquariums. Care and housing of *G. affinis* is maintained as discussed in Chapter III. Fish are acclimated for 2 weeks, then exposed to a high dose, 6.9 mM, or low dose, 0.069 mM, of 2-AF and BZ individually or combined each day as outlined in Table 1. These doses are based on carcinogenic studies in previous aquatic research [199, 310, 430, 431].

There are two control groups, a non-treated control and a vehicle control. The control group that does not have any treatment added to their food will have their food processed in the same manner as the treated food. The vehicle control group's food is treated with ethanol (EtOH) the same as the treated groups since ethanol is a vehicle for solubilizing and delivering 2-AF and BZ to their food.

Table 1  
Experimental Design

Treatment Groups	# of Replicates	Time Intervals		
No Treatment Control (N)	7	4 Weeks	8 Weeks	12 Weeks
Ethanol Vehicle Control (E)	7	4 Weeks	8 Weeks	12 Weeks
0.069 mM 2-Aminofluorene (AL)	7	4 Weeks	8 Weeks	12 Weeks
6.9 mM 2-Aminofluorene (AH)	7	4 Weeks	8 Weeks	12 Weeks
0.069 mM Benzidine (BL)	7	4 Weeks	8 Weeks	12 Weeks
6.9 mM Benzidine (BH)	7	4 Weeks	8 Weeks	12 Weeks
0.069 mM 2-Aminofluorene and Benzidine (ABL)	7	4 Weeks	8 Weeks	12 Weeks
6.9 mM 2-Aminofluorene and Benzidine (ABH)	7	4 Weeks	8 Weeks	12 Weeks

Both individually and combined, 2-AF and BZ is measured and the appropriate amounts are solubilized in 150 ml ethanol and thoroughly mixed into their food overnight on a roller mixer. The EtOH is evaporated from the treated food in a vacuum for 48 hrs. Fish are fed the treated and control doses at approximately 1% of their

averaged body weight 2 times daily for 4, 8 and 12-week time intervals. As treatments are completed, fish are randomly selected for sacrifice, liver removal and fixation, tissue sectioning and PCNA, TUNEL and H&E staining.

## Methods and Materials

### Fish and Housing

Fish were housed and maintained as described in the same section in Chapter III. Fish were dosed orally in their food in doses set forth in Table 1 using automatic fish feeders. After treatment for 4 weeks 1/3 of the fish from each tank were randomly selected, sacrificed and their livers removed and processed for IHC detection of PCNA, TUNEL labeling of DNA strand breaks and subsequent H & E staining (this is discussed in Chapter V). After 8 weeks of treatment, half of the remaining fish in the aquariums were randomly selected and processed in the same manner. The rest of the fish completed their treatment at 12 weeks and were also processed as described.

Upon completion of a treatment interval, *G. affinis* were randomly selected from each of the treatment groups and control tanks. The fish were anesthetized by immediately placing them on ice for 45 min followed by decapitation and immediate liver removal. Due the small size of the fish, the livers were removed under a dissection microscope using microsurgical tools. Once the fish livers were carefully removed, they were immediately placed in a fixative overnight for subsequent paraffin embedding and tissue sectioning.

### Tissue Sectioning of Livers

Livers were fixed in 10% neutral buffered formalin (Sigma Aldrich, St. Louis, MO) for 24 hrs, and then placed in cassettes for tissue processing for paraffin embedding. Tissue was processed in a Fisher model 266MP Histomatic tissue processor (Fisher Scientific, Pittsburgh, PA). Processed tissues were embedded in paraffin using the Fisher HistoCenter™ (Fisher Scientific, Pittsburgh, PA). The paraffin embedded blocks were placed on a surface maintained at approximately 4 °C for sectioning. Serial tissue sections were cut using a microtome, model 1512 (Ernst Leitz Wetzlar GMBH, Germany), 5 microns thin and adhered to poly-L-lysine poly prep slides (Sigma Aldrich, St. Louis, MO) and incubated at 37 °C overnight.

### PCNA Antibody and TUNEL Labeling of Tissue Sections

As discussed in their appropriate literature reviews, PCNA antibodies and TUNEL are used as biomarkers to identify proliferating cells and cell death, respectively. Both of their protocols were optimized using positive and negative controls. These controls were also used as internal controls. The positive control used for PCNA detection was *G. affinis* intestinal tissue and the negative controls omitted exposure to the primary PCNA antibody. The positive control used for TUNEL was rat mammary gland tissue 3-5 days after weaning (Serologicals Corp., Norcross, GA) and negative controls omitted exposure to the TdT enzyme. These controls were also used as internal controls.

### PCNA Antibody Labeling of Tissue Sections

The protocol used for detection of PCNA in *G. affinis* was based on a previously published protocol that had been optimized for PCNA detection in three small fish species including *G. affinis* [373]. Tissue section slides were placed in glass slide racks (20 slides/rack) and immersed in the appropriate reagents in staining jars. Slides were deparaffinized in xylenes, 2 X for 3 mins each, then hydrated in 100% EtOH, 2 X 1 min each, 95% EtOH, 2 X 1 min each and rinsed in phosphate buffered saline with 5% Tween 20 (PBS-T) for 5 min. Slides were microwaved in 3% H<sub>2</sub>O<sub>2</sub> with 0.1% sodium azide for 2 min, rested 1 min and microwaved again for 30 sec for antigen retrieval. After cooling for 15 min, slides were rinsed in 2 X for 5 min each in ddH<sub>2</sub>O followed by 5 min in PBS-T. Slides were incubated in 0.5% powdered milk for 20 min for protein blocking. Individual tissue sections were incubated in non-diluted PCNA antibody, PC10 clone (Dako, Carpinteria, CA) for 1 hr and rinsed in PBS-T for 5 min. Mouse biotinylated secondary antibody from the mouse super-sensitive detection kit (BioGenex Laboratories, San Ramon, CA) was applied to the tissue sections for 30 min and rinsed in PBS-T for 5 min. Peroxidase-conjugated streptavidin (BioGenex Laboratories, San Ramon, CA) was applied for 30 min and rinsed in PBS-T for 5 min. Diaminobenzidine (DAB) solution was prepared by dissolving 1 mg DAB (Sigma Aldrich, St. Louis, MO) in 2 ml PBS-T. Just before applying, 0.6 ul of 30% H<sub>2</sub>O<sub>2</sub> was added. DAB solution was applied for 10 min then rinsed for 2 min in running tap water. Slides were counterstained with Mayer's modified hemotoxylin (Newcomer Supply, Middleton, WI) for 1 min, rinsed in running tap water for 2 min and blued in PBS-T with 0.5% ammonium acetate (Sigma Aldrich, St. Louis, MO) for 1 min. Slides were then dehydrated in 95% EtOH, 2 x 1 min each,



100% EtOH, 2 X 1 min each, and xylenes, 2 X 3 min each. Coverslips were adhered to slides with Cytoseal mounting media (VWR Scientific, South Plainfield, NJ).

#### TUNEL Labeling of Tissue Sections

TUNEL labeling of the liver sections was carried out in accordance with the protocol from the ApopTag® Apoptosis Detection Kit (Serologicals Corp, Norcross, GA). Slight modifications to the protocol were as follows. DAB dissolved in PBS-T was used as the peroxidase substrate and was applied for 10 min. Mayer's modified hematoxylin was used as the counterstain, rinsed in running tap water for 2 min, and then blued in PBS-T with 0.5% ammonium acetate. Dehydration included immersing in 75% EtOH for 1 min, 95% EtOH for 1 min and 100% EtOH for 2 min before immersing in xylenes. Coverslips were adhered to the slides using Cytoseal XYL mounting media (VWR Scientific, South Plainfield, NJ).

#### Determination of PI Index and TUNEL Index

PCNA and TUNEL slides of liver tissue sections were observed under a microscope containing a 19 mm eyepiece graticle with a 1mm grid with 10 vertical and horizontal lines (Electron Microscopy Sciences, Ft. Washington, PA). This grid was used for tracking and counting cells. When a focal area of positive cells was observed they were included in the count on the grid, however, the majority of normal (non-positive) cells were also within in the grid. If more than one focal area of positive cells were encountered, only those that fell within the 1mm grid were counted. The grid was not moved around to include all areas with positive foci, only those within the original setting

of the grid were included in the count. This was to give a representative count of the overall tissue. Counting of PCNA labeled cells is reported in scientific literature as a PCNA index (PI), the number of PCNA positive cells per 1000 cells, and that index is used here for reporting the number of proliferating cells in each tissue section [181-183]. This index was also used for reporting the number of TUNEL positive cells in each tissue section. All positive cells were counted including those lightly stained as established in the guidelines of the Registry of Industrial Toxicology Animal-data Cell Proliferation and Apoptosis (RITA CEPA) group. The RITA CEPA group consists of 16 European companies and organizations whose primary goal is assisting its members in proliferation studies for comparison and interpretation of results in accordance with self-established standardized protocols for the subsequent formation of a cell proliferation and apoptosis database. PCNA and TUNEL positive cell counts were spot checked by an electron microscopist from the Biological Imaging Center at Western Michigan University.

Due to the death of four fish during the experiment and three paraffin blocks that would not yield good sections, their individual PI and TI counts were derived from the averages in their particular treatment group since they accounted for <5% of the samples. This was done in order to maintain sample numbers that were balanced for statistical analysis with minimal manipulation and to avoid discarding of pertinent data collected. This averaging was double-checked to ensure it did not change the original values of the means from the actual data collected.

## Results

The means and standard deviations of PI, TI and their difference are reported in Table 2 and a histogram of their individual means and standard error are located in Figures 15 - 23. It is important to note that the y-axis of these histograms are not uniform and change between the individual histograms. The histograms reflect significant increases in some of the treatment groups when compared to the others. However, these increases may be due to the contribution of outliers rather than effects from the PAA, since the standard error for those groups is typically higher than the remaining groups. Also, both 2AF and BZ are from the same metabolic class, resulting in like metabolic products, and there are no reports in literature that they enhance or decrease the effects of one another.

### PCNA Index

PCNA staining appeared restricted to the nuclei and varied in the intensity of staining as seen in Figure 24. PCNA positivity was widely distributed in some tissue sections, focally located in other tissue sections or a combination of the two. The number of PCNA positive cells varied greatly between and within dose groups. The variation between dose groups is observed in the means reported in Table 2. The PI contained several expected outliers as seen in the boxplot of Wilcoxon studentized residuals in Figure 25. PI data did not have a normal distribution as revealed by the lack of linearity in the Q-Q plot of studentized residuals in Figure 26. The desire for inclusion of outliers and the lack of a normal distribution dictated a robust non-parametric statistical analysis of the data. Therefore, Wilcoxon's robust 2-way analysis for time and treatment effects

and interaction was performed on the data and reported in Table 3. Effects of the three time intervals, 4, 8, and 12 weeks had a p-value of 3.14e-05, treatments had a p-value of 0, and the interaction between times and treatments had a p-value of 3.42e-05. Both of the effects and their interaction revealed high statistical significance.

Further statistical analysis was conducted on the differences between controls and treatments by using Fisher's paired least significant differences test. Statistically significant differences in contrasts are reported in Table 4. Significant contrasts were observed between 4 wks and 12 wks, and 8 wks and 12 wks. There were not any significant differences between the no treatment control and the EtOH vehicle control. All treatments, except the low dose (0.069mM) of 2AF, had significant contrasts with the EtOH vehicle control. Contrasts were noted between the low 2AF dose and the remaining treatment groups indicating a no-effects level for the low dose 2AF exposure with regard to cellular proliferation. The high dose of 2AF and the high dose 2AF/BZ had significant contrasts, while the low dose of BZ significantly contrasted with the high doses of BZ and 2AF/BZ. The 2AF/BZ high and low doses also had significant contrasts with one another. These differences between treatment groups reflect the increased proliferative effects, of 2AF/BZ and BZ at the low dose and 2AF at the high dose, above the already significant proliferative effects of 2AF/BZ and BZ at the high dose. Because there is so little data available on PCNA staining of fish livers and the data that exists is not standardized, a direct comparison with other results is not feasible. For example, one study with *F. grandis*, calculates the PI as PCNA positive cells/mm<sup>2</sup> of tissue [199]. Due to variation in the number of cells per mm<sup>2</sup> in the *G. affinis* livers, this method for calculating the PI would have resulted in a collection of non-standardized

data. The most standardized of methods for counting PCNA positive cells in literature is to count the number of PCNA positive cells/1000 cells.

### TUNEL Index

TUNEL staining, shown in Figure 27, was localized in the nuclei and stained at varying intensities. TUNEL positive cells were widely distributed in most tissue sections with rare focal occurrence. Over 75% (127/168) of the tissue sections examined exhibited a low TI, 0 – 3, while 20% (32/168) exhibited TIs from 4-12 and only 5% (9/168) had  $TIs > 12$ . These counts resulted in extremely discrete data. The upper 5% of the TI values resulted in outliers as plotted in the boxplot of Wilcoxon studentized residuals in Figure 28. This data did not have a normal distribution as indicated in the non-linear Q-Q plot of studentized residuals in Figure 29. For inclusion of outliers and the lack of normal distribution the non-parametric robust Wilcoxon 2-way analysis for time and treatment effects and interaction was used in the statistical analysis. However, this was problematic since the data was extremely discrete. The discrete data prohibited the robust Wilcoxon 2-way analysis from formulating a true statistical analysis as evidenced by the negative F-statistic as reported in Table 5. To overcome this difficulty, a value between  $-0.05$  and  $0.05$  was randomly added to the actual TI values to create a little 'noise'. To ensure that this manipulation of the data did not significantly change the true value of the data a comparison of the raw data and the adjusted data, using a least squares method of 2-way analysis, was employed. The results are reported in Table 5 of the raw data and Table 6 of the adjusted data. With the raw data, the least squares p-value is 0.0586 and F-statistic is 2.89 for time effects, p-value is 0.399 and F-statistic is

Table 2

Means for TUNEL Index, PCNA Index and Their Differences

Treatment Groups	Time Intervals (Weeks)	TUNEL		PCNA		PCNA – TUNEL	
		Means	Std. Dev.	Means	Std. Dev.	Means	Std. Dev.
Control	4	1.857	2.035	1.571	2.299	-0.286	1.7
	8	4.14	5.669	4.71	6.422	0.57	9.2
	12	2.0	1.414	18.14	13.619	16.14	12.72
EtOH Vehicle Control	4	0.857	0.9	5.29	6.751	4.43	1.64
	8	4.57	4.509	13.71	13.13	9.14	11.47
	12	2.429	0.787	7.86	5.87	5.43	5.44
0.069 mM 2AF	4	15.29	26.291	14.29	23.436	-1.0	11.78
	8	3.0	2.517	16.57	12.581	13.57	11.82
	12	1.286	2.563	33.3	38.9	32.0	39.32
6.9 mM 2AF	4	3.29	3.352	23.9	35.145	20.6	35.52
	8	2.0	1.826	16.14	5.27	14.14	6.07
	12	1.286	1.113	67.9	47.90	66.6	48.77
0.069 mM BZ	4	6.43	6.241	89.1	66.76	82.7	69.51
	8	3.714	1.113	13.29	12.958	9.57	12.55
	12	2.429	10.565	31.4	28.919	29.0	30.05
6.9 mM BZ	4	6.86	15.115	26.71	21.631	19.9	30.1
	8	2.143	1.773	19.43	15.619	17.29	17.0
	12	1.571	1.618	34.3	27.14	32.7	27.65
0.069 mM 2AF/BZ	4	3.14	1.069	21.86	19.557	18.71	20.31
	8	1.571	1.813	46.0	44.52	44.4	45.57
	12	3.0	1.512	37.14	18.3	34.14	18.35
6.9 mM 2AF/BZ	4	2.0	2.449	15.57	7.39	13.57	7.23
	8	1.714	1.799	15.14	12.967	13.43	14.06
	12	1.714	1.604	38.1	28.644	36.4	28.66

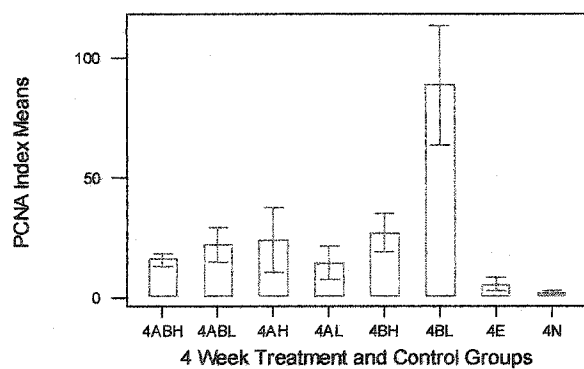


Figure 15. Histogram of PCNA Index Means and Standard Deviations of Treatment and Control Groups at 4 Weeks.

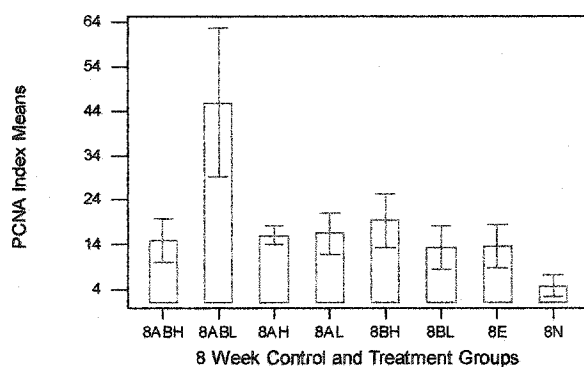


Figure 16. Histogram of PCNA Index Means and Standard Deviations of Treatment and Control Groups at 8 Weeks.

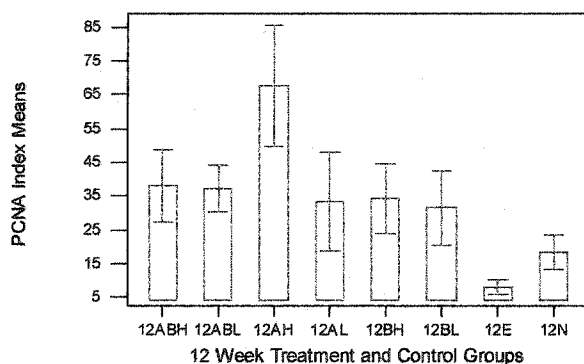


Figure 17. Histogram of PCNA Index Means and Standard Deviations of Treatment and Control Groups at 12 Weeks.

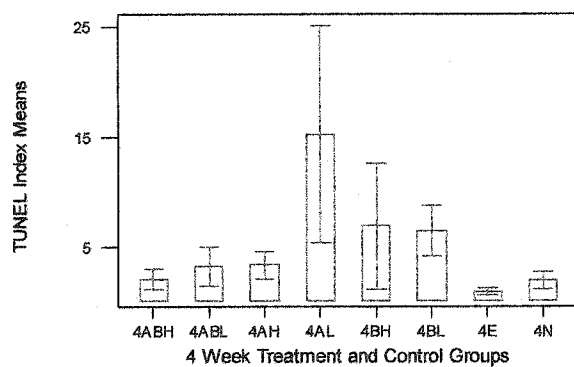


Figure 18. Histogram of TUNEL Index Means and Standard Deviations of Treatment and Control Groups at 4 Weeks.

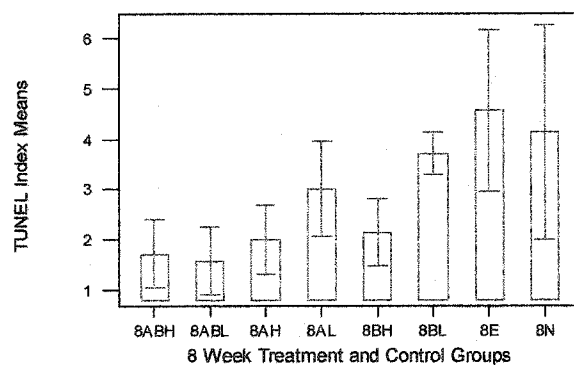


Figure 19. Histogram of TUNEL Index Means and Standard Deviations of Treatment and Control Groups at 8 Weeks.

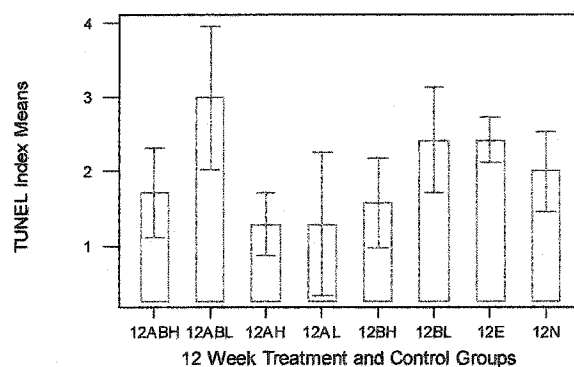


Figure 20. Histogram of TUNEL Index Means and Standard Deviations of Treatment and Control Groups at 12 Weeks.



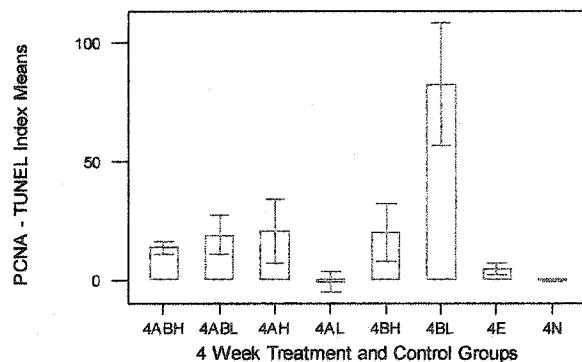


Figure 21. Histogram of PI - TI Index Means and Standard Deviations of Treatment and Control Groups at 4 Weeks.

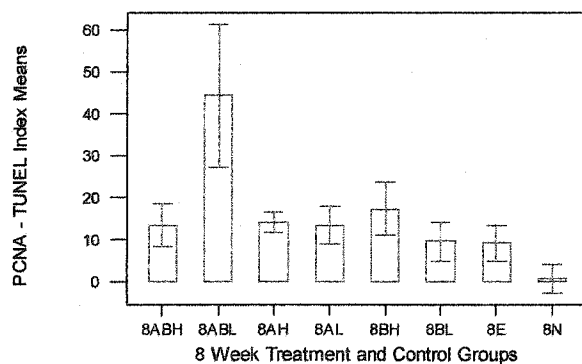


Figure 22. Histogram of PI - TI Index Means and Standard Deviations of Treatment and Control Groups at 8 Weeks.

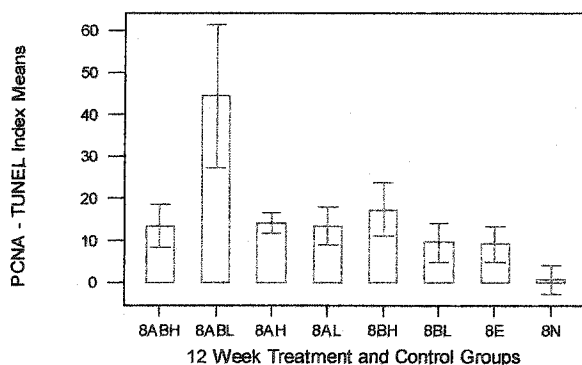


Figure 23. Histogram of PI - TI Index Means and Standard Deviations of Treatment and Control Groups at 12 Weeks.

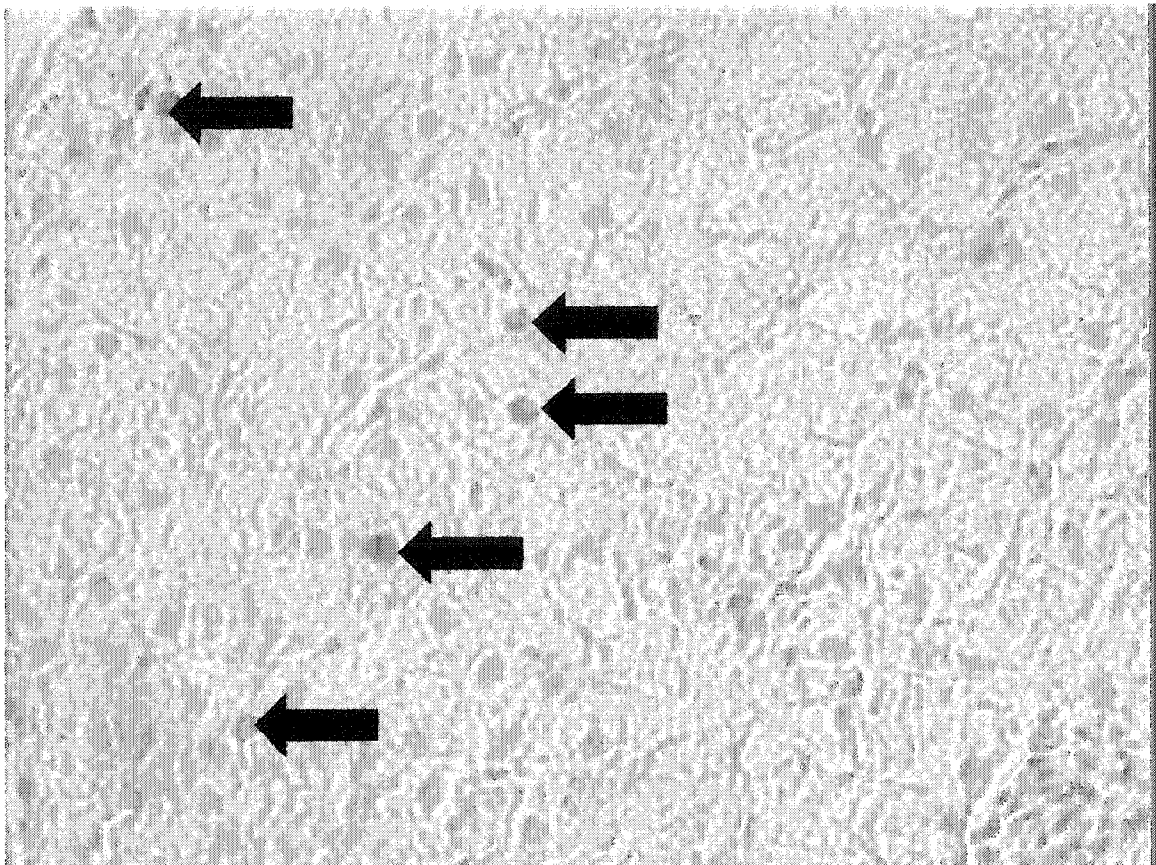


Figure 24. PCNA Positives from a *G. affinis* Liver Tissue Section.

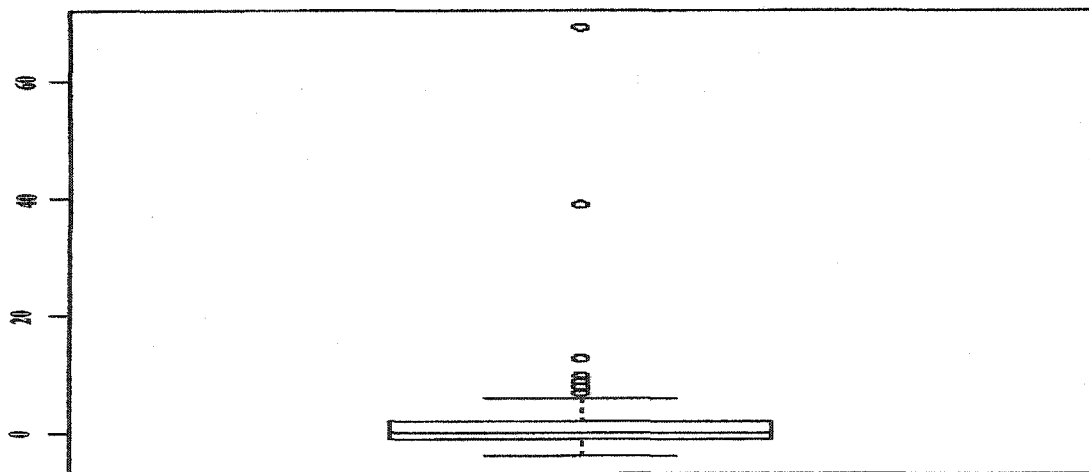


Figure 25. PCNA Boxplot of Wilcoxon Studentized Residuals.

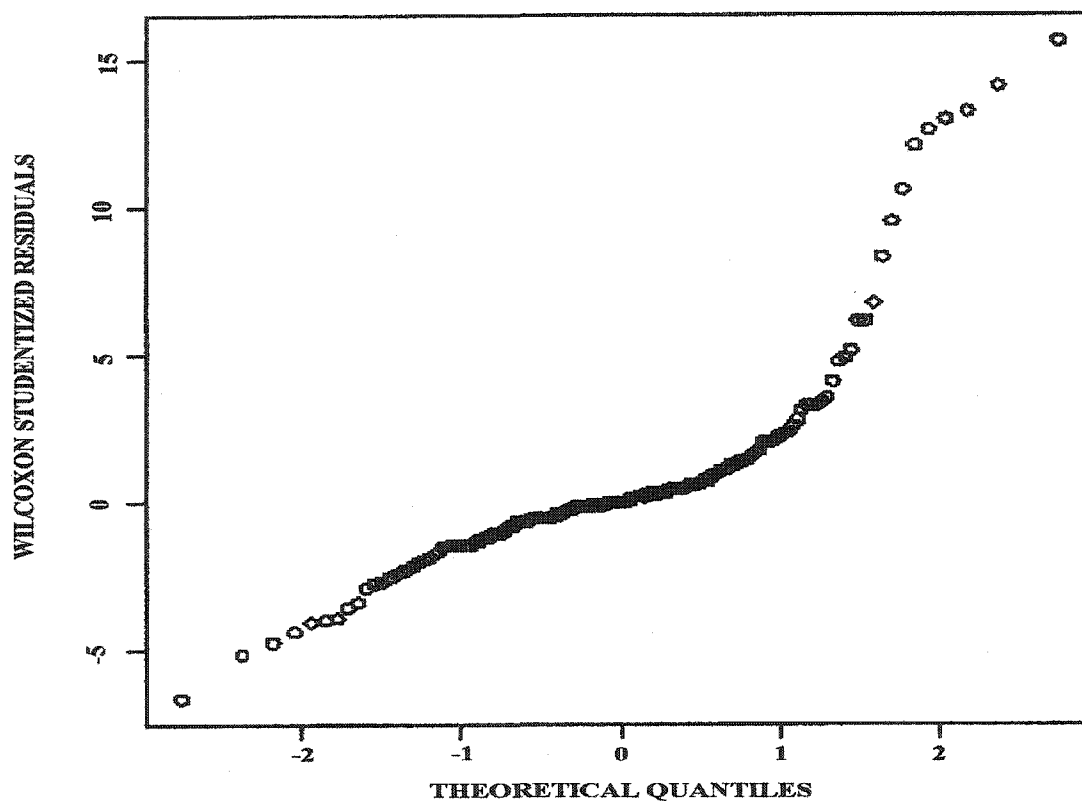


Figure 26. PCNA Q-Q Plot of Studentized Residuals.

Table 3

PCNA - Tests of Time and Treatment Effects and Interaction

Wilcoxon Robust			
Effects and Interaction	F-Statistic	df	p-value
Time Intervals	11.1528	2,144	3.14116e-05
Treatments	8.38557	7,144	0
Time Intervals/Treatment	3.64758	14,144	3.42727e-05

Table 4

PCNA - Fisher's Paired Least Significant Differences With Statistical Significance

Wilcoxon Robust Contrasts*			
Contrast	Estimate	Error Term	Interval
4 Wks vs 12 Wks	-10.8884	5.50334	(-16.3917, -5.38507)
8 Wks vs 12 Wks	-13.7978	5.50334	(-19.3012, -8.29451)
No Treatment vs 2AF Low Dose	-9.08497	8.98691	(-18.0719, -0.0980654)
No Treatment vs 2AF High Dose	-22.6851	8.98691	(-31.672, -13.6982)
No Treatment vs BZ Low Dose	-25.9979	8.98691	(-34.9848, -17.011)
No Treatment vs BZ High Dose	-15.5676	8.98691	(-24.5545, -6.58072)
No Treatment vs 2AF/BZ Low Dose	-23.1327	8.98691	(-32.1196, -14.1458)
No Treatment vs 2AF/BZ High Dose	-13.6326	8.98691	(-22.6195, -4.64572)
Ethanol Control vs 2AF High Dose	-22.0184	8.98691	(-31.0053, -13.0315)
Ethanol Control vs BZ Low Dose	-25.3312	8.98691	(-34.3181, -16.3443)
Ethanol Control vs BZ High Dose	-14.901	8.98691	(-23.8879, -5.91406)
Ethanol Control vs 2AF/BZ Low Dose	-22.4661	8.98691	(-31.453, -13.4792)
Ethanol Control vs 2AF/BZ High Dose	-12.966	8.98691	(-21.9529, -3.97906)
2AF Low Dose vs 2AF High Dose	-13.6001	8.98691	(-22.587, -4.61321)
2AF Low Dose vs BZ Low Dose	-16.9129	8.98691	(-25.8998, -7.92601)
2AF Low Dose vs 2AF/BZ Low	-14.0478	8.98691	(-23.0347, -5.06084)

Table 4 – Continued

Wilcoxon Robust Contrasts			
Contrast	Estimate	Error Term	Interval
Dose			
2AF High Dose vs 2AF/BZ High Dose	9.05247	8.98691	(0.0655584, 18.0394)
BZ Low Dose vs BZ High Dose	10.4303	8.98691	(1.44335, 19.4172)
BZ Low Dose vs 2AF/BZ High Dose	12.3653	8.98691	(3.37835, 21.3522)
2AF/BZ Low Dose vs 2AF/BZ High Dose	9.5011	8.98691	(0.51319, 18.487)

\*95% Individual Confidence Intervals

1.05 for treatment effects and p-value is 0.226 and F-statistic is 1.28 for their interaction. This least squares 2-way analysis was comparable to the adjusted data's p-value of 0.0598 and F-statistic of 2.88 for time effects, p-value of 0.397 and F-statistic of 1.05 for treatment effects and p-value. Since there were no significant changes to the data, we continued our analysis with the adjusted data.

Wilcoxon 2-way analysis of the adjusted data in Table 6 shows statistical significance for treatment effects with a p-value of 0.00556 and F-statistic of 3.01 and its interaction with time (p-value is 0.0248 and F-statistic is 1.96), but not with the time effects (p-value is 0.219 and F-statistic is 1.53). This indicates that treatments were

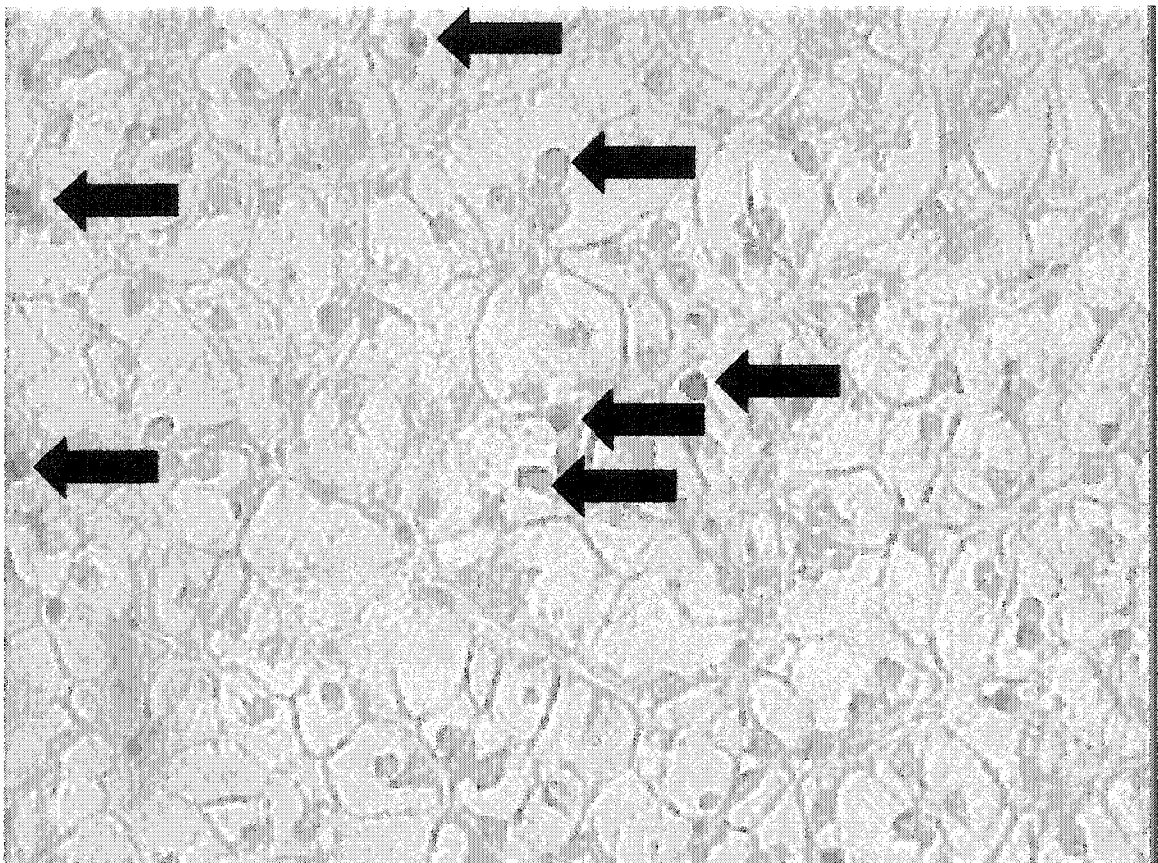


Figure 27. TUNEL Positives from a *G. affinis* Liver Tissue Section.

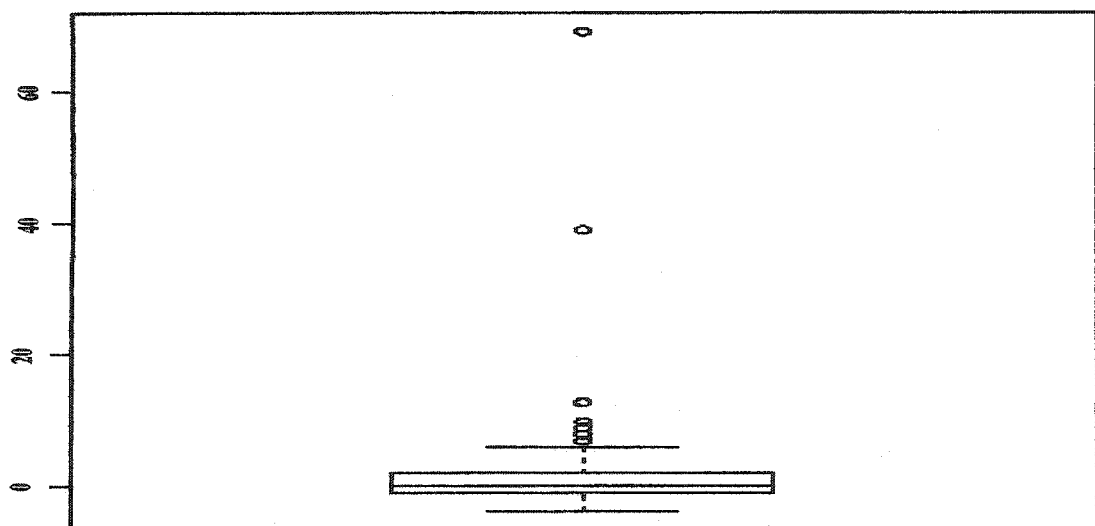


Figure 28. TUNEL Boxplot of Wilcoxon Studentized Residuals.

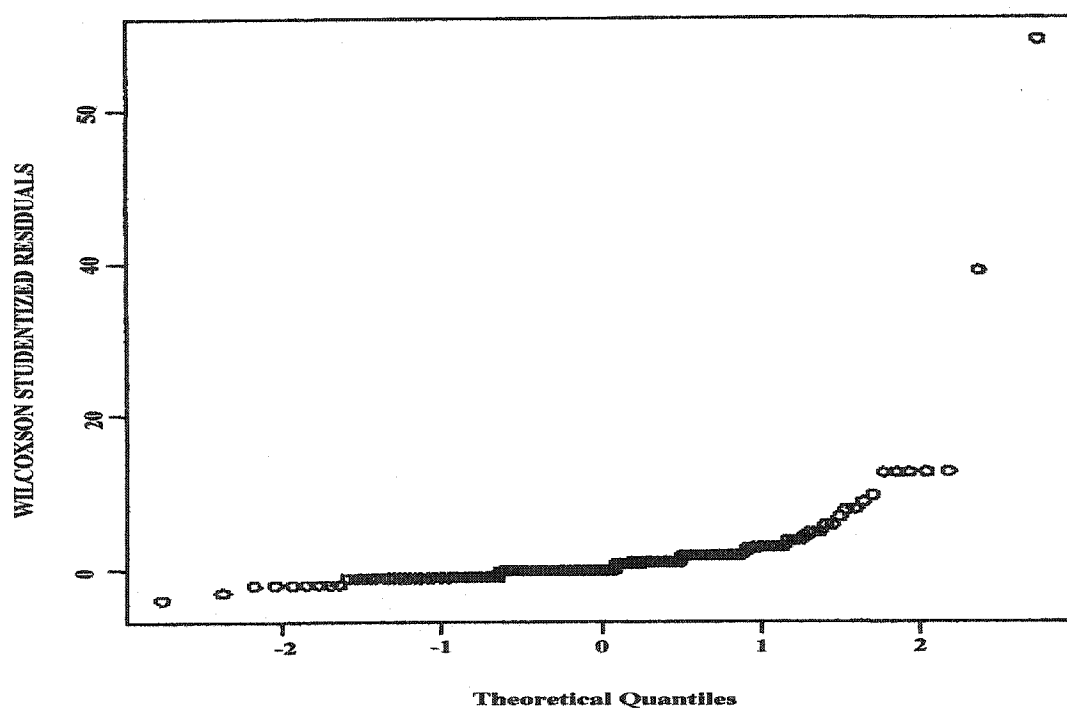


Figure 29. TUNEL Q-Q Plot of Studentized Residuals.

Table 5

TUNEL - Comparison of Tests of Time and Treatment Effects and Interactions

Effects and Interaction	Wilcoxon Robust			Least Squares		
	F-Statistic	df	p-Value	F-Statistic	df	p-Value
Time Intervals	-5.11573	2,144	1	2.89421	2,144	0.055688
Treatments	0.448136	7,144	0.87013	1.05021	7,144	0.398994
Time Intervals /Treatment	0.589617	14,144	0.0869931	1.28157	14,144	0.225583

Table 6

TUNEL - Adjusted Comparison of Tests Time and Treatment Effects and Interactions

Effects and Interaction	Wilcoxon Robust			Least Squares		
	F-Statistic	df	p-Value	F-Statistic	df	p-Value
Time Intervals	1.53316	2,144	0.219355	2.87527	2,144	0.0596451
Treatments	3.0108	7,144	0.00555927	1.05284	7,144	0.397222
Time Intervals /Treatment	1.96045	14,144	0.0248168	1.28226	14,144	0.225149

Table 7

TUNEL - Fisher's Paired Least Significant Differences With Statistical Significance

Wilcoxon Robust Contrasts*			
Contrast	Estimate	Error Term	Interval
No Treatment vs BZ Low Dose	-1.96798	0.759272	(-2.72726, -1.20871)
Ethanol Control vs BZ Low Dose	-1.32372	0.759272	(-2.08299, -0.56445)
2AF Low Dose vs BZ Low Dose	-1.66739	0.759272	(-2.42667, -0.90812)
2AF High Dose vs BZ Low Dose	-1.68261	0.759272	(-2.44188, -0.923338)
BZ Low Dose vs BZ High Dose	1.99555	0.759272	(1.23628, 2.75483)
BZ Low Dose vs 2AF/BZ Low Dose	1.67372	0.759272	(0.914451, 2.433)



Table 7 – Continued

Wilcoxon Robust Contrasts			
Contrast	Estimate	Error Term	Interval
BZ Low Dose vs 2AF/BZ High High Dose	2.01209	0.759272	(1.25282, 2.77136)

\*95% Individual Confidence Intervals

statistically significant as was their interaction with time, but that time alone did not have a statistically significant effect on the number of TUNEL positive cells.

Further analysis was conducted for determining differences between time intervals and between treatment groups. The results are reported in Table 7. Using Fisher's protected least square differences, no significant contrasts were observed between time intervals. No significant contrasts were observed between the no treatment control and EtOH vehicle control. Statistically significant contrasts were observed between the EtOH vehicle control and low dose of BZ, but not with the remaining treatment groups.

#### PCNA Index Minus TUNEL Index

The difference in cellular proliferation and death results in net cellular growth. To determine whether cellular proliferation exceeds cellular death in carcinogenesis, studies have examined the difference between PI and TI [432-434]. This difference is imperative in carcinogenic investigations since a fine balance between positive and

negative regulation of cell growth is essential in normal development and growth, whereas a disruption in this balance during carcinogenesis results in tumor formation [435]. In this study the differences between cellular proliferation (PI) and cellular death (TI) were examined and exhibited a combined mean of 5.9 for the control groups and 27.65 for the treated groups. These results are indicative of positive cellular growth, with treatment groups producing a 4.7 fold increase in cellular growth over the controls. A list of the means for the individual control groups and treatment groups are located in Table 2. Results from the differences between PI and TI contained expected outliers as demonstrated in the boxplot of Wilcoxon studentized residuals in Figure 30. This indicates that the data did not have a normal distribution. Additionally, the Q-Q plot of studentized residuals in Figure 31 indicates a lack of a linear response. Therefore, the non-parametric robust Wilcoxon 2-way analysis of time and treatment effects and interaction was used for statistical analysis. Results from this analysis are listed in Table 8. Both time intervals and treatments along with their interaction were highly statistically significant with p-values of  $3.16 \times 10^{-6}$ , 0, and  $2.85 \times 10^{-5}$  and F-statistics of 13.84, 8.67, and 3.69, respectively. Contrasts, listed in Table 9, indicate significant differences between 4 wks and 12 wks, and between 8 wks and 12 wks. No significant contrasts were noted between the no treatment control and the EtOH vehicle control. Significant contrasts existed between the EtOH vehicle control and all treatment groups except the 2AF low dose. The greatest contrast between control and treatment was with the 2AF/BZ low dose combination.

## Discussion

A hallmark of tumor formation is unregulated cell growth. This is evidenced in numerous studies examining the proliferative rates of neoplastic tissue. Several PCNA immunoreactivity studies have been conducted on human livers exhibiting chronic liver diseases and hepatocellular carcinomas [193, 195, 196, 199, 434, 436-438]. Normal liver tissue exhibited a minimal PCNA levels, while hepatocellular carcinomas (HCC) exhibited increasing PCNA levels that correspond with Grade. For example, one study indicated the PCNA levels in HCC Grades 1, 2, 3 and 4 were 12.2%, 17.5%, 53.7% and 83.9%, respectively [438]. In another study, normal liver tissue resulted in PCNA levels of 0.78%, while the HCC of Edmondson's type I, II and III revealed PIs of 4.83%, 6.65% and 30.6%, respectively. This PI increase is also reported in progressive chronic liver diseases, where PI increases were observed as the disease state progresses until the stage of cirrhosis, where it decreased [437]. In the fish, *Fundulus grandis*, the potent carcinogens 2AF and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were administered and the PCNA levels were assayed. Exposed fish had statistically significant elevated liver PCNA levels in both exposure groups ( $p = 0.003$ ). Studies with rats exposed to 2AAF as an initiator, promoter, or both, demonstrate apoptotic cell death, regenerative proliferation, morphological alterations and tumor formation [439-445]. This progression has also been observed in azo-based dye exposures [439-441]. Although cellular proliferation is a major facet in tumor development, it is also important to consider the regulation of cell growth and cell death. Reductions in the rate of cell death can result in increased cellular growth rates in the absence of proliferative changes [435].

Since *G. affinis* is an excellent sentinel species for biomonitoring of environmental carcinogens, we investigated the effects of the arylamine metabolic class of carcinogens on proliferation in their livers. The arylamines, 2AF and BZ have been shown to form hepatic neoplastic lesions in several rodent models tested, including 2AF in the fish model, *Fundulus grandis* [342, 343, 446-452].

To examine the early cellular growth patterns preceding neoplastic formation resulting from exposure to 2AF and/or BZ in *G. affinis*, both proliferation and death of liver cells were assayed after 4, 8, and 12-week exposure intervals. The working hypothesis is that an increase in cellular proliferation and/or a decrease in cellular death would occur due to arylamine exposure in the early stages of their neoplastic development as has been observed in rodents. Cell proliferation was assessed using PCNA antibody reactivity, cell death using the TUNEL method, and cell growth by the differences in PI and TI. Results of the PCNA immunohistochemistry assay indicated significant increases in proliferation that were associated with exposure intervals, treatments and their interaction, Table 3. Although significant increases were observed in the higher doses of BZ and 2AF/BZ, Table 4, increases were most pronounced for the low doses of BZ and its combination 2AF/BZ, along with 2AF at the higher dose. The proliferative effects of 2AF at 0.069mM (1 ppm) may be too gradual for statistical significance, since the means increased substantially from the 4 and 8 wk points to 12 wks, Table 2. This was observed in rats treated with 50 ppm 2AAF where foci developed much more gradually than at higher doses [442]. With regard to TUNEL detection for assaying cell death, treatments were statistically significant as was their interaction with time, Table 6. The only increase with statistical significance was demonstrated for the

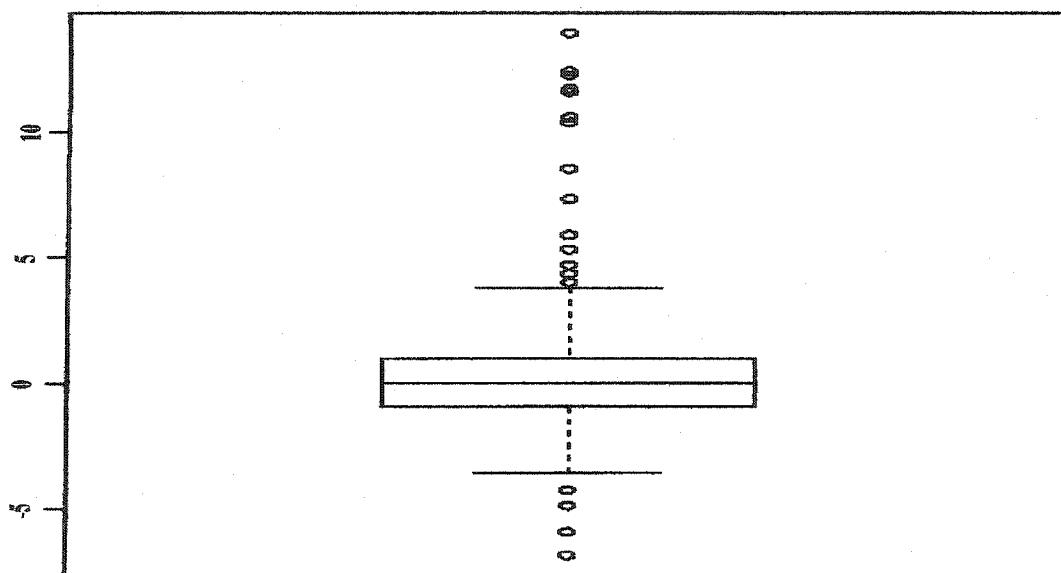


Figure 30. PCNA and TUNEL Differences Boxplot of Wilcoxon Studentized Residuals.

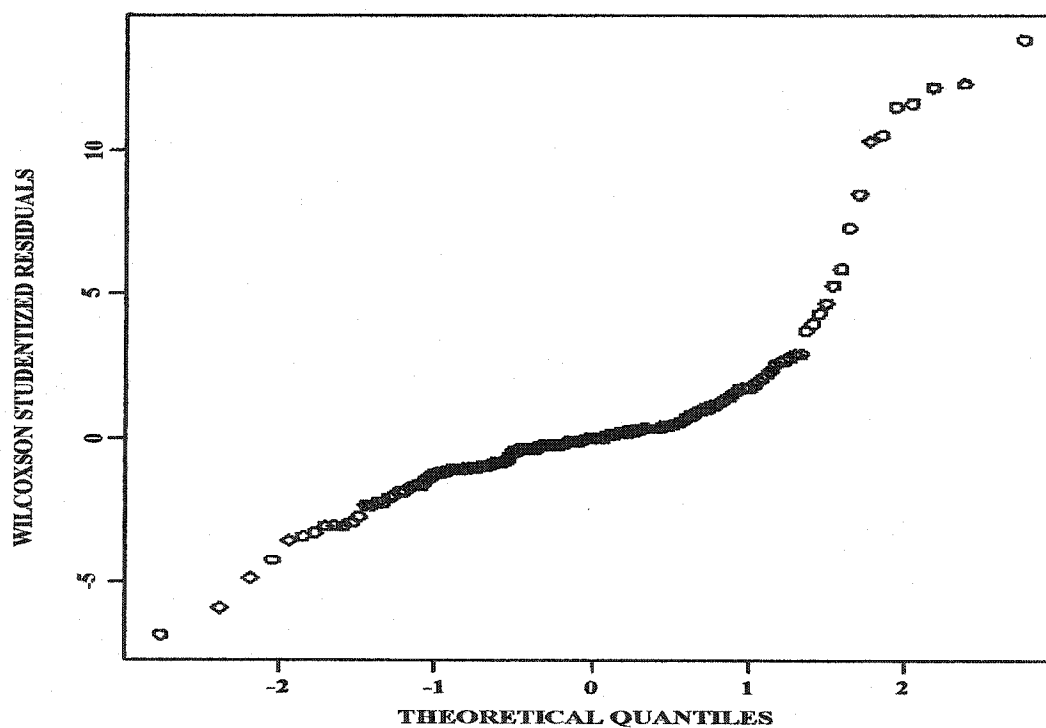


Figure 31. PCNA and TUNEL Differences Q-Q Plot of Studentized Residuals.

Table 8

PCNA and TUNEL Differences - Test of Time and Treatment Effects and Interaction

Wilcoxon Robust			
Effects and Interaction	F-Statistic	df	p-value
Time Intervals	13.8364	2,144	3.15905e-06
Treatments	8.67168	7,144	0
Time Intervals/Treatment	3.6933	14,144	2.8491e-05

Table 9

PCNA and TUNEL Differences - Fisher's Paired Least Significant Differences With Statistical Significance

Wilcoxon Robust Contrasts*			
Contrast	Estimate	Error Term	Interval
4 Wks vs 12 Wks	-12.8567	5.34615	(-18.2029, -7.51055)
8 Wks vs 12 Wks	-14.4132	5.34615	(-19.7594, -9.06706)
No Treatment vs 2AF High Dose	-22.965	8.73023	(-31.6952, -14.2347)
No Treatment vs BZ Low Dose	-22.3636	8.73023	(-31.0938, -13.6333)
No Treatment vs BZ High Dose	-16.3462	8.73023	(-25.0764, -7.61594)
No Treatment vs 2AF/BZ Low Dose	-23.2858	8.73023	(-32.016, -14.5556)

Table 9 – Continued

Wilcoxon Robust Contrasts			
Contrast	Estimate	Error Term	Interval
No Treatment vs 2AF/BZ High Dose	-13.9874	8.73023	(-22.7177, -5.25721)
Ethanol Control vs 2AF High Dose	-22.3341	8.73023	(-31.0644, -13.6039)
Ethanol Control vs BZ Low Dose	-21.7327	8.73023	(-30.463, -13.0025)
Ethanol Control vs BZ High Dose	-15.7154	8.73023	(-24.4456, -6.98513)
Ethanol Control vs 2AF/BZ Low Dose	-22.655	8.73023	(-31.3852, -13.9248)
Ethanol Control vs 2AF/BZ High Dose	-13.3566	8.73023	(-22.0869, -4.6264)
2AF Low Dose vs 2AF High Dose	-16.7878	8.73023	(-25.518, -8.05758)
2AF Low Dose vs BZ Low Dose	-16.1864	8.73023	(-24.9166, -7.45619)
2AF Low Dose vs BZ High Dose	-10.169	8.73023	(-18.8993, -1.4388)
2AF Low Dose vs 2AF/BZ Low Dose	-17.1087	8.73023	(-25.8389, -8.37843)
2AF High Dose vs 2AF/BZ High Dose	8.97751	8.73023	(0.247284, 17.7077)
2AF/BZ Low Dose vs 2AF/BZ High Dose	9.29836	8.73023	(0.568134, 18.0286)

\*95% Individual Confidence Intervals

low dose BZ exposure over the 4, 8 and 12 wk exposures when contrasted with the controls. The controls were not significantly different from one another in all assays.

This early transient increase in cell death was also observed in the rodent studies. This early cell death and subsequent increase in proliferation is the subject of several studies into the early proliferative effects of 2AAF in rats [442, 444, 453-456]. Evidence is accumulating that 2AAF induces a stress response where apoptosis is triggered by altered mitochondrial permeability transition effects and a compensatory regenerative proliferation follows as part of an adaptive response [442]. One of the most sensitive parameters for this adaptive response was the inhibition of mitochondrial permeability transition pores that are involved in inducing apoptosis. They exhibited 40% to 100 % inhibition by 8 wks in a dose-dependent manner [442, 457].

To determine whether the cellular proliferation exceeds cell death, the differences between the PI and TI were examined. The difference between PI and TI revealed the low dose of 2AF/BZ as the most statistically significant, while the high dose of 2AF and low dose of BZ followed in significance, Table 9. This indicates the net cellular growth of the arylamine exposed groups is positive and increased when compared to controls. This aligns with the increased proliferative state and decreased apoptosis in livers of rodents when exposed to 2AAF [442, 444, 453, 454]. Although the differences in PI and TI were closely aligned, the order of significance for contrasts changed, Tables 4 and 9. These results indicate that when examining cell proliferation alone or cell death alone, the results can be somewhat misleading with regard to net cellular growth. Studies examining tumor growth and apoptosis suggest that failure to consider apoptosis in cell growth may account for the discrepancies between the actual and expected growth of tumors calculated from cell proliferation rates [435, 458]. Thus, when taking into



account the balance of proliferating cells and dying cells, whether apoptotic or necrotic, a more precise measurement of cellular growth is achieved.

With that considered, this study demonstrates the statistical significance of the polycyclic arylamines, 2AF and BZ and their combination in overall cellular growth in *G. affinis* livers. Effects from their exposure indicate increased net cellular proliferation in all exposures except the low dose of 2AF. When cells are dividing the potential for neoplastic formation also increases. This was demonstrated in rat hepatocytes and human fibroblasts where clastogenesis was shown to be cell cycle dependent and maximally susceptible when cells were at the G1/S border [160]. The studies conducted on rats revealed an early increase in cell death (2 weeks) followed by a delayed increase in cell proliferation (4 weeks) and subsequent tumor formation with exposure to 50, 100, 200, 400, and 800 ppm 2AAF [442, 453]. *G. affinis* exposed to 2AF and BZ revealed an early increase in cell death at 4 weeks than diminishes at 8 and 12 weeks with increased proliferation at 4 weeks that is significantly increased by 12 weeks. The results from this investigation with both 2AF and BZ at 0.069mM (1 ppm) and 6.9mM (100 ppm) are aligned with results from the rat studies and *Fundulus grandis* study. The 2AF study on the fish *Fundulus grandis* reported significant differences in PCNA index, and PCNA/TUNEL ratio with slight increases but not statistically significant differences in TUNEL index during exposure to 6.9mM (100 ppm) 2AF [199].

The results from this study additionally indicate the utility of PCNA and TUNEL immunohistochemistry for the detection of net cellular proliferation due to exposure of environmental carcinogens in field studies using sentinel species such as *G. affinis*. So far, the two metabolic classes of environmental carcinogens that have been tested on *G.*

*affinis* result in increased proliferation that can be detected and differentiated from non-exposed groups [27, 199].

## CHAPTER V

### HISTOPATHOLOGY OF 2-AMINOFLUORENE AND BENZIDINE TREATED *G. AFFINIS* LIVERS

#### Purpose of Study

The objective of histological studies in *G. affinis* liver tissue sections is to determine any gross morphological differences between treatment groups and controls during the 4, 8 and 12-week time intervals. The major morphological features examined here are preneoplastic proliferative states, formation of foci and neoplastic formations. These features are important in carcinogenic studies, as they represent the fundamental changes that occur in tissues when exposed to environmental carcinogens. Histological studies of azo-based dyes and 2AAF exposure in rodents have reported proliferation of oval cells in livers with in the early stages of chemically induced carcinogenesis and subsequent tumor formations that are related to this altered state [440, 447, 459-462]. In this study, a well-established protocol of hematoxylin and eosin (H&E) staining are used to discriminate normal and morphologically altered foci.

#### Experimental Design

The experimental design is basically identical to the experimental design in Chapter IV with regard to preparation, dosing and administration of 2AF and BZ, and processing of *G. affinis* livers. Slides of the liver tissue sections are stained with H&E and examined under a microscope at 40 X for morphological aberrations. The tissue sections are stained with hematoxylin for nucleic acids and eosin for protein. H&E

staining has been used extensively in studies to identify morphological features such as apoptosis, infections, chromatin texture and malignant melanomas just to name a few [463-467]. The morphological features of previous cytological studies on the liver of *G. affinis* are used as guidelines in the histological analysis [27, 380, 468]. Diagnoses of altered cellular foci and tumor formation are verified by an ACVP certified pathologist experienced in *G. affinis* liver histology.

Additionally, two groups of the PCNA stained slides, the EtOH vehicle controls and the 4 week BZ low dose, were re-examined using DIC microscopy to determine the morphological characteristics of the PCNA positive foci. This was to verify whether the foci observed in the PCNA positive focal regions had the same morphological characteristics as those observed in the H&E stained sections.

### Methods and Materials

The fish were maintained and housed as stated in Chapter III. Processing of fish and their livers for preparation of serial tissue sections are described in Chapter IV in its corresponding sections.

Tissue section slides were placed in glass slide racks (20 slides/rack) and immersed in the appropriate reagents in staining jars for H&E staining. Slides were deparaffinized in xylenes, 2 X for 3 mins each, then hydrated in 100% EtOH, 15 dips, 95% EtOH, 15 dips and 70% EtOH, 10 dips, then rinsed for 3 min in running tap water. Slides were stained with Mayer's modified hemotoxylin (Newcomer Supply, Middleton, WI) for 5 min, rinsed in running tap water for 2 min and blued in PBS-T with 0.5% ammonium acetate (Sigma Aldrich, St. Louis, MO) for 1 min. Sections were

counterstained with eosin (Sigma Aldrich, St. Louis, MO) for 3 min and rinsed in running tap water for 2 min. Slides were then dehydrated in 70% EtOH, 3 dips, 95% EtOH, 5 dips, 100% EtOH, 2 X 3 min each, and xylenes, 2 X 3 min each. Coverslips were adhered to slides with Cytoseal XYL mounting media (VWR Scientific, South Plainfield, NJ).

## Results

Livers of *G. affinis* and other teleost fish are not lobular in architecture. Instead their liver architecture is an arrangement of tubules of hepatocytes in which the tubules are composed of 5-7 hepatocytes in a concentric arrangement [469]. Histopathological examination of H&E stained *G. affinis* liver tissue in both the controls and treated groups exhibited varying degrees of vacuolation. Vacuolation was much more prominent in the PAA exposed groups, than in the controls. They typically appeared as clear intracytoplasmic vacuoles and were distributed throughout the section. Pigmented macrophage aggregates (PMA) were observed in both the controls and treated groups, but were more frequently observed in the treated groups. Granulomas were identified in both controls and treated groups, however they were rare by comparison in the controls. Although difficult to differentiate at times, granulomas contained densely packed pigmented macrophages encapsulated by small basophilic nuclei in epithelial appearing cells. An example of PMA and granuloma can be seen in Figure 32, the upper arrow points to spindle cell proliferation, the middle arrow to a granuloma and the lower arrow to a PMA. Both PMA and granulomas are inflammatory lesions and may be due to

Table 10

Histopathological Examination of *G. affinis* Lives After Exposure to 2AF and BZ

Treatment Group	Time (Wks)	Oval to Spindle Shaped Cell Altered Foci	Spindle Cell Tumors
No Treatment Control	4	0/6	-
	8	1/7	-
	12	1/6	-
EtOH Vehicle Control	4	0/7	-
	8	1/6	-
	12	1/7	-
0.069 mM 2AF	4	3/6	-
	8	3/6	-
	12	2/6	-
6.9 mM 2AF	4	1/5	-
	8	3/7	-
	12	3/6	-
0.069 mM BZ	4	2/7	-
	8	2/7	1/7
	12	3/7	-
6.9 mM BZ	4	1/7	-
	8	1/7	-
	12	3/7	-
0.069 mM 2AF/BZ	4	0/6	-
	8	2/7	-
	12	3/6	-
6.9 mM 2AF/BZ	4	4/7	-
	8	2/7	-
	12	2/6	-

an undiagnosed infection or due to phagocytosis of cellular debris resulting from necrosis or apoptosis. No indication of parasitic invasion was observed, however, microbial infection and phagocytosis of cellular debris cannot be ruled out.

The appearance of altered cellular foci occurred predominately in the treated groups, although 2 cases were observed in each of the control groups. Table 10 lists the number of the oval to spindled shaped basophilic duct-like cells comprising the altered foci and tumor observed in the treated groups of *G. affinis*. The areas of altered foci were small, basophilic clusters of oval to spindle shaped cells typically located by nearby vessels and without compression of surrounding tissue. These basophilic foci of spindle cell proliferation are shown in Figures 32 and 33, bottom arrow and only arrow, respectively. These cells had enlarged nuclear to cytoplasmic ratios and were deeply basophilic in comparison with adjacent parenchyma. These foci differed from the appearance of the parenchyma and bile duct cells of the control livers. The normal parenchyma, hepatocytes, were much larger cells with smaller nuclear to cytoplasmic ratios. The normal bile duct cells were arranged as a single row of small basophilic cells in a circular or tubular fashion, whereas the cells contained in the basophilic foci, were not uniformly distributed as a single layer of concentric cells. Instead, they appeared as varying numbers of cells in close proximity to one another without a duct-like structure. To determine whether these were also the cells observed in the PCNA positive focal regions of the PCNA stained sections, two representative groups, the 4 week EtOH vehicle control and BZ low dose group, of PCNA stained sections were observed using DIC microscopy. The PCNA positive focal regions were selected on these sections, there

morphology was examined. The morphology of the PCNA positive focal sites in the treated groups revealed a higher nuclear to cytoplasmic ratio with oval to spindle shaped cells that were smaller and more condensed than the surrounding parenchyma. This indicates that these oval to spindle shaped cells in the basophilic foci were proliferating in the foci examined. The ratio of PCNA positive cells to total cells counted in two focal sites for the 4 weeks EtOH vehicle controls totaled 13 PCNA positive cells/75 cells total and their morphology had lower nuclear to cytoplasmic ratios. This same value for the 4 week BZ low dose was 83 PCNA positive cells/657 cells total in 12 focal sites. Previous exposure studies have indicated the occurrence of these small basophilic foci in fish after exposure to MAM-Ac, diethylnitrosamine (DEN), or creosote [27, 365, 470]. These areas of basophilic altered cellular foci occurred in 0%, 15% and 15% of controls and 29%, 32% and 42% of treated fish at 4, 8 and 12 weeks, respectively. By treatment group, these foci appeared in 42% of 2AF groups, 29% of BZ groups and 33% of 2AF/BZ groups. Only one tumor was observed and this was a spindle cell tumor in the 0.069 mM BZ group at 8 weeks.

## Discussion

The results from histological examination of *G. affinis* livers after exposure to 2AF, BZ and their combination at low and high doses are similar to those studies of azo-based dyes and 2AAF exposure in rats, mice and fish [27, 440, 450, 453, 471, 472]. Those studies have all indicated an increase in oval cell (bile duct) proliferation after exposure and subsequent formation of tumors. In this study, 10% of the control groups exhibited basophilic oval/spindle cell proliferation. That was substantially



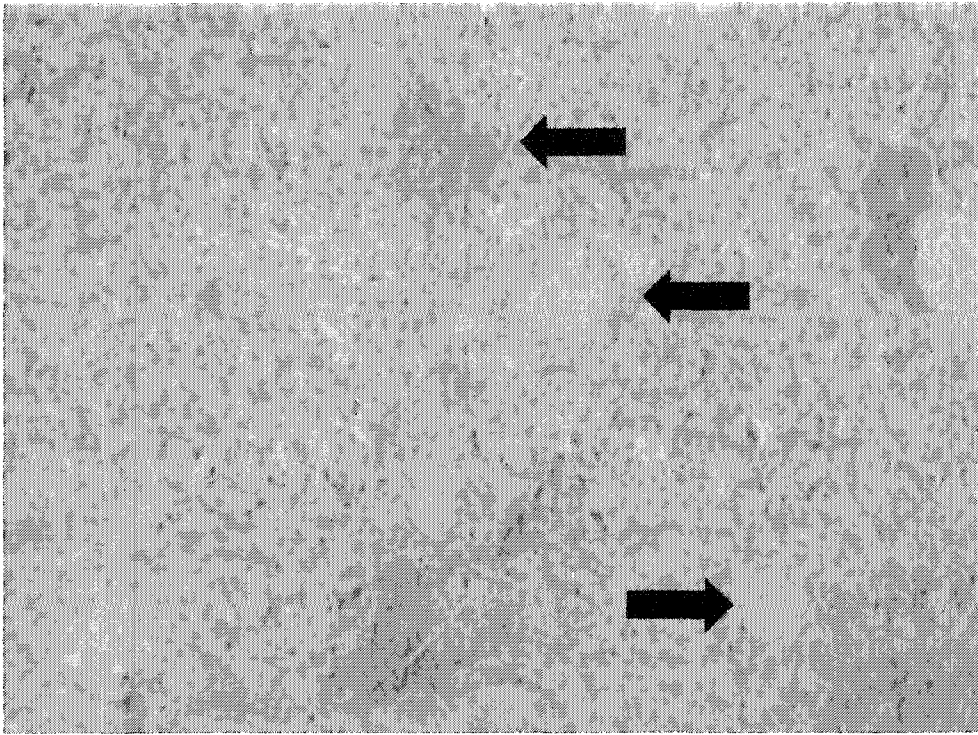


Figure 32. Altered Foci, Granuloma and PMA, respectively, in *G. affinis* Liver.

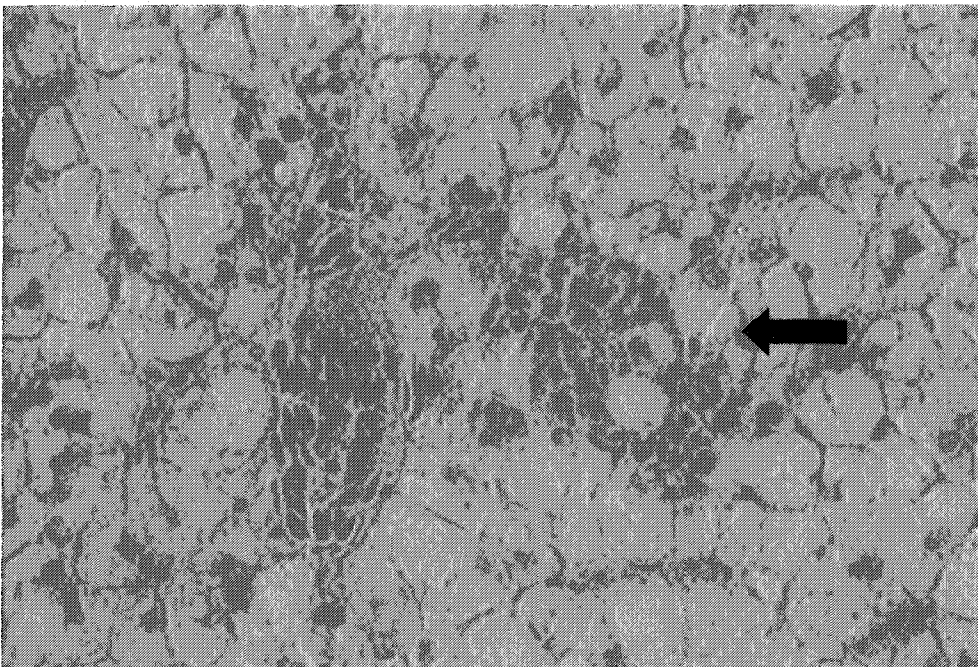


Figure 33. Basophilic Foci of Spindle Cell Proliferation in *G. affinis* Liver.

increased in the treated groups anywhere from a 2.9 to 4.2 fold over the controls. The 2AF exposure groups had the greatest incidence of basophilic altered cellular foci, occurring in 42% of the 2AF hepatic tissue examined, followed by 33% of 2AF/BZ and 29% of the BZ. Although these were early studies, a spindle tumor was observed in an 8 week 0.069 mM BZ exposed liver.

A great deal of experimentation has taken place with livers and hepatocytes in determining if these areas of altered cellular foci comprised of bile duct like oval cells are the preneoplastic origin of subsequent neoplasms that are consistently observed during exposures to azo-based dyes and 2AAF [27, 440, 450, 453, 456, 471-474]. A large array of antibodies to hepatocytes, oval cells/bile duct, shared-hepatocytes and bile ducts, and neoplastic antigens in F-344 rats were assayed in primary hepatocellular carcinomas induced by exposure to a single dose of *N*-nitrosodiethylamine (DENA) followed by 14 days of 2.5 mg 2AAF exposure. Their results indicated that the antigenic phenotype expressed in 20% of the primary hepatocellular carcinomas (PHC) contained both oval cell- and hepatocyte-associated antigens. This was not observed in normal livers where hepatocytes expressed hepatocyte-associated antigens and bile duct cells expressed oval cell-associated antigens only. These same antigens were also detected in foci and nodules, but in lesser amounts [445]. Several other studies have also shown the antigenic properties of both oval cells and hepatocytes in hepatocellular carcinomas and tumors [471, 475, 476]. In *G. affinis*, these basophilic bile duct-like oval cells were the only type of altered foci observed. While there was only one tumor observed in this study, it was a basophilic spindle cell tumor. This is aligned with the growing evidence that

proliferation of these oval cells and their subsequent formation of foci may be a precursor in PHC, tumors, and nodules.

Investigations into the early proliferative state of these oval cells upon exposure to 2AAF, have revealed that although it is a regenerative process resulting from a adaptive response to earlier cell death, this compensatory regeneration is different from those induced by partial hepatectomy. Hepatocytes proliferating from a partial hepatectomy do not involve intra- or extra-hepatic stem cells, only hepatocytes, while intra-hepatic precursor cells (oval cells) proliferate and generate lineage only when hepatocyte proliferation is blocked or delayed as seen with 2AFF [461]. 2AAF was shown to inhibit proliferation of non-initiated hepatocytes by blocking cells in the G1 phase, suggesting a direct toxic effect [472]. Here, significant increases in oval cell proliferation were observed in the 2AF and BZ exposed groups. This is reflective of compensatory proliferation of oval cells rather than normal regeneration of hepatocytes. Further evidence of differential proliferation of cells during chemical exposure was demonstrated in a study with 2AAF that induced a mitogenic response in ductal and nondescript periductular cells within 24 hrs after administration. Levels of three transcription factors and two genes regulated by these factors were assayed and 2AAF exposed livers had increased expression of two of the transcription factors and both of the genes they regulate. In contrast, the bile duct proliferation induced by bile duct ligation, did not affect the levels of any of these factors assayed [462]. Therefore, based on these previous studies, the results from arylamine exposure in *G. affinis* livers are closely aligned with those in mammals where early, reversible cell death is followed by proliferation of

oval cells resulting in altered cellular foci of basophilic spindle/oval cell composition and ultimately tumor formation.

## CHAPTER VI

### CONCLUSIONS

A need exists for testing environmental carcinogens in small aquatic species.

Small fish species are well suited to act as sentinels for monitoring of harmful chemicals in ponds, streams, lakes, etc. Typically aquatic ecosystems are monitored by collecting water or sediment samples from specific sites and evaluating the samples for acute toxicity. However, this doesn't reflect the dynamic change occurring in aquatic ecosystems that organisms encounter such as tide, temperature and sediment disturbance that may modify exposure effects [477]. Additionally, the demonstrated capacity for neoplastic development in aquatic species is useful in comparison studies for investigating common mechanisms in carcinogenesis [29, 360, 470]. For example, exposure studies with diethylnitrosamine (DEN) have demonstrated neoplasms in Medaka and Mangrove rivulus along with increases in the expression of Fos, Ras, Myc and p53 cancer related genes [361, 478]. Other chemicals such as MAM-Ac have induced tumor formation in *G. affinis*, and 2AF and MNNG induced tumors in *Fundulus grandis* [27, 199]. Another aspect is the ease in housing in large numbers of small fish with minimal expense when compared to traditional rodent models. This is important for both fiscal limitations and statistical significance.

Here, *G. affinis* was selected as the small fish species because they develop neoplasms in response to carcinogen exposure, are geographically diverse, tolerate a wide range of waters from optimal to poor, reside in a localized vicinity and are easy to house and maintain [35, 377, 468, 479-482]. *G. affinis* form neoplasms in response to the

alkylating metabolic class of carcinogens, however more metabolic classes need to be examined [27]. To test the utility of the *G. affinis* in carcinogenesis studies, molecular tools were investigated for use in assaying the effects of exposure to the PAA metabolic class of carcinogens. The PAA's examined here are the much-studied 2AF and BZ. Both azo-based dyes, of which BZ is a component, and 2AAF, a metabolite of 2AF, exert non-genotoxic and genotoxic effects in the livers of rodents. They inhibit the proliferation of non-initiated hepatocytes and enhance the proliferation of oval cells. This ultimately leads to an altered liver architecture and subsequent tumor formation [119, 445, 454, 457, 461]. 2AF, has also been shown to induce proliferation in the small fish species, *Fundulus grandis* [199].

Therefore, the molecular tools investigated here are antibodies against antigens associated with cellular proliferation and programmed cell death. These antibodies were tested for specificity in *G. affinis*. Cellular proliferation was represented by the PCNA antibody and various stages of apoptosis by p53 and cleaved caspase-3 and PARP antibodies as discussed in their literature reviews. However, in order to test the specificity of these antibodies on *G. affinis* proteins, non-transformed, proliferating hepatocytes induced to undergo apoptosis, with no or minimal necrosis, are necessary for use as internal controls. The AML12 line of epithelial-like proliferating hepatocytes was tested for this purpose using camptothecin for apoptosis induction. Results from flow cytometric analysis, fluorescence microscopy and DIC microscopy demonstrated that this proliferating line of hepatocytes could be induced to undergo apoptosis with negligible necrosis. This allowed for the use of the AML12 hepatocytes as internal controls for

western blot analysis of PCNA, p53 and cleaved caspase-3 and PARP antibody specificity to *G. affinis*.

The antibodies were tested using western blotting with protein extracts from carbon tetrachloride treated and control *G. affinis*. The results of this analysis indicated specificity of the PCNA antibody for *G. affinis* PCNA. Unfortunately, western blots were inconclusive for the remaining antibodies, p53 and cleaved caspase-3 and PARP. While the PCNA antibody is sufficient for detection of proliferation, detection of cellular death would require the use of a different biomarker. Therefore, the TUNEL technique was tested. TUNEL labels DNA strand breaks associated with apoptosis, however, it has also been demonstrated to detect necrosis [291-294]. Testing of the TUNEL technique was successful and was employed in this study to measure cellular death.

To test the effects of 2AF and BZ exposure on cellular proliferation and death in *G. affinis*, doses of 0.069 mM and 6.9 mM individually or combined was added in their diets for time intervals of 4, 8 and 12 weeks. Liver tissue sections were labeled individually with PCNA antibody, TUNEL, and H&E. Results revealed a slight transient increase in cell death at 4 weeks that decreased in weeks 8 and 12. Cellular proliferation was increased at all time intervals, especially by week 12. H&E staining revealed substantially increased proliferation of basophilic oval to spindle shaped bile duct-like cells. These same basophilic oval cells were also observed in altered areas of cellular foci. All treatment groups had significant increases of the altered foci. At 8 weeks a basophilic spindle cell tumor had formed in the low dose BZ group.

Comparisons of these results with those in mammalian studies of arylamines are aligned and may have common mechanisms. Investigations into arylamine

carcinogenicity have established an early increase in apoptotic and/or necrotic cell death that decreases shortly thereafter to normal levels as was observed in *G. affinis*. In rodents, this is in response to a disruption of the biochemical homeostasis in the liver by 2AAF metabolites as evidenced by the number of changes in carbohydrate metabolism and oxidative phosphorylation enzyme activity levels [442, 444, 457]. The apoptotic response alters the activation of the mitochondrial permeability transition pores that induce apoptosis. At first they are activated, and shortly thereafter, inhibited during the early stages of 2AAF carcinogenesis [455]. In an adaptive response to the loss of cells during this early stage, regenerative proliferation takes place. However, this regenerative proliferation is not like the type that occurs during partial hepatectomy (PH). PH results in the proliferation of normal hepatocytes, whereas, 2AAF results in the proliferation of bile duct-like oval cells and the inhibition of normal hepatocyte proliferation [442, 445, 454, 461]. This compensatory regeneration alters the normal architecture of the liver. It has been hypothesized that the altered architecture of the liver is a prerequisite for the expansion of initiated foci. This is based on the proliferation of bile duct-like cells that produce reticulin fibers and disrupt microcirculation, along with resistance of mitochondrial permeability transition pore activation. Also, an increased proportion of GST-P-positive foci begin appearing at a time when the liver reaches a certain degree of alteration [444, 453, 455].

In summary, 2AF and BZ exposed *G. affinis* produce a mammalian-like response to 2AF and BZ exposure with varying degrees of the same response. All treatment groups had a greater proliferative response and only a transient death response that resulted in positive net cellular growth. More oval cell proliferation, altered foci and



tumor formation occurred in the treated groups when compared with the controls. These results are much like the results of numerous studies of 2AAF and azo-based dye exposure in rodents and humans. This study further advances the potential utility of *G. affinis* as an excellent sentinel species candidate for biomonitoring of environmental carcinogens in aquatic ecosystems. The use of PCNA antibodies and TUNEL method are excellent biomarkers for cellular proliferation and death. Future investigations using varying metabolic classes of carcinogen and non-genotoxic chemical exposures to *G. affinis*, will aid in the advancement of their biomonitoring potential. Additionally, examination of cellular death at earlier time points and foci and tumor formation at later time points will further define the carcinogenic process in this species. In retrospect, the use of whole mounts rather than individual organs, and additional screening of molecular tools would be beneficial in future toxicology and carcinogenesis studies with *G. affinis*.

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## APPENDIX A

### Institutional Animal Care and Use Committee Approval Form

Date Received 12-18-00  
 IACUC Number 99-06-02  
 First Renewal Request /  
 Second Renewal Request \_\_\_\_\_

WESTERN MICHIGAN UNIVERSITY  
 YEARLY RENEWAL FORM APPLICATION TO USE  
 VERTEBRATE ANIMALS FOR RESEARCH OR TEACHING

GENERAL INFORMATION: Fill in all appropriate information

Jay C. Means Chemistry 7-2923  
 Principal Investigator/Instructor Department Campus Phone

\_\_\_\_\_  
 Co-Principal/Student Investigator Department Campus Phone

Title of Project/Course Investigation of point mutations in Gambusia affinis

PRINCIPAL INVESTIGATOR/INSTRUCTOR DECLARATION

I assure that I have obtained IACUC approval prior to implementing this project and that there are no changes in the protocol submitted in the original application to use vertebrate animals for research or teaching. I understand that if at any time changes are made in the use of animals as described in the original application, a letter or amended protocol must be filed for review. I assure that the activities do not unnecessarily duplicate previous experiments.

Signatures:

Jay C. Means 12-4-2000  
 Principal Investigator/Instructor Date

Susan 12-4-2000  
 Co-Principal/Student Investigator Date  
 (If PI not a faculty member)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

[Signature] 12/5/00  
 IACUC Chairperson Date

PLEASE MAIL COMPLETED APPLICATION TO:

Research Compliance Coordinator  
 Western Michigan University  
 327E Walwood Hall  
 Kalamazoo, MI 49008  
 (616) 387-8293

IAC-E

## APPENDIX B

### Individual TUNEL Index, PCNA Index and (PI – TI) Index Counts

Time and Treatment	TUNEL Index	PCNA Index	(PI - TI) Index	Time and Treatment	TUNEL Index	PCNA Index	(PI - TI) Index
4N	0	0	0	4ABL	0	7	7
4N	6	6	0	4ABL	1	54	53
4N	2	0	-2	4ABL	2	22	20
4N	2	0	-2	4ABL	3	22	19
4N	0	3	3	4ABL	1	3	2
4N	1	0	-1	4ABL	1	41	40
4N	2	2	0	4ABL	14	4	-10
4E	0	1	1	4ABH	2	12	10
4E	2	9	7	4ABH	0	13	13
4E	2	4	2	4ABH	1	9	8
4E	0	0	0	4ABH	0	15	15
4E	1	3	2	4ABH	1	29	28
4E	1	19	18	4ABH	7	22	15
4E	0	1	1	4ABH	3	9	6
4AL	15	0	-15	8N	0	0	0
4AL	1	14	13	8N	10	2	-8
4AL	1	5	4	8N	14	4	-10
4AL	73	55	-18	8N	4	2	-2
4AL	1	2	1	8N	0	3	3
4AL	1	11	10	8N	0	3	3
4AL	15	13	-2	8N	1	19	18
4AH	2	9	7	8E	6	2	-4
4AH	2	101	99	8E	13	26	13
4AH	1	1	0	8E	1	6	5
4AH	0	22	22	8E	1	0	-1
4AH	3	4	1	8E	1	12	11
4AH	5	6	1	8E	5	36	31
4AH	10	24	14	8E	5	14	9
4BL	4	33	29	8AL	7	39	32
4BL	3	25	22	8AL	5	4	-1
4BL	17	28	11	8AL	1	18	17
4BL	1	141	140	8AL	4	9	5
4BL	0	156	156	8AL	1	25	24
4BL	12	63	51	8AL	0	4	4
4BL	8	178	170	8AL	3	17	14
4BH	41	3	-38	8AH	1	21	20
4BH	1	66	65	8AH	1	22	21
4BH	0	22	22	8AH	1	15	14
4BH	0	45	45	8AH	1	7	6
4BH	3	12	9	8AH	2	18	16
4BH	3	16	13	8AH	6	12	6
4BH	0	23	23	8AH	2	18	16

Time and Treatment	TUNEL Index	PCNA Index	(PI - TI) Index	Time and Treatment	TUNEL Index	PCNA Index	(PI - TI) Index
8BL	5	37	32	12AL	1	5	4
8BL	2	18	16	12AL	0	11	11
8BL	4	20	16	12AL	0	30	30
8BL	4	7	3	12AL	0	118	118
8BL	5	10	5	12AL	7	26	19
8BL	3	1	-2	12AL	0	10	10
8BL	3	0	-3	12AL	1	33	32
8BH	4	13	9	12AH	0	79	79
8BH	0	52	52	12AH	0	164	164
8BH	0	27	27	12AH	1	58	57
8BH	4	10	6	12AH	2	35	33
8BH	1	14	13	12AH	2	11	9
8BH	3	12	9	12AH	3	60	57
8BH	3	8	5	12AH	1	68	67
8ABL	1	17	16	12BL	2	13	11
8ABL	1	54	53	12BL	5	33	28
8ABL	0	135	135	12BL	5	7	2
8ABL	5	5	0	12BL	2	22	20
8ABL	0	58	58	12BL	0	94	94
8ABL	3	42	39	12BL	1	27	26
8ABL	1	11	10	12BL	2	24	22
8ABH	1	8	7	12BH	3	43	40
8ABH	0	12	12	12BH	0	81	81
8ABH	1	21	20	12BH	2	49	47
8ABH	2	11	9	12BH	0	38	38
8ABH	3	4	1	12BH	2	17	15
8ABH	0	42	42	12BH	0	2	2
8ABH	5	8	3	12BH	4	10	6
12N	3	40	37	12ABL	8	21	13
12N	3	31	28	12ABL	3	37	34
12N	1	12	11	12ABL	4	51	47
12N	1	18	17	12ABL	3	69	66
12N	2	8	6	12ABL	1	30	29
12N	0	0	0	12ABL	0	15	15
12N	4	18	14	12ABL	2	37	35
12E	4	17	13	12ABH	1	15	14
12E	3	7	4	12ABH	1	6	5
12E	2	14	12	12ABH	1	96	95
12E	2	3	1	12ABH	2	37	35
12E	2	0	-2	12ABH	5	39	34
12E	2	7	5	12ABH	0	36	36
12E	2	7	5	12ABH	2	38	36