2006

Cell Cycle Cyclin Expression of Atrazine Treated HepG2 Cells Using Western Blot Analysis

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CELL CYCLE CYCLIN EXPRESSION OF ATRAZINE TREATED HEPG2 CELLS USING WESTERN BLOT ANALYSIS

A Thesis

Submitted

in Partial Fulfillment

of the Requirements of the Designation

University Honors

And

B.A. – Honors Research Emphasis

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University of Northern Iowa

December 2006

ABSTRACT

Atrazine is one of the most widely used herbicides in the United States. It is frequently used for general weed control on both industrial and agricultural land. However, atrazine has been associated with many harmful non-target effects in animals and humans. Due to its prevalence in the environment, the United States Environmental Protection Agency (USEPA) has set the maximal contaminant level of atrazine in drinking water at 3 ppb (USEPA, 2003). One of the non-target effects of atrazine is slowed growth of both normal and immortalized human cells. Previous work from our lab demonstrated that exposing normal human fibroblasts to 0.8 parts per billion (ppb) or 0.8 μg/L of atrazine for 72 hours caused a statistically significant decrease in cell growth compared to unexposed cells (Dhanwada et al., 2003) and it took 12.5 ppb atrazine to show a statistically significant decrease in immortalized human hepatoma HepG2 cell growth (Powell and Dhanwada, 2006).

To understand the observed slow down in cell growth after atrazine exposure, the current study attempted to determine if the expression of cell cycle proteins that regulate passage through the cell cycle is being altered in atrazine exposed cells compared to unexposed cells. Three cyclins were studied: A, B, and E. Cyclin expression in atrazine-exposed cells (50, 100, 300, 500ppb) compared to control (0 ppb) HepG2 cells was quantitated under three experimental conditions, 24-hour atrazine exposure, 48-hour atrazine exposure, and 24 hour cell synchronization followed by 24-hour atrazine exposure. After the indicated time, cells were harvested for nuclear proteins, and Western blot analysis was performed.

The results of the current study show that cyclin A, B, and E expression patterns are altered in atrazine-exposed cells compared to control depending on the time of atrazine exposure. Results suggested that either a G_1 or a G_2 block in the cell cycle was occurring upon atrazine exposure.

This study by: April Dawn Rand

Entitled: Cell Cycle Cyclin expression of Atrazine Treated HepG2 Cells using Western Blot Analysis

has been approved as meeting the thesis requirement for the Designation University Honors.

12/6/06
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Entitled: Cell Cycle Cyclin expression of Atrazine Treated HepG2 Cells using Western Blot Analysis

has been approved as meeting the thesis requirement for the Degree of B.A. Biology - Honors Research Emphasis

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Background Review

Atrazine is one of the most commonly used agricultural pesticides in the United States. It belongs to the selective triazine herbicide family, which also includes the herbicides cyanazine, simazine, and metribuzin. Atrazine is applied both before and after planting to control broadleaf and grassy weeds in corn, sorghum, sugarcane, pineapple, macadamia nuts, and citrus fruits. It is also used as a general weed control on industrial and nonagricultural land (Cox, 2001). First registered for use as a pesticide in 1958, atrazine is currently the most widely used agricultural pesticide with an estimated production of 76 to 87 million pounds annually. The heaviest atrazine use per unit area occurs in Delaware, Iowa, Illinois, Indiana, Ohio, and Nebraska (USEPA, 2003). Atrazine, applied as an aerosol, kills plants by binding to the cell membrane where photosynthesis occurs, inhibiting this process. This herbicide is moderately resistant to degradation since it persists in soil growing season after season and has a high to medium mobility in soil (Cox, 2001). Considering its high rate of use, it is not unusual to find atrazine as a common water contaminant. It has been found in groundwater, rivers, high mountain lakes, drinking water supplies, rain, and fog (Uhler, 2001) allowing it to be ingested by both animals and humans. The U.S. Environmental Protection Agency (EPA) has set maximum contaminant levels (MCL) allowed in drinking water at 3 parts per billion (ppb, μg/L) atrazine (USEPA, 2003).

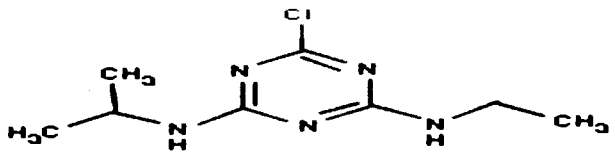

Figure 1: Chemical structure of atrazine. The triazine ring of this compound makes it fairly resistant to degradation with persistence in soil longer than one year in cold and dry conditions (Gold, 2006).

Many studies have been done to determine the effects of atrazine on animals and humans and the mechanisms through which these effects occur. One such study showed the effects of atrazine on North American native leopard frogs; *Rana pipens* (Hayes et al., 2003). Laboratory raised *R. pipens* were studied, followed by an examination of wild *R. pipens* from a variety of habitats with both low and high reported atrazine use. In the lab, *R. pipens* larvae were treated with concentrations of 0, 0.1, and 25 ppb atrazine for approximately 3 days until complete tail reabsorption. It was found that 36% and 12% of males treated with 0.1 and 25 ppb atrazine respectively suffered from gonadal dysgenesis or infertility. Additionally, 29% and 8% of male larvae treated with 0.1 and 25 ppb atrazine respectively displayed varying degrees of sex reversal characterized by testicular lobules containing oocytes. These effects were not seen in atrazine exposed females (Hayes et al., 2003). Thus, lower doses (0.1 ppb) of atrazine exposure had greater effects than higher doses (25 ppb) of atrazine exposure implying that a linear dose response is not seen with atrazine exposure and that intermediate concentrations of atrazine may actually be the most effective at causing abnormalities in male *R. pipens* (Hayes et al., 2003).

Field studies showed similar effects to the lab analyses in *R. pipens*. `Sites with atrazine sales of less than 0.4 kg/km^2 were sampled as uncontaminated areas, while sites

with atrazine sales of greater than 0.4 kg/km^2 were sampled as contaminated areas. Testicular oocytes were displayed in males from 7 of the 8 sites sampled, all of which were associated with atrazine contaminant levels that exceeded 0.2 ppb. These sites also displayed sex reversal similar to the abnormalities induced in the lab by 0.1 ppb atrazine exposure (Hayes et al., 2003).

Interestingly, this study showed the persistence of this herbicide in soil for long periods of time. It was observed that even though atrazine has a relatively short half life (8 days), atrazine concentrations of greater than 0.2 ppb were still present in irrigation ditches 10 months later, indicating atrazine can persist for long periods. Additionally, atrazine levels varied from 15.2 ppb, well over the EPA limit of 3 ppb, to 0.8 ppb over a 24 hour period as a result of evaporation and irrigation. Runoff from cornfields into adjacent wildlife protection areas also resulted in atrazine contamination above 15 ppb (Hayes et al., 2003).

An epidemiological study was done to correlate pesticide contamination and reproductive abnormalities in humans (Munger et al., 1997). Municipal water supplies in Iowa surveyed from 1986 to 1987 showed that the Rathbun water system serving 12 communities in southern Iowa had persistently high levels of herbicide contamination, including cyanazine, metolachlor, chloroform, bromodichloromethane, and dibromochloromethane, and the most notable being atrazine at a mean level of 2.2 ppb. This level of atrazine was below the EPA's allowable limit. Analyses were done to determine the correlation between drinking water from this contaminated water system and the rates of low birth weight, prematurity and intrauterine growth retardation (IUGR). Low birth weight was defined as birth weight less than 2,500 grams, births less than 37

weeks of gestation were considered premature, and IUGR was defined as weight less than the 10^{th} percentile for gestational age. It was found that the rate of birth of babies with IUGR was 1.8 times greater in the Rathbun served communities in comparison to the other 38 communities in the same county with other sources of drinking water. Only a marginal increase in the rate of low birth weights or premature births was seen in Rathbun in comparison to other communities. Socioeconomic factors, such as maternal smoking, prenatal care, and income were ruled out as contributing factors. Water contamination was suggested as a factor in the increased rate of IUGR, but because so many herbicides were present, the independent contribution of each herbicide could not be directly determined (Munger et al., 1997). However, it is important to note that none of the pesticides were above EPA levels and thus this water supply would not automatically be referred for cleanup.

The above study correlating pesticide exposure and reproductive abnormalities suggest that there may be an effect on cells when exposed to pesticides. Several studies have been done to examine the effects of pesticides on the cell cycle. To understand how these pesticides may function on cells, a primer on the cell cycle is presented.

Cell growth in eukaryotic cells is dependent on its cell cycle, a series of events that occur when one cell gives rise to two, new identical daughter cells. Each cell cycle consists of two distinct phases - interphase and cell division. Interphase consists of three phases – Gap 1 (G_1), Synthesis (S), and Gap 2 (G_2) and cell division consists of mitosis (M) and cytokinesis. Generally, many cells take 18-24 hours to complete one cell cycle. Cells spend the majority of time in interphase, growing and preparing for cell division. In the G_1 and G_2 phases, cells grow and duplicate their contents. In the S phase, DNA is

replicated. After interphase, mitosis begins and cells divide their DNA (mitosis) and cytoplasm (cytokinesis) between 2 daughter cells (Becker et al., 2006).

Progression through each phase of the cell cycle is driven by a series of protein kinases which are called cyclin dependent kinases or cdks. These cdks exhibit enzymatic activity only when they are bound to a protein called a cyclin and the complete cdk-cyclin is activated. Cyclin concentrations oscillate between high and low levels and thus, cyclins control the activity of specific cdks at specific phases in the cell cycle. Levels of specific cyclins (A, B, D, and E) begin to increase at a particular phase of the cell cycle, eventually reaching a critical threshold. At this level, it binds to a specific cdk and forms a cdk-cyclin complex that is not activated. The inactive cdk-cyclin complex is activated after a series of phosphorylation and dephosphorylation events on the complex. Once activated, the cdk-cyclin complex can trigger the onset of a specific phase (G_1, S, G_2, or M) in the cell cycle through its kinase activity. After reaching peak levels and forming an active complex to get through a particular cell cycle phase or checkpoint, cyclin concentrations drop and rise again when the cell nears that specific phase of the cell cycle once again. Figure 2 illustrates how the different cyclin levels vary during the cell cycle leading to progression from one phase to the next (Weinberg, 2007).

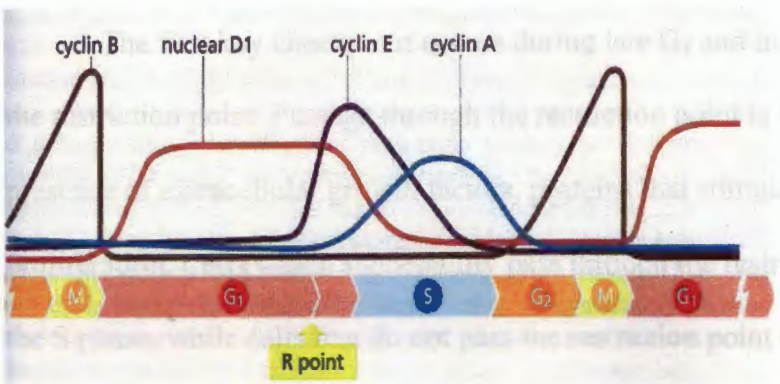

Figure 2: Fluctuation of cyclin levels during the cell cycle (Weinberg, 2007). During the cell cycle, cyclin E levels rise at the end of G_1 to get cells past the restriction point and fall during the S phase. Cyclin A levels peak in the middle of the S phase and then being to fall, and cyclin B levels rise through the G_2 phase peaking in the M phase and then decline.

Progression through the cell cycle is regulated at several key transition points. Passage through transition points or checkpoints ensures that each phase of the cell cycle is carried out at the appropriate time and in the appropriate sequence and that one phase is completed before another phase begins. The determination of whether or not the cell will continue to proceed through the cell cycle is largely determined by chemical signals which reflect the cell's internal state and external environment and the appropriate levels of a specific active cdk-cyclin complex. Figure 3 shows the three important checkpoints in the eukaryotic cell cycle (Becker et al., 2006).

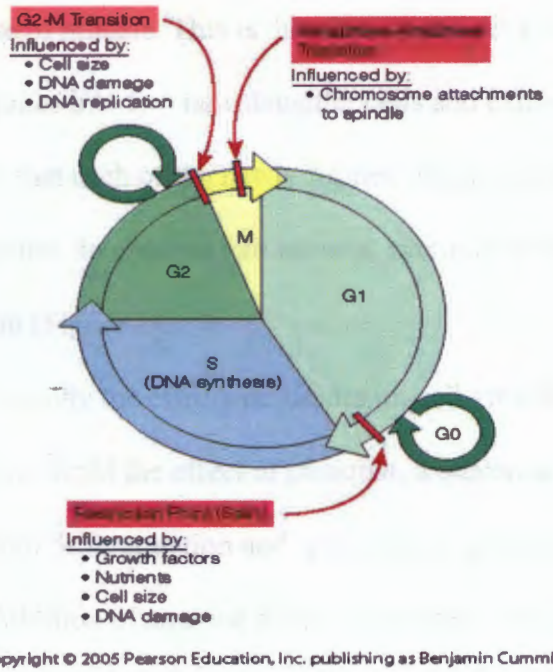

Figure 3: Key checkpoints in the cell cycle. Checkpoints are found between the G_1/S transition, G_2/M transition and within M (Becker et al., 2006).

The first key checkpoint occurs during late G_1 and in animal cells is referred to as the restriction point. Passage through the restriction point is largely determined by the presence of extracellular growth factors, proteins that stimulate or inhibit cell proliferation. Cells which successfully pass through the restriction point are committed to the S phase, while cells that do not pass the restriction point enter the G_0 phase. In G_0, cells wait for a signal that will allow them to re-enter into the G_1 phase and pass the

restriction point. In G_1, cyclin D and E levels increase to ensure the formation of active cdk-cyclin complexes to allow cells to pass through the restriction point into S phase (Figure 2).

The second key checkpoint occurs between the G_2/M boundary, the point where cells commit to mitosis. Cells can be arrested at this point if cell division is not necessary, regulating the rate of cell division. In this phase, cyclin A has dropped and cyclin B is rising to ensure passage through this checkpoint (Figure 2).

The third key checkpoint of the cell cycle occurs between anaphase and metaphase of mitosis. This is the point where cells make the commitment to segregate chromosomes into two new daughter cells and exit mitosis. This checkpoint acts to guarantee that each of the newly formed daughter cells receives a complete set of chromosomes. In order to exit mitosis, cyclin B levels must be low to get past this checkpoint (Figure 2).

To study the effects pesticides on cell growth and differentiation, Magnelli et al. (1989) considered the effect of paraquat, a quaternary nitrogen compound, and atrazine on erythroid differentiation and spontaneous growth in mouse erythroleukaemic cells (MEL). Addition of atrazine alone to the MEL cells caused a 50% growth inhibition at 8 μg/ml (or 8 parts per million) atrazine. Addition of paraquat to the MEL cells caused a 50% growth inhibition at 15 μg/ml (ppm) paraquat. Growth inhibition was not cell specific, since the same concentrations of both atrazine and paraquat also inhibited the spontaneous growth of mouse fibroblasts. Growth inhibition was completely reversed after the removal of both herbicides (Magnelli et al., 1989) While this study shows that both atrazine and paraquat inhibit cell proliferation, the concentration of atrazine (8ppm)

and paraquat (15ppm) are 30 to 50 times higher than levels would be in the environment after spraying and run off into water supplies after a heavy rain fall event.

Another study examined the effects of cell cycle deregulation in BALB/c 3T3 cells with 1,2-dibromoethane (DBE) and folpet (Santucci et al., 2003). DBE, a halogenated organic, and folpet, a carboximide, are two widely used agricultural pesticides that pose a high risk as environmental pollutants. Murine BALB/c 3T3 cells were transformed with these pesticides and cell cycle studies were done to compare parental cells with DBE and folpet transformed cells (Santucci et al., 2003). Results suggested that transformation with these pesticides caused a loss of regulatory control over cell proliferation. Transformation involves a change in the cell's genotype resulting in the altered regulation of the cell cycle. Cell transformation by both DBE and folpet caused a significant reduction in the growth of cells in the G_1 phase under normal conditions with fewer transformed cells in G_1. However, the difference in the number of cells in the G_1 phase between the parental and transformed DBE and folpet cells was enhanced after exposure to the DNA damaging effects of low dose ionizing radiation (IR). After 16 hours of IR exposure, there was a growth arrest of parental BALB/c 3T3 cells. However, the pesticide transformed cells proceeded from G_1 to S phase regardless of the IR exposure, but were arrested later between the G_2/M boundary.

When cyclin expression was measured, it was found that cyclin D3 and E3 were both over expressed in the pesticide transformed cells under steady state conditions. The increased expression of these two cyclins suggested that cells proceeded through the G_1 phase and into the S phase without an intact, functional G_1/S checkpoint. This would be expected of transformed, continuously growing cancer cells. However, the most

significant difference occurred with cyclin A, which was over expressed even after IR exposure. This suggested perhaps these pesticide transformed cells, unlike parental BALB/c 3T3 cells, would not undergo a G_2 arrest since normal cells that arrest in G_2 are shown to have declining cyclin A levels (Niculescu et al., 1998). In contrast, these pesticide transformed cells that had high levels of cyclin A did arrest in G_2, thus had an altered G_2/M checkpoint (Santucci et al., 2003). Therefore, pesticide transformed murine cells have lost their ability to regulate their proliferation at either the G_1/S checkpoint and/or the G_2/M checkpoint.

Another study using sea urchin embryos, the organism on which cyclins were first discovered, was used to test the effects of the pesticide glyphosate (Round Up) on cell growth (Marc et al., 2004). Many pesticides contain glyphosate as an active ingredient; however, it is currently not considered to be a great health or environmental concern. However, Marc et al. (2004) showed that glyphosate caused cell cycle dysfunction in sea urchin embryos. Glyphosate (10mM) delayed the first cell cycle by 30 minutes and prevented the activation of the protein kinase cdk1/cyclin B, a cyclin complex necessary for entry into mitosis. The abundance of cdk1 remained constant and the association of cyclin B with cdk1 was not disrupted. However, the cyclin B expression pattern change associated with cdk1 activity was delayed, thus the complex took longer to become activated since expression of cyclin B was delayed. Phosphorylation analysis found that formulated glyphosate exposure prevented the activating dephosphorylation of cdk1 on tyrosine and thus the late activation of the cdk1/cyclin B complex impeded cell cycle progression at the G_2/M transition. However, it was found that glyphosate had no direct effect on the phosphatase responsible for dephosphorylation (Marc et al., 2004). This

study further tested formulated glyphosate on DNA synthesis using radio labeled thymidine incorporated into DNA (Marc et al., 2004). Control embryos incorporated thymidine at 20 minutes post fertilization and incorporation increased rapidly until reaching a plateau at 50 minutes post fertilization. In contrast, embryos treated in formulated glyphosate showed an approximate 75% inhibition of DNA synthesis. It was then concluded that the inhibitory effects of formulated glyphosate on DNA synthesis lead to the delayed entry of treated embryos into the M phase (Marc et al., 2004). Although adverse effects of formulated glyphosate in the cell cycle were seen in these experiments, it was seen at millimolar concentrations when applied for a short time to sea urchin embryos. Soil and water concentrations of glyphosate are generally in the nanomolar range. As of yet, no indication that the glyphosate has any genotoxic effects at these lower environmentally relevant levels (Marc et al., 2004).

Our laboratory has been looking at the effects of atrazine exposure on human cells. Growth effects of low dose atrazine exposure have shown that there are a fewer number of cells growing after atrazine exposure when compared to control cells. Work from our lab has demonstrated that atrazine slows the growth of human cells. Both normal and immortalized human cells have been used. Exposing normal human fibroblasts to 0.8 ppb of atrazine for 48 or 72 hours caused a statistically significant decrease in cell growth compared to non-exposed cells (Dhanwada et al., 2003). This level was 3.75X lower than the maximum contaminant levels of atrazine allowable in drinking water (3 ppb). Similar results were also found in an immortalized liver cell line, HepG2. However, it took 12.5 ppb atrazine to show a statistically significant decrease in the HepG2 cell growth (Powell and Dhanwada, 2006). The HepG2 cells are an immortal

human hepatoma cell line and are not normal primary cells like the fibroblasts, which may be why it takes higher levels of atrazine to inhibit growth. However, 12.5 ppb is still within an environmentally relevant range of atrazine concentrations.

From the growth analysis, it was thought that one of two things may be happening to result in fewer cells after atrazine exposure. Either the cells were being killed due to the toxic effects of atrazine, or they were actually taking longer to grow in the atrazine containing media, resulting in fewer cells. We have previously shown that neither apoptosis nor necrosis was occurring in atrazine exposed cells at these low levels of atrazine (Manske et al., 2003). Thus, to determine if in fact cell growth is being slowed, the current study attempts to determine if the expression of cyclin proteins that regulate passage through the cell cycle is being altered in atrazine exposed cells compared to unexposed cells leading to a slow down in cell growth. We exposed HepG2 cells to increasing concentrations of atrazine, harvested nuclear protein, and performed Western blot analysis to determine if cyclin A, B, or E expression is altered in atrazine exposed cells compared to control cells.

Materials and Methods

Cells

The HepG2 cell line, a non-tumorigenic human hepatocellular carcinoma, (American Type Culture Collection, Manassas, VA) was grown in Eagle's Minimum Essential medium (EMEM) with Earle's Balanced Salt Solution (BSS) and 2mM L-glutamine (Gibco) supplemented with 1.0mM sodium pyruvate (Gibco), 0.1mM nonessential amino acids (Gibco), 1.5 g/L sodium bicarbonate, 10% fetal bovine serum (FBS) (Hyclone), and 1% penicillin-streptomycin, pH 7.3 (Gibco). These cells were maintained at 37°C with 5% CO_2. When 80% confluent, cells were generally split 1:6. This would occur once a week. To split cells, old medium was removed and cells were washed with calcium-magnesium free 1X phosphate buffered saline (CMF-PBS) (Bio Whittaker). Trypsin-EDTA (0.25%) (Gibco) was then added to dislodge cells from dishes. Trypsin action was stopped by the addition of 10% serum-containing medium to the cells. Cells were then resuspended by pipetting. A 1:6 dilution of the cell suspension was removed and added to new 100mm tissue culture plates.

Atrazine exposure of HepG2 cells

Within 24 hours of splitting cells, atrazine containing medium was added. Cell culture medium was removed from each plate and medium containing varying concentrations of atrazine was added. The atrazine medium was made from a 6.96 ppm stock solution and appropriate dilutions were made and added to each dish. The dilutions used were 0 parts per billion (ppb) - the control, 50ppb, 100ppb, 300ppb, and 500ppb atrazine. The dishes were then put back into the 37°C with 5% CO_2 for 24 or 48 hours.

Atrazine quantitation

Atrazine (Chem Services, Wechester, PA) was dissolved in serum and antibiotic free media. The stock solution was quantitated by liquid chromatography- mass spectroscopy (LC-MS). Atrazine standards dissolved in acetonitrile were made and quantitated on the LC-MS (Agilent 1100 Series) with a C8 column (Agilent) using 60% acetonitrile and 40% water. Atrazine standards of 2.5, 5, 7.5, 10, 12.5, 15, and 20 parts per million (ppm or mg/L) were used to generate a standard curve. A linear regression line was generated and used to determine the concentration of the experimental atrazine solution. This was the stock atrazine solution used at a concentration of 6.96ppm (~32.2μm).

Cell synchronization

To determine the effects of cell synchronization on cyclin expression patterns in atrazine exposed HepG2 cells, these cells were exposed to aphidocolin. This chemical causes the cells to stop at the late G_1/early S phase in the cell cycle since it reversibly inhibits DNA polymerase. Cell synchronization followed the same treatment for the cells except that after splitting cells, 20μl of 2mg/ml (4μg/ml) aphidocolin (Acros) was added to all experimental cells, including control. A solvent control received 20μl of DMSO. The final DMSO concentration was 0.2%. After 24 hours, the aphidocolin containing media was removed and cells were washed twice with CMF-PBS. Atrazine containing media was then added to cells for 24 or 48 hours and proteins were extracted.

Extraction of nuclear proteins

HepG2 cells were harvested to obtain nuclear proteins from the atrazine exposed cells. After 24 or 48 hours of atrazine exposure, cell culture medium was removed and cells were washed two times with CMF-PBS. 500µl of buffer A (10mM HEPES, 10mM KCl, 0.1mM EDTA, 1mM DTT, 0.5 mM PMSF, 5µl of 10µg/µl of aprotinin, pepstatin, and leupeptin, 200µl 10% IGEPAL) was added to each dish for 10 minutes. Each plate was scraped and transferred to labeled microcentrifuge tubes and placed on ice. Centrifugation was done for 3 minutes at 14,000 rpm. The supernatant (cytoplasmic proteins) was discarded, while the nuclear pellet portion was set aside. 150µl of buffer B (20mM HEPES, 1.4M NaCl, 1mM EDTA, 10% glycerol, 1mM DTT, 0.5 mM PMSF, and 3.3µl of 10µg/µl of aprotinin, leupeptin, and pepstatin) was added to each pellet and resuspended. These nuclear protein containing tubes were then wrapped in parafilm and placed in a wrist shaker (Burrell) for 2 hours at 4°C. After 2 hours of vigorous shaking, protein solutions were centrifuged for 5 minutes at 14,000 rpm. The supernatant (nuclear proteins) was transferred to new tubes and stored at -80 °C. This protocol was adapted from Mirmira (http://faculty.virgina.edu/mirmira/resources_files/Protocols/Nuclear%20 Extracts.htm, 2006).

Protein quantitation

Protein quantitation was done by Bradford analysis (Bio-Rad). Stock solution of BSA (2mg/ml) and buffer B (of the protein extraction protocol) were used to make dilutions for standards and samples. Five replicates were prepared of six concentrations of BSA, including 0, 0.2, 0.4, 0.6, 0.8, and 1mg/ml BSA. Buffer B and nuclear protein extract samples were used to make 5 replicates of 1:5 dilutions of the control protein as

well as proteins from treated cells. 200 µl of 1:4 diluted Bio Rad protein assay dye reagent (Bradford) was added to each well and then incubated for 15 minutes at room temperature. Plates were read at 595 nm in a spectrophotometer. Using the program SoftMax Pro 4.3.1 LS, a standard curve was generated using linear regression and concentration for each sample was calculated. The assays with R^2 values of 0.98 and higher were used.

SDS-PAGE gel

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis or SDS-PAGE gel was made using vertical gel apparatus and gel casting accessories (BioRad). A 10% separating gel was used (0.375 M Tris-HCl, pH 8.8, 10% acrylamide, 0.1% SDS, 0.05% Ammonium persulfate, and 0.05% TEMED) and allowed to polymerize. The stacking gel (0.125 M Tris-HCl, pH 6.8, 5% acrylamide, 0.1% SDS, 0.05% Ammonium persulfate and 0.1% TEMED) was made, added, and allowed to polymerize.

Fifteen µg of nuclear extract was loaded onto the SDS-PAGE gel along with 6X loading buffer (4x Tris-Cl/SDS, pH 6.8, 30% glycerol, 10% SDS, 0.012% bromophenol blue) after boiling samples for 5 minutes. 5X Electrophoresis running buffer (0.025M Tris base, 0.192M glycine, 0.1% SDS) was diluted to 1X and proteins were separated at a constant voltage of 100V for 1.5 hours at 4°C.

Western Blot analysis

The gels were removed from the gel box apparatus, measured and placed in transfer buffer (25mM Tris, 192 mM glycine, 20% methanol) for 30 minutes on a shaker table to remove SDS. While the gels were equilibrating in the transfer buffer, filter paper and polyvinylidine fluoride (PVDF) membrane were placed in transfer buffer and kept at

4°C till gel was equilibrated. After 30 minutes, wet transfer was used to transfer proteins to PDVF membrane for 1.5 hours at a constant voltage of 100V at 4°C. To assess transfer efficiency, gels (after transfer) were stained overnight with Coomassie Blue (10% (vol/vol) acetic acid, 0.006% (wt/vol) Coomassie brilliant blue G-250) by first fixing in isopropanal fixing solution (25% (vol/vol) isopropanol, 10% (vol/vol) acetic acid) for 20 minutes. The gels were then placed in 10% acetic acid for de-staining for a day and dried onto filter paper.

The protein containing membranes were put into blocking solution (10%glycerol, 5% dried milk powder, 0.2% Tween-20 in TBS (20mM Tris, 500mM NaCl, pH 7.5)) either overnight at 4°C or at room temperature for one hour. Blocking solution was then removed and two quick washes (1-2 minutes) were done with T-TBS (0.2% Tween-20 in TBS). Primary antibody, in blocking solution, directed against either cyclin A, B, or E (1:200(A, B), 1:100 (E), Santa Cruz Biotechnology) was added to the membranes for one hour at room temperature. The primary antibody solution was then removed and two quick washes were done with T-TBS, followed by a 15 minute wash in T-TBS followed by three 5 minute washes in T-TBS. The secondary antibody, (1:5000, goat anti-rabbit-HRP conjugate, Santa Cruz Biotechnology) was added to the membrane for one hour at room temperature. The secondary antibody was removed and two quick washes were done with T-TBS followed by a 15 minute wash in T-TBS and three 5 minute washes in T-TBS. Proteins were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences) followed by scanning and data analysis with the STORM 860 phosphoimager (Amersham Biosciences).

The STORM scanner control program was used to scan membranes using fluorescence, channel 2 Blue (450nm), under normal sensitivity, with a PMT voltage of 800nm. The backwards orientation was selected and a pixel size of 200 microns was used. This was followed by quantitation of the blot using the Image Quant 5.2 program to determine relative densities of each protein band.

Statistical Analysis

Quantitation data from Image Quant 5.2 was entered into a Microsoft Excel spreadsheet. The averages from all tests were calculated and plotted against increasing atrazine concentration for each cyclin protein. Each cyclin experiment was repeated 3-4 times for averages calculated. Standard deviation, standard error, and student T Test analyses were determined using the averages from each cyclin experiment. The student T Test was two tailed and compared samples of varying atrazine levels to control. The standard error was used for error bars on the scatter plot graph and samples with p values of less then 0.05 were found to be statistically significant from control.

Results

To determine if the expression of cell cycle proteins is being altered in atrazine exposed cells compared to unexposed cells, Western blot analysis was performed. In this project, we attempt to determine why there was an observed decrease in cell growth by measuring the levels of specific proteins that regulate passage through the cell cycle in atrazine treated and untreated control cells.

Atrazine quantitation

LC-MS chromatography was performed with prepared concentrations of pure atrazine. A linear regression curve was determined (Figure 1) and the concentration of stock atrazine solution was determined to be 6.96 ppm. This stock solution was used to make all dilutions used during the experiments.

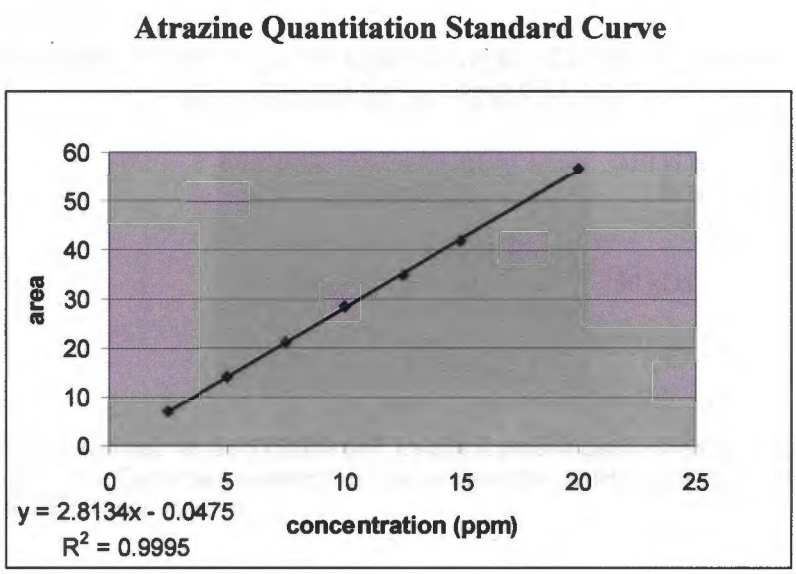

Figure1: Atrazine standard quantitation curve determined from the area of peaks of atrazine standards at 2.5, 5, 7.5, 10, 12.5, 15, and 20ppm. A best fit linear regression with the equation y = 2.8134 – 0.0475 was used to determine the concentration of the stock atrazine which was 6.96 ppm (R^2 = 0.9995).

Quantitation of cyclin levels after 24-hour atrazine exposure without cell synchronization

HepG2 cells were exposed to increasing concentrations of atrazine (0, 50, 100, 300, 500ppb) for 24 hours. Blots were treated either with anti-cyclin A, B, or E primary antibody. Figure 2 shows a representative blot using anti-cyclin A on HepG2 nuclear proteins harvested after 24 hours atrazine exposure. The intensity level of each band was quantitated using the Image Quant 5.2 program. A ratio was determined by comparing the density of the atrazine treated samples to untreated control proteins. The average for all of the trials was calculated as well as the standard deviation and standard error. Student T Test analysis was done for all trials. A p value of less than 0.05 was considered to be statistically significant from control.

Western Blot of HepG2 cells exposed to atrazine for 24 hours, treated with cyclin A, and scanned on the STORM 860

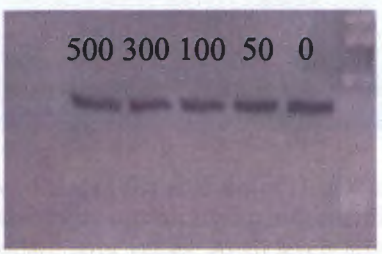

Figure 2: Western Blot scanned on the STORM 860. Cyclin A protein can be seen at around 60kDa. Wells containing varying levels of atrazine concentration can be seen (from left to right- 500, 300, 100, 50, 0ppb atrazine, and molecular weight markers).

HepG2 cells exposed to increasing concentrations of atrazine for 24 hours showed no significant difference in cyclin A levels in comparison to control (Figure 3). In contrast, HepG2 cells exposed to increasing concentrations of atrazine showed a statistically significant decrease in cyclin E expression levels for 50, 100, 300, and

500ppb compared to control. Figure 4 shows decreased levels of cyclin E expression at all tested concentrations of atrazine. Cyclin B expression also showed no statistically significant differences in comparison to control (Figure 5). Tables 1-3 show the ratio of the density of specific proteins of atrazine treated cells compared to control. Both cyclin A and cyclin E had 3 separate experiments while cyclin B had 2 experiments.

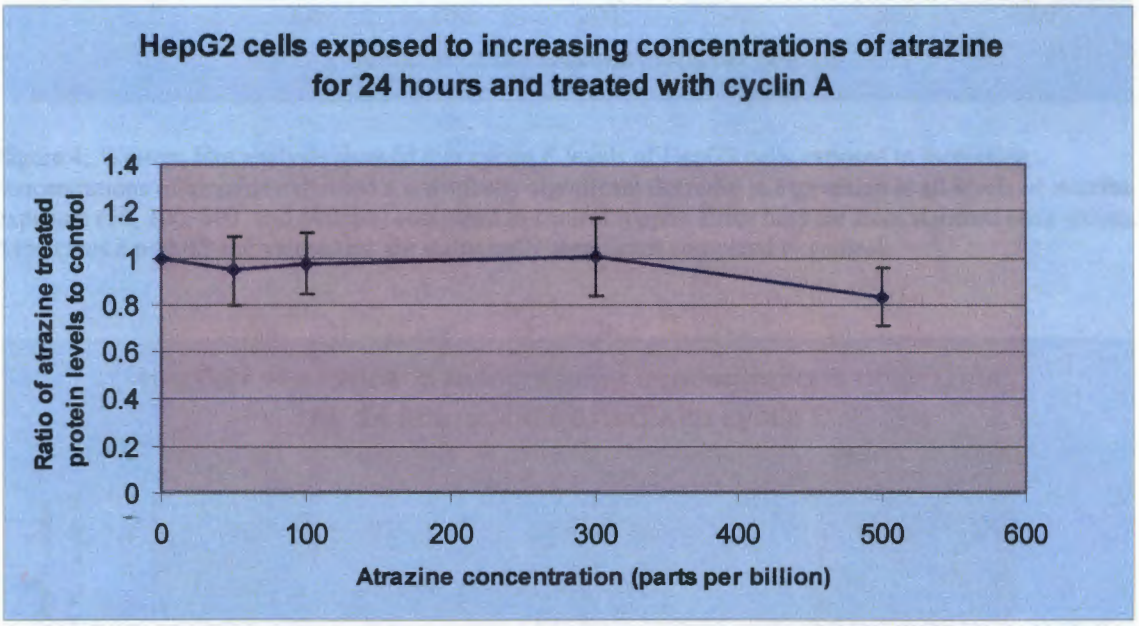

Figure 3: Western blot analysis showed that cyclin A levels of HepG2 cells exposed to increasing concentrations of atrazine did not exhibit any statistically significant differences compared to control (0ppb) cells. Error bars are from standard error values. At all levels tested $p>0.05$.

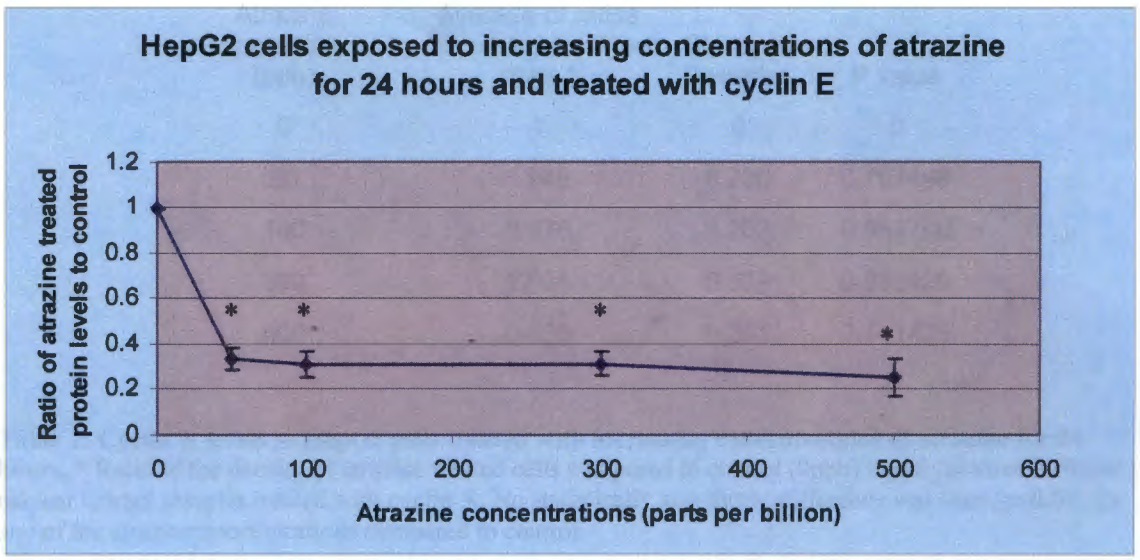

Figure 4: Western blot analysis showed that cyclin E levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited a statistically significant decrease in expression at all levels of atrazine exposure (50, 100, 300, and 500ppb) compared to control (0ppb). Error bars are from standard error values. * indicates a $p<0.05$ and values that are statistically significant compared to control.

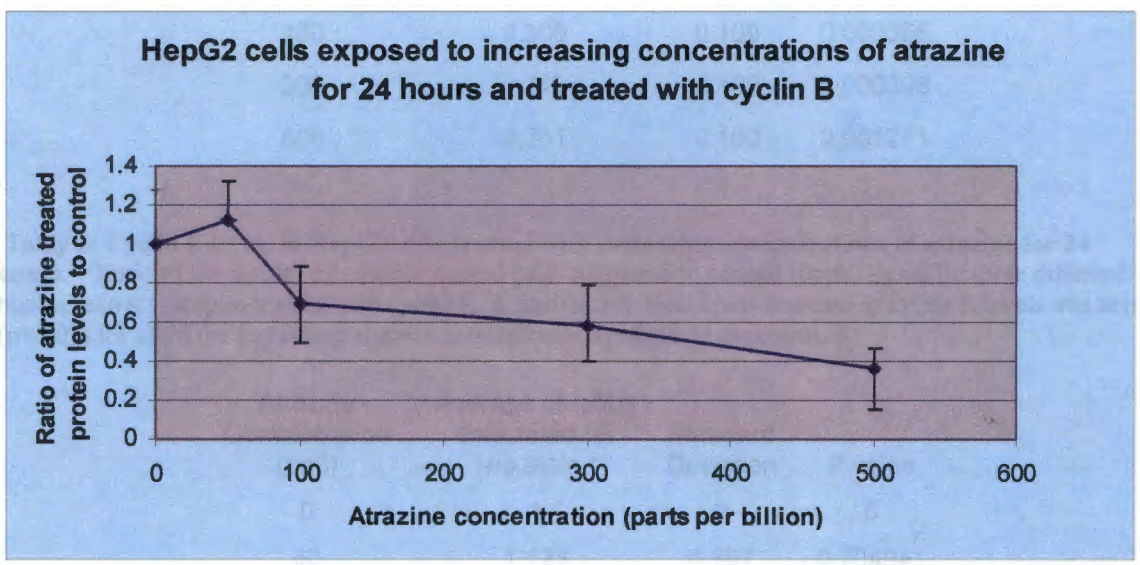

Figure 5: Western blot analysis showed that cyclin B levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited no statistically significant difference at any level of atrazine exposure (50, 100, 300, and 500ppb) compared to control (0ppb). Error was depicted as standard error. At all levels tested $p>0.05$.

Atrazine Concentration (ppb)	Average of ratios calculated for three trials *	Standard Deviation	P value
0	1	0	0
50	0.946	0.296	0.767449
100	0.976	0.263	0.883700
300	1.005	0.332	0.980429
500	0.833	0.251	0.141425

Table 1: Cyclin A levels in HepG2 cells treated with increasing concentrations of atrazine for 24 hours. * Ratio of the density of atrazine treated cells compared to control (0ppb) signal for three different nuclear extract samples treated with cyclin A. No statistically significant difference was seen ($p<0.05$) for any of the atrazine concentrations compared to control.

Atrazine Concentration (ppb)	Average of ratios calculated for three trials *	Standard Deviation	P value
0	1	0	0
50	0.333	0.094	0.000252
100	0.309	0.109	0.000396
300	0.312	0.109	0.000398
500	0.251	0.160	0.001271

Table 2: Cyclin E levels in HepG2 cells treated with increasing concentrations of atrazine for 24 hours. * Ratio of the density of atrazine treated cells compared to control (0ppb) signal for three different nuclear extract samples treated with cyclin E. A statistically significant decrease in cyclin E levels was seen ($p<0.05$) for all of the increasing atrazine concentrations compared to control.

Atrazine Concentration (ppb)	Average of ratios calculated for two trials *	Standard Deviation	P value
0	1	0	0
50	1.123	0.397	0.704941
100	0.695	0.335	0.326963
300	0.583	0.410	0.287038
500	0.362	0.216	0.052981

Table 3: Cyclin B levels in HepG2 cells treated with increasing concentrations of atrazine for 24 hours. * Ratio of the density of atrazine treated cells compared to control (0ppb) signal for two different nuclear extract samples treated with cyclin B. A statistically significant difference was not seen ($p<0.05$) for any of the atrazine-exposed cells compared to control cells.

Quantitation of cyclin levels after 48-hour exposure without cell synchronization

Increasing concentrations of atrazine (0, 50, 100, 300, 500ppb) were exposed to HepG2 cells for 48 hours. Figure 6 shows a representative blot using anti-cyclin B on HepG2 nuclear proteins harvested after 48 hours atrazine exposure. A ratio was determined by comparing the density of the atrazine treated samples to untreated control proteins. The average for all of the trials was calculated as well as the standard deviation and standard error. Student T Test analysis was done for all trials. A p value of less than 0.05 was considered to be statistically significant from control.

Western Blot of HepG2 cells exposed to atrazine for 48 hours, treated with cyclin B, and scanned on the STORM 860

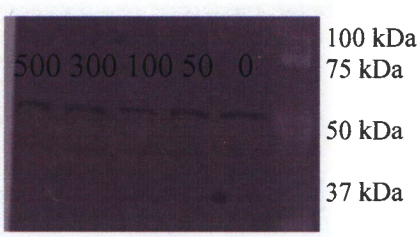

Figure 6: Western Blot scanned on the STORM 860. Cyclin B protein can be seen at around 55kDa. Wells containing varying levels of atrazine concentration can be seen (from left to right- 500, 300, 100, 50, 0ppb atrazine, and molecular weight markers).

Cyclin A expression in HepG2 cells exposed to increasing concentrations of atrazine for 48 hours showed a statistically significant decrease in protein levels at 50ppb atrazine exposed cells in comparison to control (Figure 7). However, at 500ppb, the highest concentration of atrazine, cyclin A showed an increase in protein levels, but not to statistically significant levels compared to control. This was slightly in contrast to the 24-hour atrazine exposed cells which did not show any statistically significant differences at any concentration of atrazine exposure (Figure 3).

When HepG2 cells were exposed to increasing concentrations of atrazine for 48 hours and assayed for cyclin E expression, there was no statistically significant decrease in cyclin E expression levels in comparison to control (Figure 8). Cyclin B had a significant decrease in expression at all levels of atrazine exposure (50,100, 300, 500ppb) (Figure 9). Tables 4-6 show the ratio of the density of specific proteins of atrazine treated cells compared to control. Cyclins A, E, and B each had 3 separate experiments.

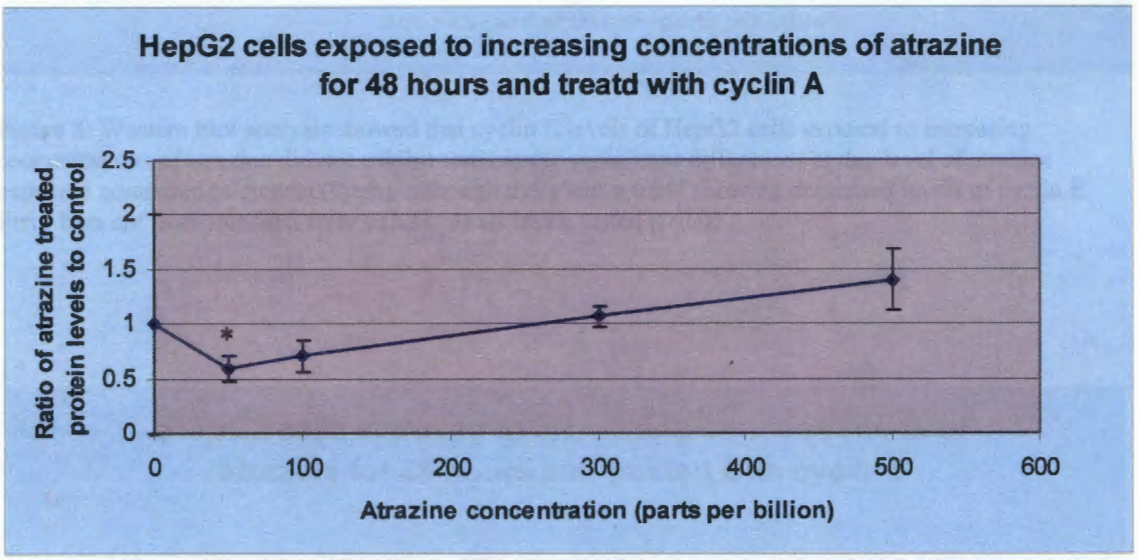

Figure 7: Western blot analysis showed that cyclin A levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited a statistically significant decrease at 50ppb of atrazine exposure compared to control (0ppb). Error bars are from standard error values. * indicates a $p<0.05$ values that are statistically significant compared to control.

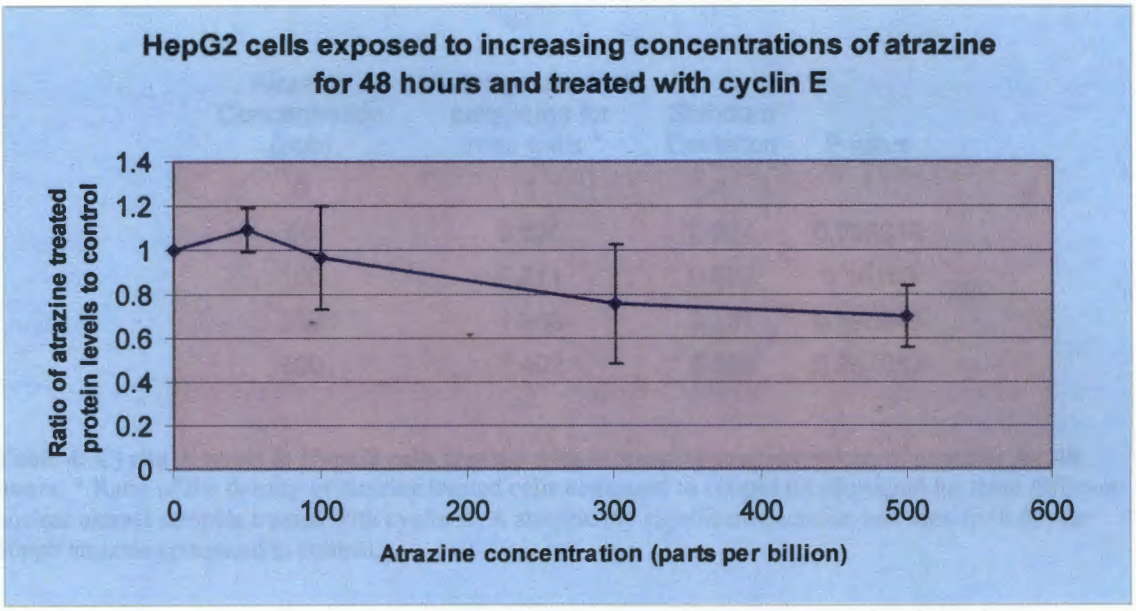

Figure 8: Western blot analysis showed that cyclin E levels of HepG2 cells exposed to increasing concentrations of atrazine did not exhibit statistically significant differences at any level of atrazine exposure compared to control (0ppb), although there was a trend showing decreased levels of cyclin E. Error bars are from standard error values. At all levels tested $p>0.05$.

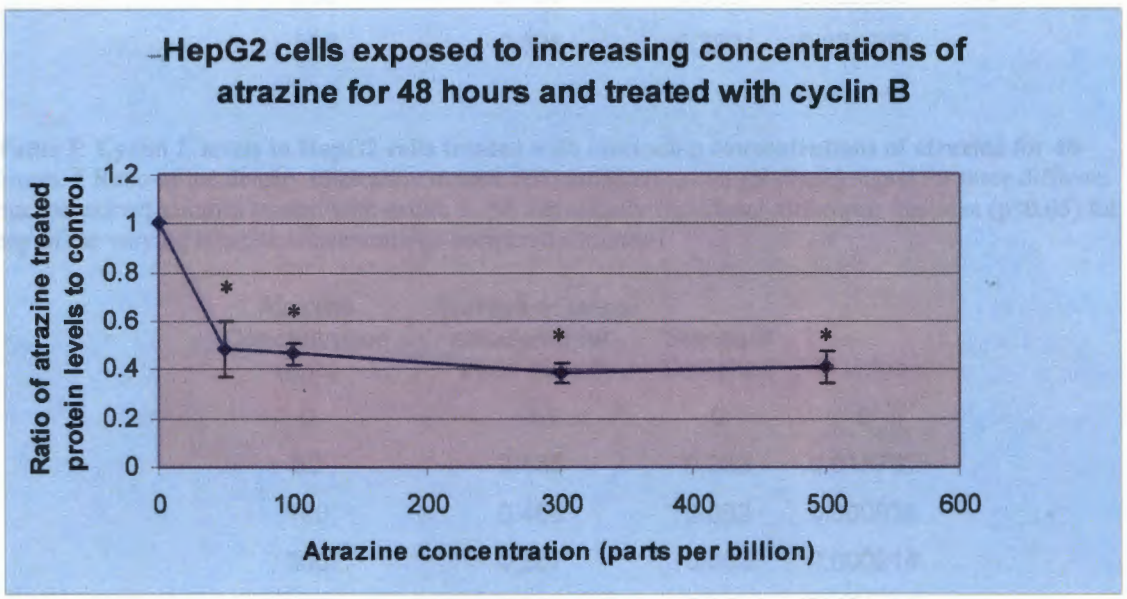

Figure 9: Western blot analysis showed that cyclin B levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited a statistically significant decrease in protein levels at all levels of atrazine exposure (50, 100, 300, and 500ppb) compared to control (0ppb). Error bars are from standard error values. * indicates a $p<0.05$ and values that are significantly significant compared to control.

Atrazine Concentration (ppb)	Average of ratios calculated for three trials *	Standard Deviation	P value
0	1	1	1
50	0.595	0.234	0.039915
100	0.711	0.292	0.16153
300	1.063	0.187	0.590245
500	1.402	0.560	0.281042

Table 4: Cyclin A levels in HepG2 cells treated with increasing concentrations of atrazine for 48 hours. * Ratio of the density of atrazine treated cells compared to control (0ppb) signal for three different nuclear extract samples treated with cyclin A. A statistically significant decrease was seen ($p<0.05$) for 50ppb atrazine compared to control.

Atrazine Concentration (ppb)	Average of ratios calculated for three trials *	Standard Deviation	P value
0	1	0	1
50	1.091	0.202	0.478188
100	0.967	0.460	0.907946
300	0.758	0.543	0.482968
500	0.698	0.280	0.134966

Table 5: Cyclin E levels in HepG2 cells treated with increasing concentrations of atrazine for 48 hours. * Ratio of the density of atrazine treated cell compared to control (0ppb) signal for three different nuclear extract samples treated with cyclin E. No statistically significant difference was seen ($p<0.05$) for any of the varying atrazine concentrations compared to control.

Atrazine Concentration (ppb)	Average of ratios calculated for three trials *	Standard Deviation	P value
0	1	0	0
50	0.485	0.233	0.018702
100	0.465	0.092	0.000535
300	0.387	0.083	0.000214
500	0.414	0.130	0.001465

Table 6: Cyclin B levels in HepG2 cells treated with increasing concentrations of atrazine for 48 hours. * Ratio of the density of atrazine treated cells compared to control (0ppb) signal for three different nuclear extract samples treated with cyclin B. A statistically significant difference was seen ($p<0.05$) for all of the varying atrazine concentrations (50,100, 300, 500ppb) compared to control.

Quantitation of cyclin levels after 24-hour cell synchronization exposure and 24-hour atrazine exposure

HepG2 cells were synchronized with aphidicolin for 24 hours followed by exposure to increasing concentrations of atrazine (0, 50, 100, 300, 500ppb) for 24 hours. Figure 10 shows a representative blot using anti-cyclin E antibody on HepG2 nuclear extract in which cells were synchronized with aphidicolin for 24 hours followed by exposure to varying levels of atrazine for 24 hours. A ratio was determined by comparing the density of the atrazine treated samples to untreated control proteins. The average for all of the trials was calculated as well as the standard deviation and standard error. Student T Test analysis was done for all trials. A p value of less than 0.05 was considered to be statistically significant.

Western Blot of HepG2 cells synchronized for 24 hours and exposed to atrazine for 24 hours, treated with cyclin E, and scanned on the STORM 860

Figure 10: Western Blot scanned on the STORM 860. Cyclin E protein can be seen at around 53kDa. Wells containing varying levels of atrazine concentration can be seen (from left to right- 500, 300, 100, 50, 0ppb atrazine, and molecular weight markers).

Synchronized HepG2 cells exposed to increasing concentrations of atrazine for 24 hours showed no significant difference in cyclin A expression levels when atrazine treated cells were compared to control cells (Figure 11). Synchronized HepG2 cells exposed to increasing concentrations of atrazine showed that cyclin E protein levels had

decreased to statistically significant expression levels in the 100, 300, and 500 ppb atrazine exposed cells compared to control cells (Figure 12). When cyclin B levels were measured in HepG2 cells synchronized with aphidicolin for 24 hours and exposed to increasing concentrations of atrazine for 24 hours there was a statistically significant decrease in cyclin B protein in the 50, 300, and 500 ppb atrazine exposed cells seen in Figure 13. Tables 7-9 show the ratio of the density of specific proteins of atrazine treated cells compared to control. Both cyclin A and cyclin E had 3 separate experiments, while cyclin B had four experiments.

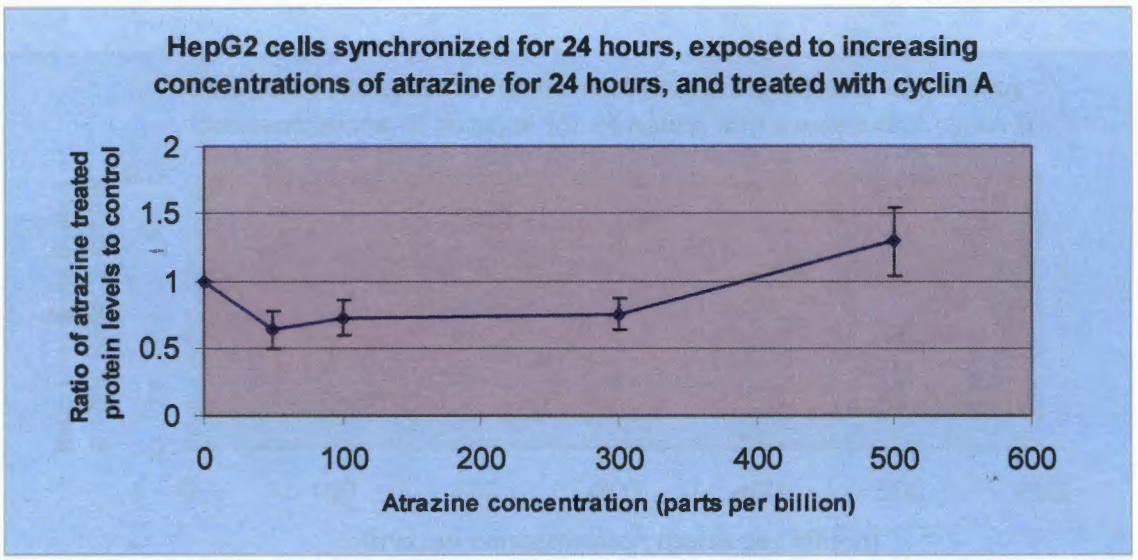

Figure 11: Western blot analysis showed that cyclin A levels of HepG2 cells synchronized for 24 hours and exposed to increasing concentrations of atrazine for 24 hours exhibited no statistically significant difference at any level of atrazine exposure compared to control (0ppb). Error bars are from standard error values. At all levels tested $p>0.05$.

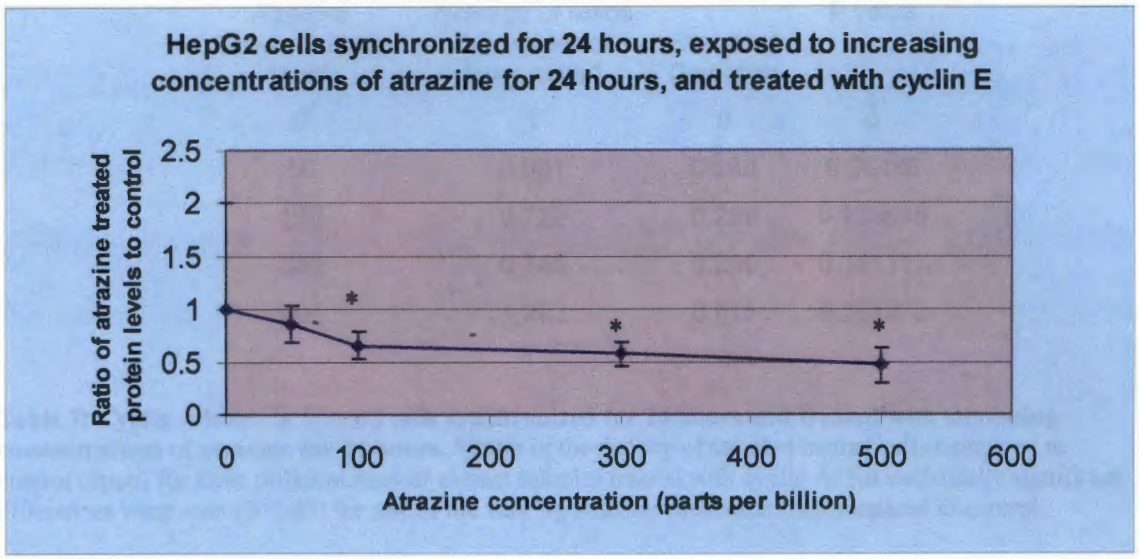

Figure 12: Western blot analysis showed that cyclin E levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited a statistically significant decrease at the 100, 300, and 500ppb atrazine exposure compared to control (0ppb). Error bars are from standard error values. * indicates a $p<0.05$ and values that are significantly significant compared to control.

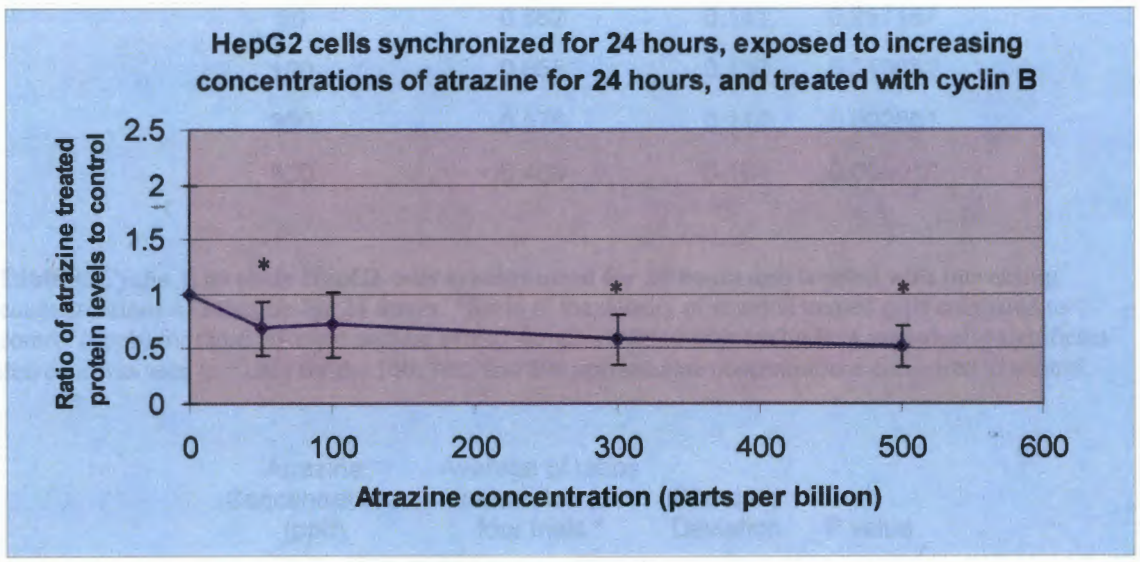

Figure 13: Western blot analysis showed that cyclin B levels of HepG2 cells synchronized for 24 hours and exposed to increasing concentrations of atrazine for 24 hours exhibited a statistically significant decrease in the 50, 300, and 500 ppb atrazine exposed cells compared to control (0ppb). Error bars are from standard error values. * indicates a $p<0.05$ and values that are significantly significant compared to control.

Atrazine Concentration (ppb)	Average of ratios calculated for three trials *	Standard Deviation	P value
0	1	0	0
50	0.631	0.280	0.08486
100	0.722	0.256	0.133646
300	0.748	0.239	0.141117
500	1.292	0.515	0.381372

Table 7: Cyclin A levels in HepG2 cells synchronized for 24 hours and treated with increasing concentrations of atrazine for 24 hours. *Ratio of the density of atrazine treated cells compared to control (0ppb) for three different nuclear extract samples treated with cyclin A. No statistically significant differences were seen ($p<0.05$) for any of the varying atrazine concentrations compared to control.

Atrazine Concentration (ppb)	Average of ratios calculated for three trials *	Standard Deviation	P value
0	1	1	1
50	0.862	0.181	0.257167
100	0.655	0.132	0.010652
300	0.576	0.110	0.002601
500	0.469	0.164	0.004918

Table 8: Cyclin E levels in HepG2 cells synchronized for 24 hours and treated with increasing concentrations of atrazine for 24 hours. *Ratio of the density of atrazine treated cells compared to control (0ppb) for three different nuclear extract samples treated with cyclin E. A statistically significant decrease was seen ($p<0.05$) for the 100, 300, and 500 ppb atrazine concentrations compared to control.

Atrazine Concentrations (ppb)	Average of ratios calculated for four trials *	Standard Deviation	P value
0	1	1	1
50	0.69	0.247	0.045842
100	0.722	0.295	0.10816
300	0.583	0.224	0.009699
500	0.522	0.188	0.002278

Table 9: Cyclin B levels in HepG2 cells synchronized for 24 hours and treated with increasing concentrations of atrazine for 24 hours. * Ratio of the density of atrazine treated cells compared to control (0ppb) for four different nuclear extract samples treated with cyclin B. A statistically significant decrease was seen ($p<0.05$) for the 50, 300, and 500 ppb atrazine concentrations compared to control.

Discussion

Many studies have been done showing the negative effects that pesticides, like atrazine, have on animal and human health. Atrazine, being one of the most widely used pesticides, is commonly found as a contaminant in ground water supplies (Uhler, 2001). The goal of this study was to determine the non-target effects of the herbicide atrazine on the growth of HepG2 cells.

Work from our lab has shown that human cells exposed to low doses of atrazine had fewer cells in culture after atrazine exposure when compared to control cells. It was hypothesized cell growth is being slowed down in these cells leading to the presence of fewer cells. This study attempts to determine if the expression of cyclin proteins is altered in atrazine exposed cells compared to unexposed cells.

The results of the current study have shown that cyclin A, B, and E expression patterns are altered in atrazine-exposed cells. Results varied with atrazine concentration exposed to cells (50, 100, 300, 500ppb), exposure of herbicide to cells (24 hour versus 48 hour) and whether cells were synchronized prior to atrazine exposure. In general, cyclin A expression was similar to control, but trended toward decreased levels, cyclin B expression decreased, and cyclin E expression decreased. Table 10 shows the results obtained for cyclin A, B, and E expression patterns.

Treatment	Cyclin E	Cyclin A	Cyclin B	Possible result
24 hour atrazine exposure	Decreased at all levels	No change compared to control	No change compared to control	G_1 block
24 hour atrazine exposure with synchronization	Decreased at all levels except 50ppb	No change, but trended to decrease at all except 500ppb	Decreased at 50, 300, and 500 ppb	G_1 block
48 hour atrazine exposure	Trended to decrease at all levels, but not statistically significant	Decreased at 50ppb, but otherwise no change	Decreased at all levels	G_2 block

Table 10: Results of cyclin A, B, and E expression patterns under the three HepG2 cell treatments. In general, cyclin E expression levels showed a decrease in comparison to control, cyclin A expression levels showed no change in comparison to control, and cyclin B expression levels showed a decrease in comparison to control.

Cyclin A, B, and E expression patterns after various exposure times to atrazine

HepG2 cells exposed to atrazine for 24 hours showed no statistically significant change in cyclin A or B expression patterns in comparison to control cells. However, both did show a general trend of decreased expression (Figures 3, 5). In contrast, a statistically significant decrease was seen in cyclin E expression patterns at all concentrations of atrazine exposure (50, 100, 300, 500ppb) in comparison to control cells (Figure 4). In combination, these results present a possible explanation for decreased cell growth. Cyclin E levels must reach a critical concentration in the G_1 phase to allow for activation of the G_1 cdk-cyclin complex and passage into S phase. In atrazine treated cells, cyclin E levels are found at decreased levels compared to control while cyclin A and B levels are similar to control. This suggests that atrazine treated cells are not able to pass through the G_1 phase and thus are arrested. Additionally, cyclin A and B levels did

not differ from control. This would be expected if the cells were being blocked in the G_1 phase.

While the 24-hour atrazine exposure represents approximately one cell cycle, the 48 hour atrazine treatment represents cells undergoing two cell cycles. Results showed cyclin A, B, and E levels trending toward a general decrease in cyclin expression as atrazine concentration increased. HepG2 cells exposed to atrazine for 48 hours showed cyclin E to have no statistically significant differences in expression compared to control cells, but the cells trended toward a general decrease in cyclin expression (Figure 8). There was a decrease in cyclin A expression at 50 ppb, but otherwise there was no change in cyclin A expression compared to control cells (Figure 7). However, cyclin B expression was decreased significantly at all levels of atrazine exposure (50, 100, 300, 500ppb) (Figure 9). These results present an explanation that is in contrast to the 24 hour exposed cells. In this case, atrazine exposure for 48 hours suggests a G_2 block as these cells showed a significant decrease in expression of cyclin B compared to control while there was no significant decrease with cyclin E or A. If the cells were blocked in the G_1 phase then a decrease in cyclin E levels should occur. Interestingly, cyclin E levels do tend to decrease however, not significantly.

Another treatment that was performed was to synchronize HepG2 cells with aphidicolin for 24 hours prior to atrazine exposure so that all cells began at the same phase of the cell cycle (G_1 phase) upon atrazine addition. The results from this treatment are a combination of the results obtained for the 24 and 48 hour unsynchronized treatments. Synchronized HepG2 cells exposed to atrazine for 24 hours showed that cyclin A expression was not significantly different from control however, there was a

trend of decreased cyclin A expression (Figure 11). However, cyclin E and B expression patterns significantly decreased. Cyclin E levels decreased at all atrazine concentrations except 50ppb (Figure 12) while cyclin B expression also decreased significantly at all atrazine concentrations except 100 ppb (Figure 13). These results suggest that a G_1 block may be present. The synchronization process should theoretically arrest all of the cells in G_1, so that all cells begin their cycle at the same time. If a G_1 block was occurring in atrazine treated cells, we would expect to see low levels of cyclin E and the great majority of cells would arrest in G_1 compared to control. The cells that were not blocked in G_1 would proceed through the cell cycle, however, the levels of cyclin A and B would be decreased compared to control since fewer numbers of cells are able to progress through the cell cycle compared to control cells as our results show.

Thus, in combination, our results suggest that atrazine exposure does in fact have an effect on cell cycle progression by altering levels of the proteins that are required to regulate passage through the cell cycle. From the 24 and 48 hour unsynchronized experimental results, we are unable to specifically determine if cells are undergoing a G_1 or a G_2 block. However, the 24 hour synchronized experiment strongly suggests that a G_1 block is present in these cells. Additional experiments have to be performed to provide definitive answers to our question.

Future work

This study identified several hypotheses for how cell growth is slowed in atrazine-exposed cells, including both a G_1 and G_2 block. However, additional experiments need

to be done to determine the mechanism by which cell growth is being slowed in atrazine-exposed cells.

A key experiment that needs to be performed, and was proposed for this project, is to conduct a time course experiment in which cells would be harvested at shorter timepoints following atrazine exposure. Because of a lack of time, it was not included in this study. The time course experiment proposed was to expose HepG2 cells to atrazine for varying lengths of time (1, 4, 8, 12, 24, 48 hours) prior to harvest of nuclear proteins. Because the cell cycle generally take 18-24 hours in HepG2 cells, a time course experiment would allow the ability to determine cyclin expression patterns at specified times in the cell cycle, which could be correlated with a specific phases of the cell cycle. Generally G_1 phase takes 8-10 hours, S phase takes ~6 hours, G_2 phase takes 4-6 hours, and M phase takes ~2 hours. Therefore, if cells were exposed to atrazine for 4 hours, the cell cycle would most likely be in the mid- G_1 phase, in which cyclin B is low, cyclin A is low, and cyclin E is low whereas at 8 hours, cells would be in late G_1, and we would expect higher levels of cyclin E with low levels of B and slowly rising levels of cyclin A. By doing a time course experiment and studying cyclin expression during short time points, we could determine when atrazine specifically is affecting the cells and at which phase in the cell cycle cyclins are being blocked.

An additional area of future work is to study cyclin D expression. Cyclins A, B, and E were used to determine if atrazine exposed cells have changing patterns of cyclin expression compared to control, leading to the slow down of cell growth. However, cyclin D expression also plays a major role in directing passage through the cell cycle. Figure 2 shows how cyclin D expression increases at the end of the M phase and

continues to stay increased until the beginning of the S phase where it begins to decline. It can also be seen in Figure 2 that when cyclin B levels are high, cyclin D levels are low, when cyclin E levels are high, cyclin D levels are also high, with the two cyclins declining around the beginning of the S phase, and when cyclin A levels are high, cyclin D levels are low. In future work, it will be important to include cyclin D in the experiments. With the presence of cyclin D it will be easier to identify whether a block is occurring at the end of G_1 phase, where cyclin D levels are high, or if a block is occurring at the end of the S phase, where cyclin D levels are low.

ACKNOWLEDGEMENTS

Funds for this study were provided by the SOAR Award from the College of Natural Sciences at the University of Northern Iowa.

Additional support provided by the Department of Biology and Chemistry at the University of Northern Iowa.

Special thanks to Dr. Kavita Dhanwada for her guidance, Dr. Peter Berendzen for being on the thesis committee, and Dr. Jeff Elbert for his assistance on the LC-MS chromatography.

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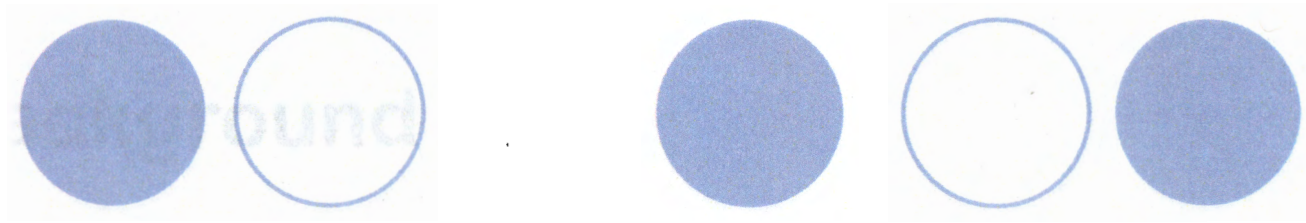


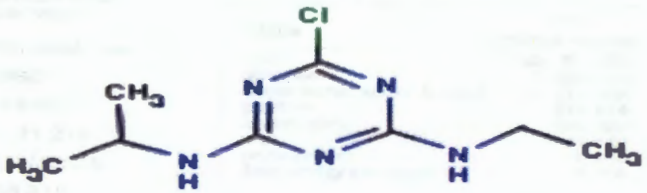
Cell cycle cyclin expression of atrazine-treated HepG2 cells using Western Blot analysis

April D. Rand and Kavita R. Dhanwada
Department of Biology and Chemistry

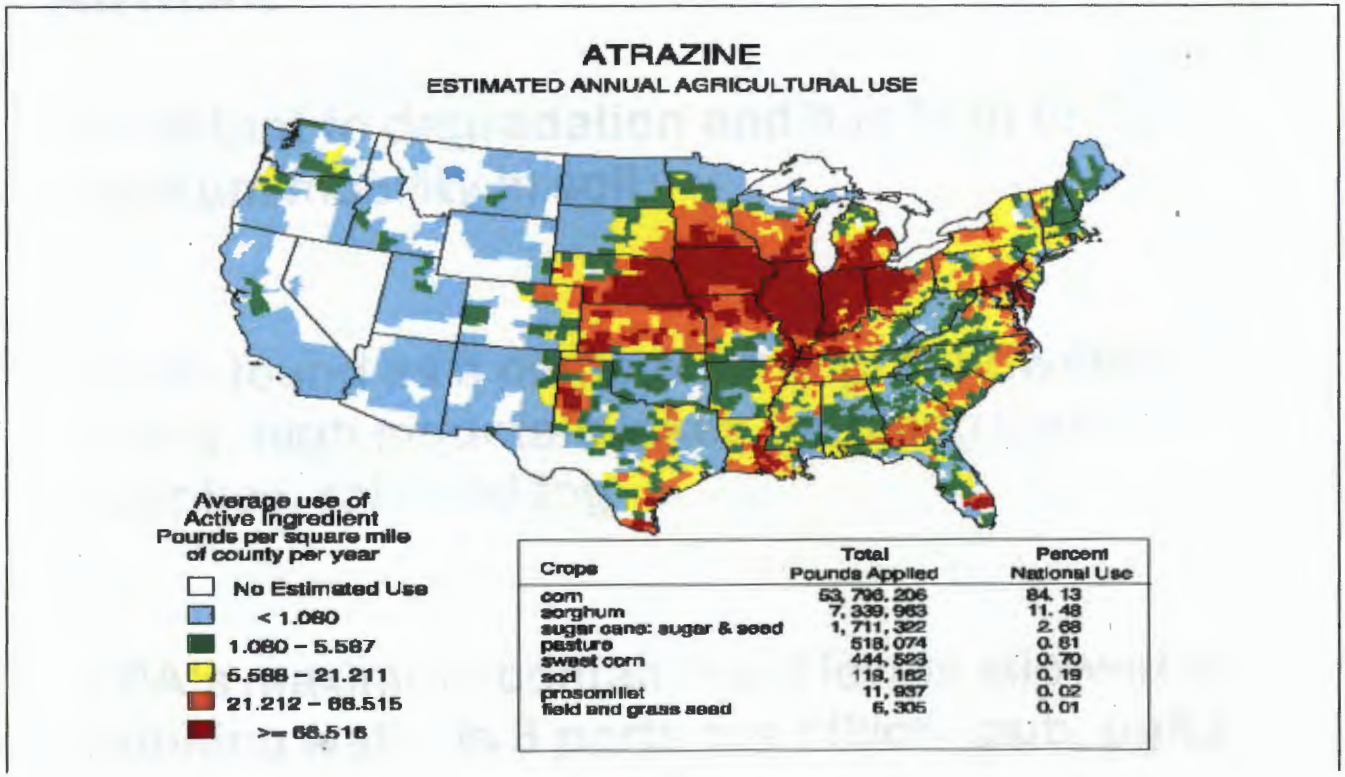
Background

Atrazine is one of the most commonly used pesticides in the U.S.

- Applied before and after planting to control broadleaf and grassy weeds
- Belongs to the triazine family



Background



Crops	Total Pounds Applied	Percent National Use
corn	53, 796, 206	84. 13
sorghum	7, 339, 963	11. 48
sugar cane: sugar & seed	1, 711, 322	2. 68
pasture	518, 074	0. 81
sweet corn	444, 523	0. 70
sod	119, 182	0. 19
prosomillet	11, 937	0. 02
field and grass seed	6, 305	0. 01

Background

Atrazine

Resistant to degradation and has high to medium mobility in soil

Often found as a contaminant- groundwater, rivers, high mountain lakes, drinking water supplies, rain and fog

EPA's maximum contaminant levels allowed in drinking water is 3 parts per billion (ppb, µg/L)

Effects of Atrazine

Target effects:

-inhibits photosynthesis

Non-target effects:

-correlations between exposure and cancer, immune system effects, and reproductive problems

-suggested to act as environmental estrogen

-can act as an endocrine disruptor

Effects of Atrazine

Atrazine and human health

Epidemiological study looking at pesticide contamination of drinking water and low birth weight babies

Rathbun water system serving 12 communities in Iowa had persistently high levels of herbicide contamination- including atrazine

All pesticide levels were below the EPA's acceptable levels

Found that rates of birth with IUGR was 1.8 times greater in Rathbun served communities

Our Lab

We are interested in looking at the effects of atrazine exposure on human cells at environmentally relevant levels

Previous work in our lab

Growth effects on cells:

Demonstrated that atrazine slows the growth of both normal and immortalized human cells

Normal human fibroblast cells exposed for 72 hours to 0.8 ppb atrazine caused a statistically significant decrease in cell growth

HepG2, a liver cell line, showed a statistically significant decrease at 12.5 ppb atrazine

Previous work in our lab

What is happening?

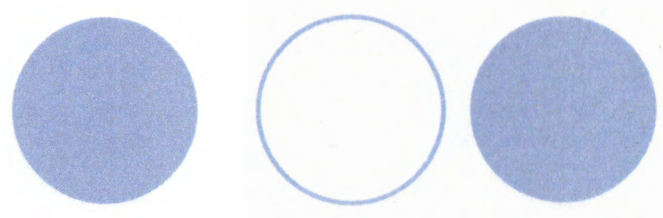
1) Cells are being killed due to the toxic effects of atrazine

- Have shown that no apoptosis or necrosis was occurring

2) Cells are taking longer to grow

- Topic of the current study

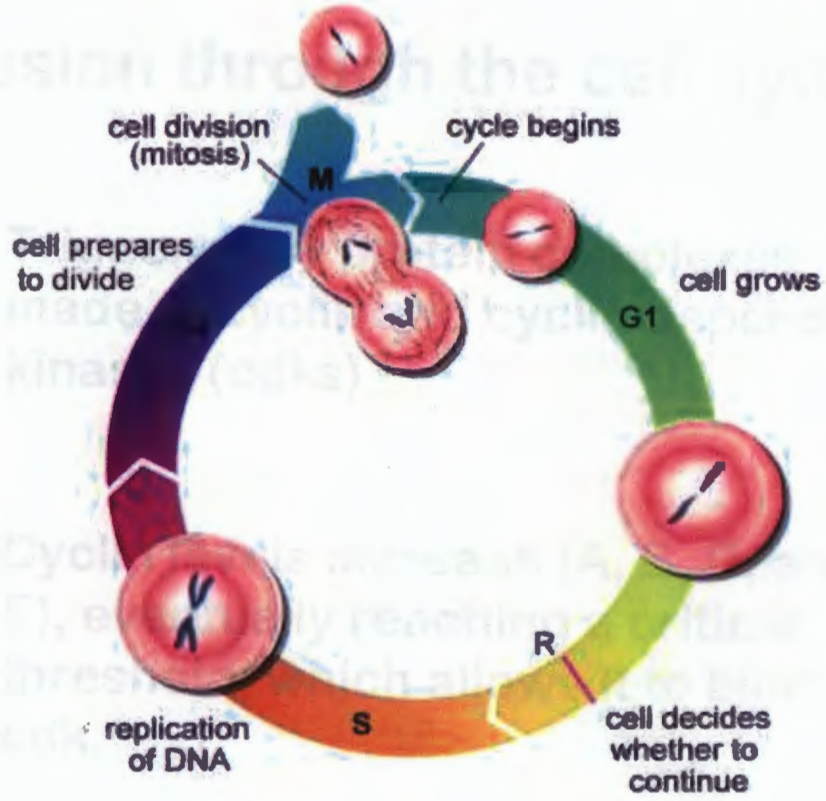
Current study



To determine if cell growth is in fact being slowed in atrazine exposed cells…

Expression of cell cycle proteins, cyclins, that regulate passage through the cell cycle was studied in atrazine exposed HepG2 cells

Cell Cycle


cell division
(mitosis)
M
cycle begins
cell prepares
to divide
cell grows
G1
R
replication
of DNA
S
cell decides
whether to
continue

Cell Cycle

Progression through the cell cycle

Triggered by protein complexes made of cyclin and cyclin dependent kinases (cdks)

Cyclin levels increase (A, B, D, and E), eventually reaching a critical threshold, which allows it to bind to cdk.

Cell Cycle

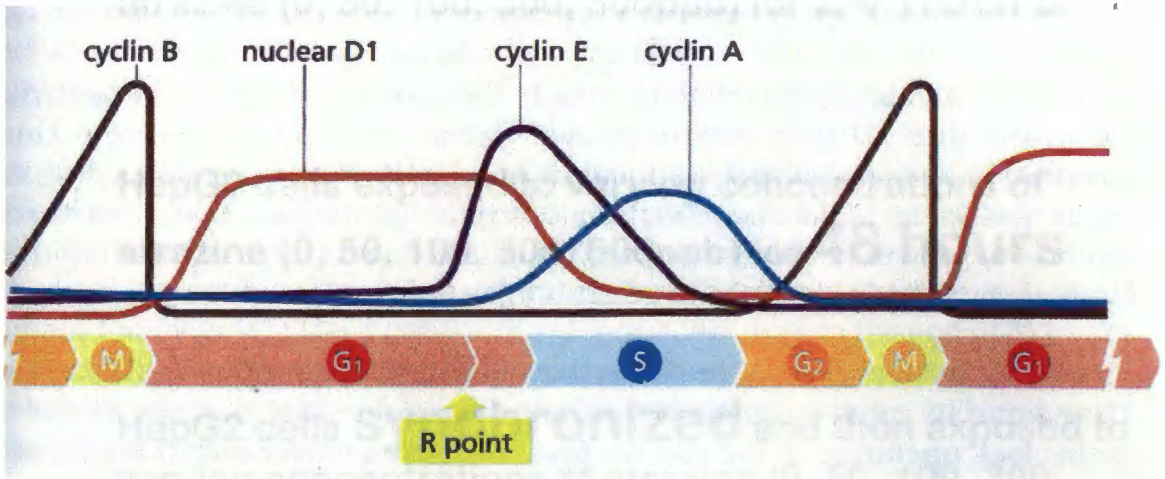
Progression through the cell cycle

An inactive cdk-cyclin complex becomes active after a series of phosphorylation and dephosphorylation events.

Once activated the cdk-cyclin complex can trigger passage through a specific phase (G1, S, G2, or M) in the cell cycle through its kinase activity.

Cell Cycle

Cyclin levels vary throughout the cell leading to progression through the cell cycle



Current Study

Three main parts

HepG2 cells exposed to varying concentrations of atrazine (0, 50, 100, 300, 500ppb) for **24 hours**

HepG2 cells exposed to varying concentrations of atrazine (0, 50, 100, 300, 500ppb) for **48 hours**

HepG2 cells **synchronized** and then exposed to varying concentrations of atrazine (0, 50, 100, 300, 500ppb) for **24 hours**

Methods

HepG2 Cells- a non-tumorigenic human hepatocellular carcinoma

Grown in 10% serum containing media at 37°C with 5% CO_2 until reaching 80% confluence

Day 1) Cells were plated

Day 2) Cells were exposed to varying concentrations of atrazine dissolved in cell media for 24 or 48 hours
- 0 (control), 50, 100, 300, and 500 ppb atrazine

or

Cells were exposed to aphidocolin for 24 hours, then exposed to varying concentrations of atrazine for 24 hours

Methods

Day 3) After 24 or 48 hours atrazine exposure, protein was extracted from the cells

- Protein quantitation was done using the Bradford assay

Methods

Day 4) Protein extract was run in an SDS-PAGE followed by Western Blot analysis

-primary antibody used was either anti-cyclin A, B, or E

-secondary antibody used was goat anti-rabbit-HRP conjugate

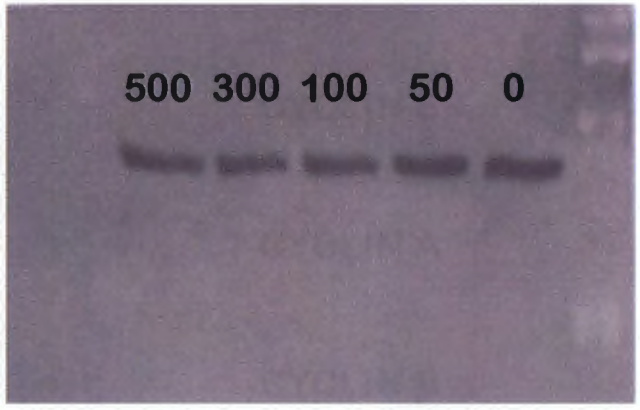
-proteins visualized using an enhanced chemiluminescence kit

-Scanning done on STORM 860

-Data analysis done on Image Quant 5.2 program

Results

Western Blot of HepG2 cells exposed to atrazine for 24 hours, treated with cyclin A, and scanned on the STORM 860



Cyclin A protein can be seen at around 60kDa. Wells containing varying levels of atrazine concentration can be seen (from left to right- 500, 300, 100, 50, 0ppb atrazine, and molecular weight markers).

Results

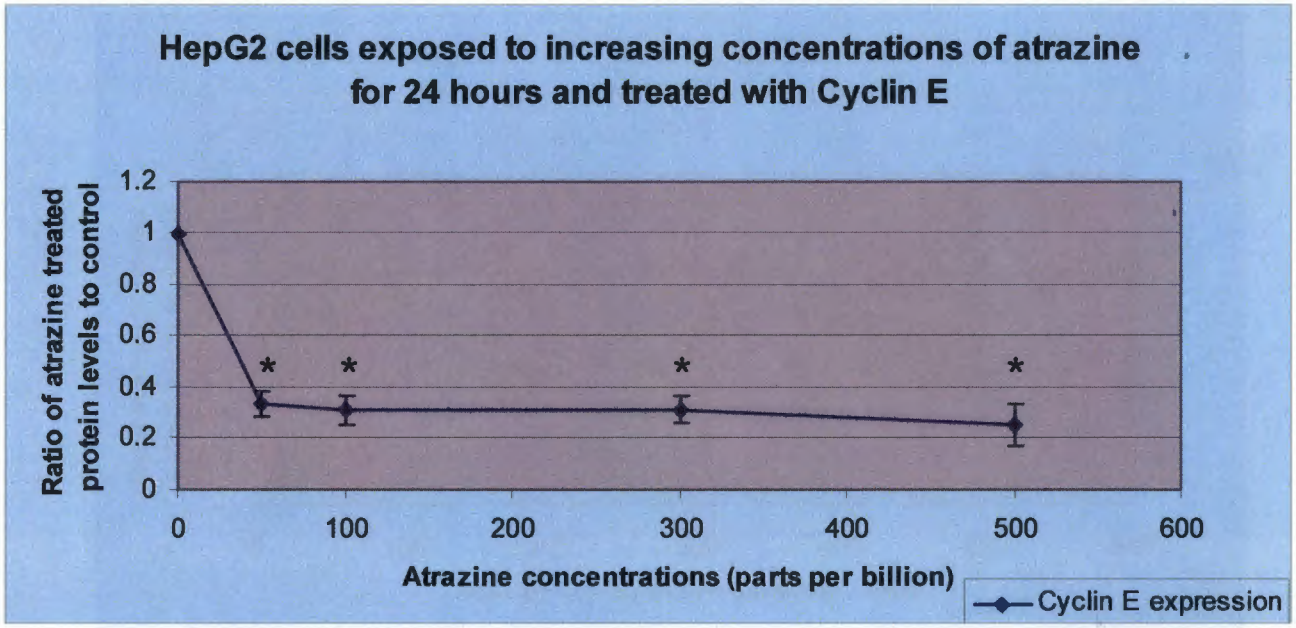
HepG2 cells exposed to varying concentrations of atrazine (0, 50, 100, 300, 500ppb) for 24 hours

CYCLIN E

CYCLIN A

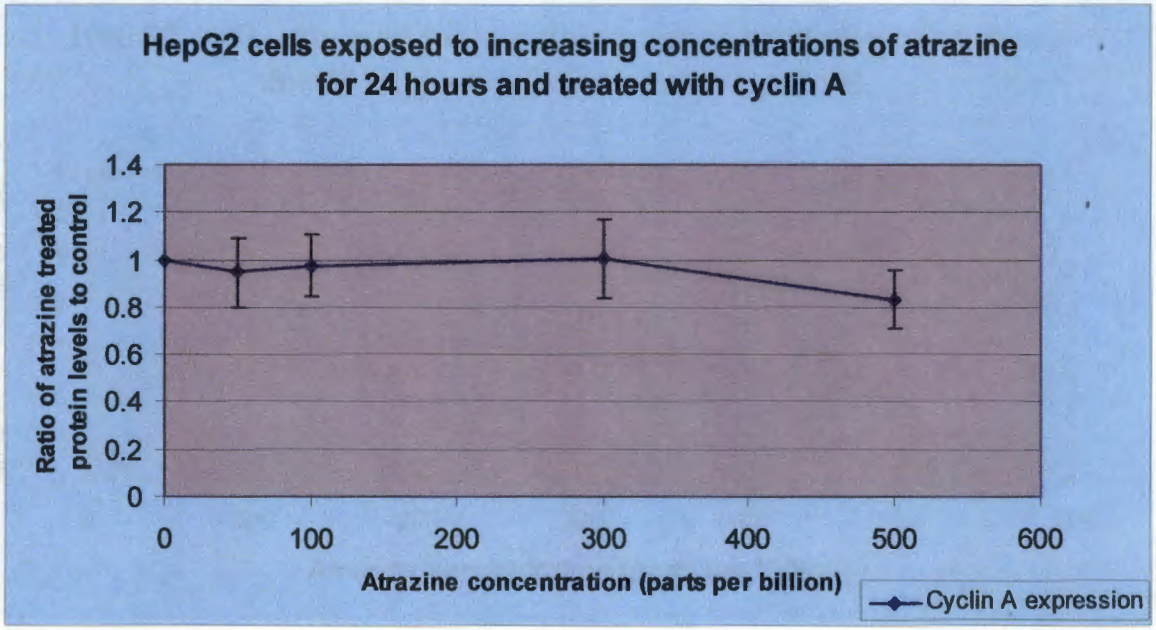
CYCLIN B

CYCLIN E- 24 atrazine exposure



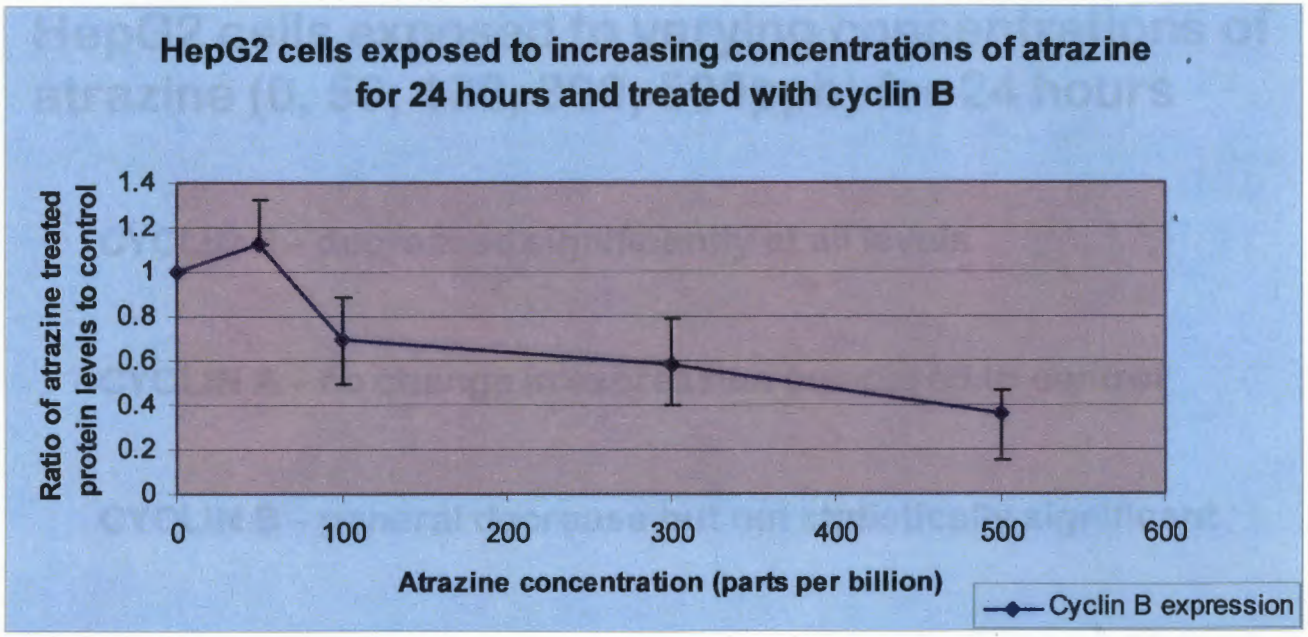
Cyclin E levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited a statistically significant decrease in expression at all levels of atrazine exposure (50, 100, 300, and 500ppb) compared to control (0ppb).

CYCLIN A- 24 hour atrazine exposure



Cyclin A levels of HepG2 cells exposed to increasing concentrations of atrazine did not exhibit any statistically significant differences compared to control (0ppb) cells.

CYCLIN B- 24 hour atrazine exposure



Cyclin B levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited no statistically significant difference at any level of atrazine exposure (50, 100, 300, and 500ppb) compared to control (0ppb).

Results

HepG2 cells exposed to varying concentrations of atrazine (0, 50, 100, 300, 500ppb) for 24 hours

CYCLIN E – decreased significantly at all levels

CYCLIN A – no change in expression compared to control

CYCLIN B – general decrease but not statistically significant

Results

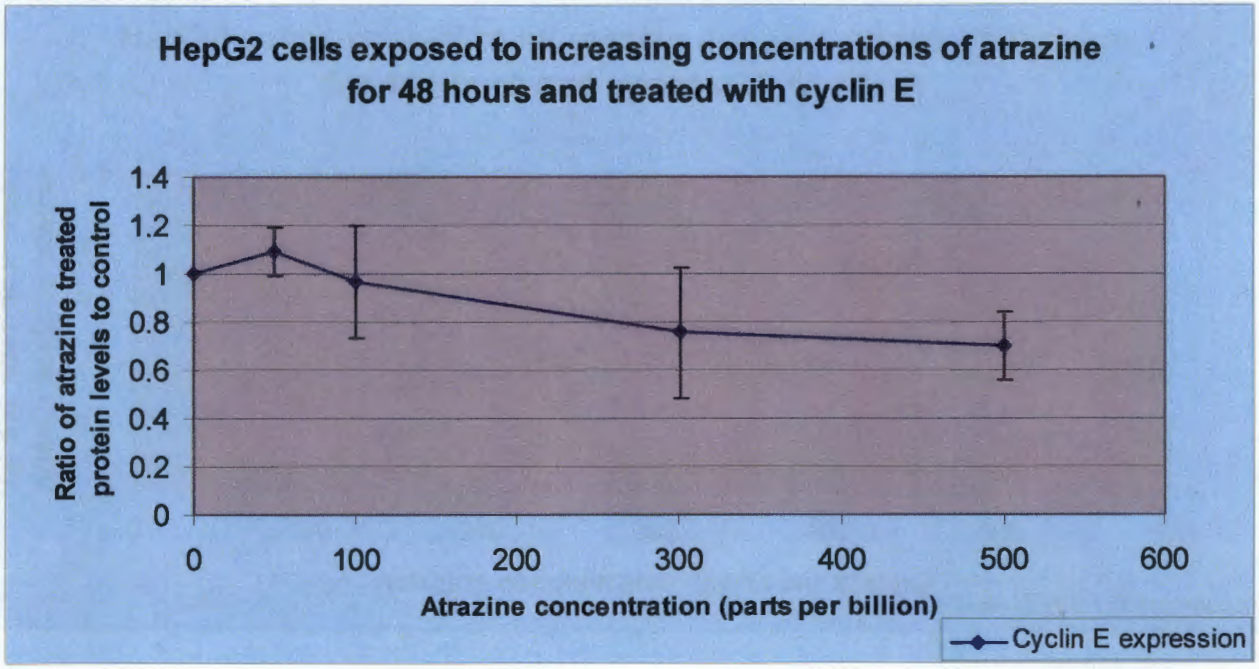
HepG2 cells exposed to varying concentrations of atrazine (0, 50, 100, 300, 500ppb) for 48 hours

CYCLIN E

CYCLIN A

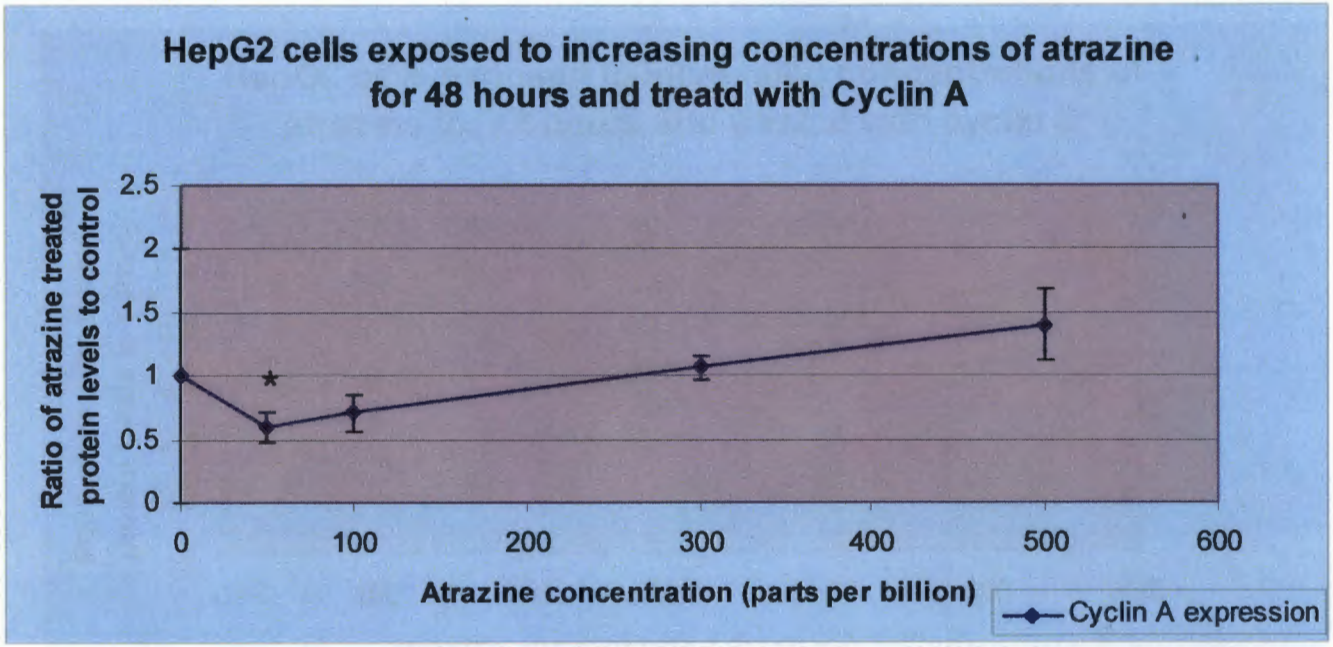
CYCLIN B

CYCLIN E- 48 hour atrazine exposure



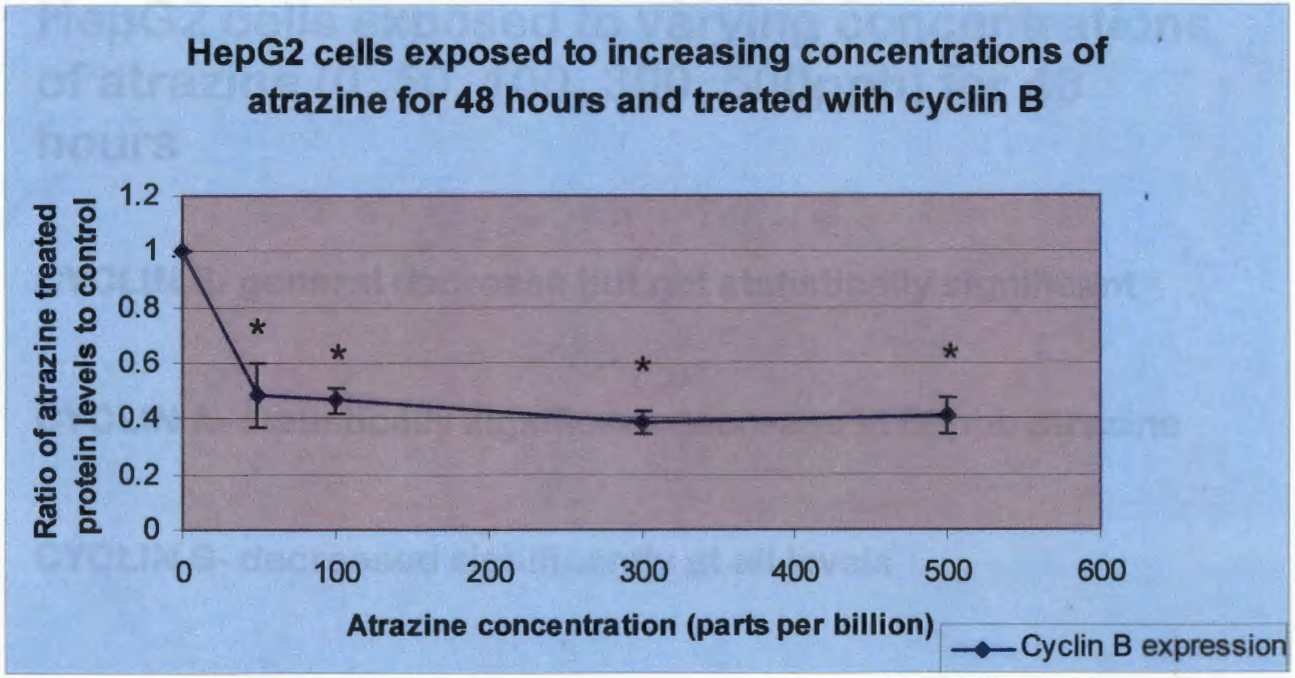
Cyclin E levels of HepG2 cells exposed to increasing concentrations of atrazine did not exhibit statistically significant differences at any level of atrazine exposure compared to control (0ppb), although there was a trend showing decreased levels of cyclin E.

CYCLIN A- 48 hour atrazine exposure



Cyclin A levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited a statistically significant decrease at 50ppb of atrazine exposure compared to control (0ppb).

CYCLIN B- 48 hour atrazine exposure



Cyclin B levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited a statistically significant decrease in protein levels at all levels of atrazine exposure (50, 100, 300, and 500ppb) compared to control (0ppb).

Results

HepG2 cells exposed to varying concentrations of atrazine (0, 50, 100, 300, 500ppb) for 48 hours

CYCLIN E- general decrease but not statistically significant

CYCLIN A- statistically significant decrease at 50ppb atrazine

CYCLIN B- decreased significantly at all levels

Results

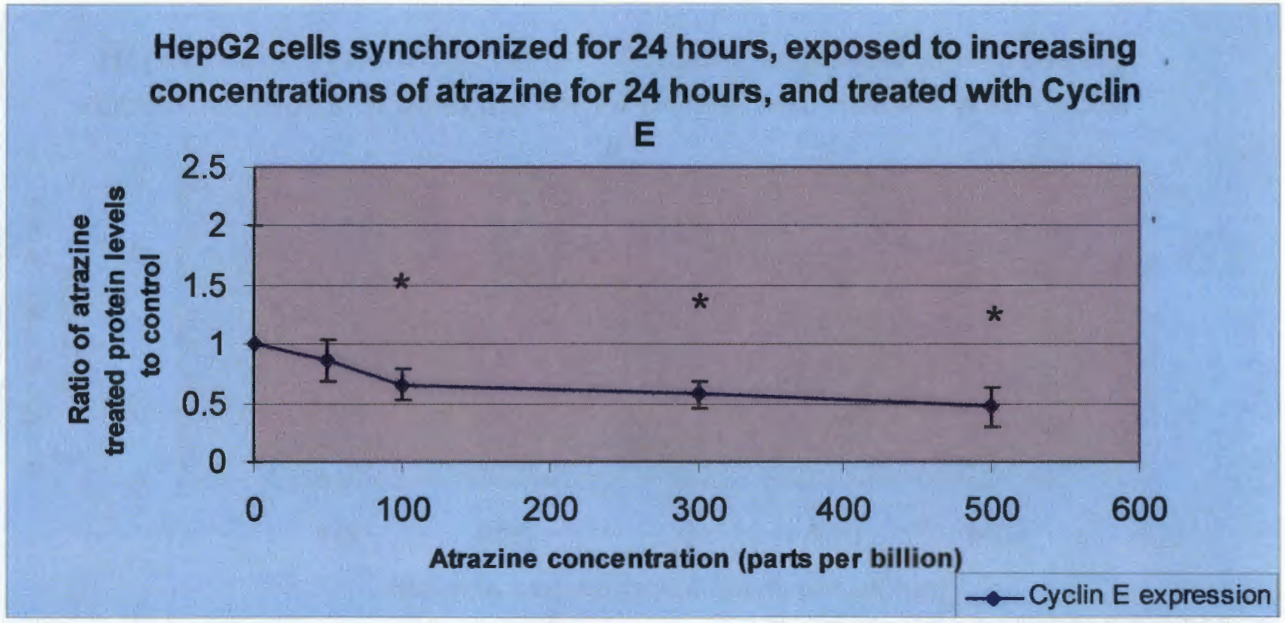
HepG2 cells synchronized and then exposed to varying concentrations of atrazine (0, 50, 100, 300, 500ppb) for 24 hours

CYCLIN E

CYCLIN A

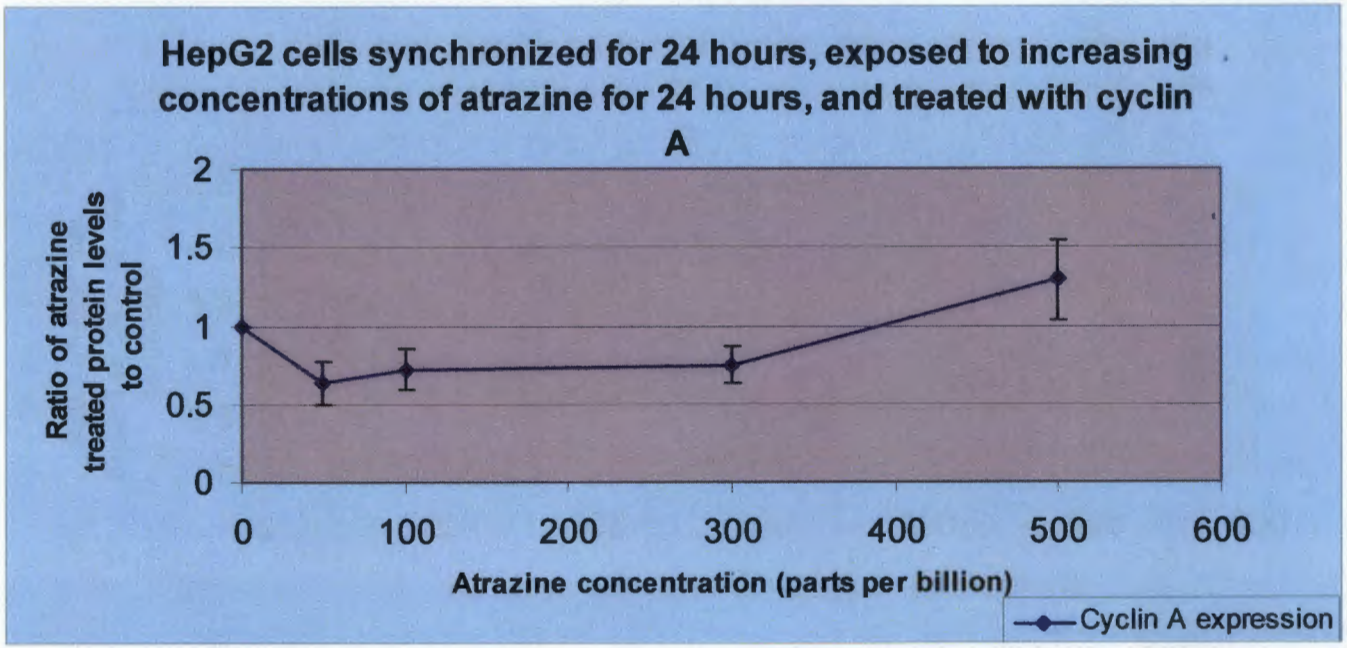
CYCLIN B

CYCLIN E- 24 hour synchronization and 24 hour atrazine exposure



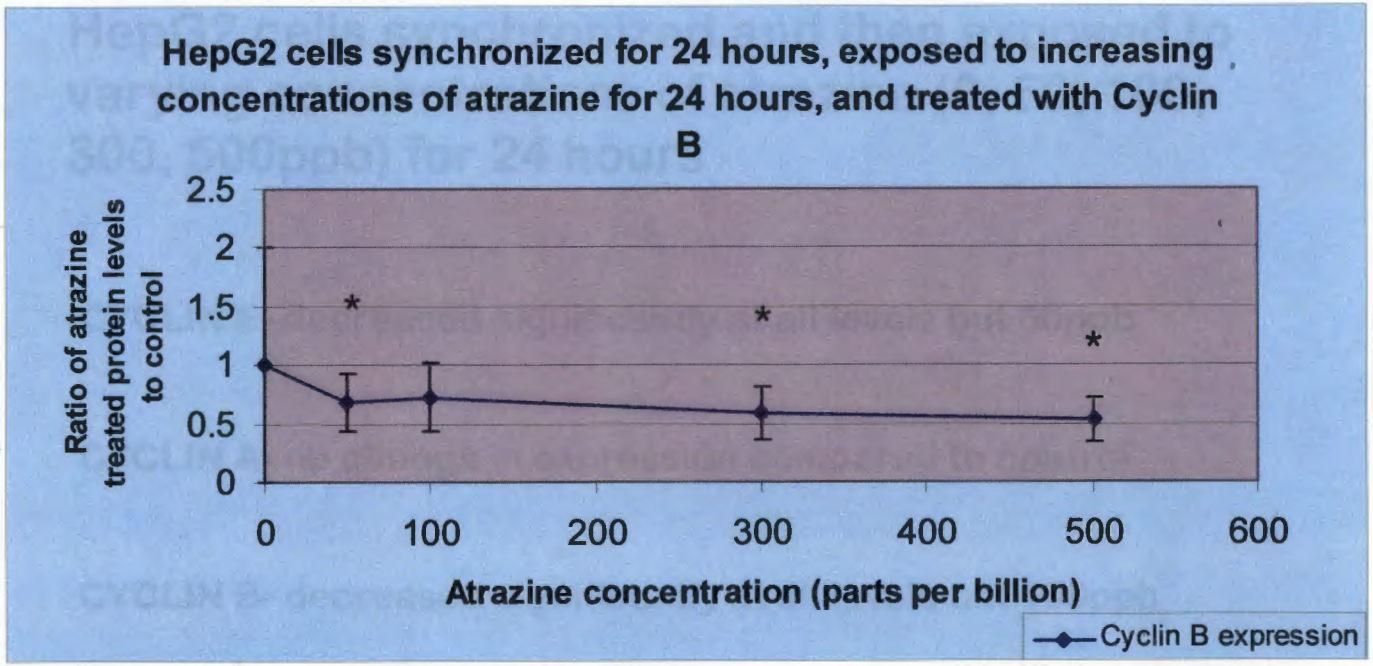
Cyclin E levels of HepG2 cells exposed to increasing concentrations of atrazine exhibited a statistically significant decrease at the 100, 300, and 500ppb atrazine exposure compared to control (0ppb). Error bars are from standard error values. * indicates a $p<0.05$ and values that are significantly significant compared to control.

CYCLIN A- 24 hour synchronization and 24 hour atrazine exposure



Cyclin A levels of HepG2 cells synchronized for 24 hours and exposed to increasing concentrations of atrazine for 24 hours exhibited no statistically significant difference at any level of atrazine exposure compared to control (0ppb). Error bars are from standard error values. At all levels tested, the p value was greater than 0.05 ($p>0.05$).

CYCLIN B- 24 hour synchronization and 24 hour atrazine exposure



Cyclin B levels of HepG2 cells synchronized for 24 hours and exposed to increasing concentrations of atrazine for 24 hours exhibited a statistically significant decrease in the 50, 300, and 500 ppb atrazine exposed cells compared to control (0ppb).

Results

HepG2 cells synchronized and then exposed to varying concentrations of atrazine (0, 50, 100, 300, 500ppb) for 24 hours

CYCLIN E- decreased significantly at all levels but 50ppb

CYCLIN A- no change in expression compared to control

CYCLIN B- decreased significantly at all levels but 100ppb

Discussion

Factors studied:

atrazine concentration exposed to cells (50, 100, 300, 500ppb)

treatment of the cells (24 hour versus 48 hour exposure) and

whether cells were synchronized prior to atrazine exposure

Discussion

Consistently found that expression patterns in the three cyclins studied, cyclins E, A, and B, were **changing** in the atrazine exposed cells in comparison to control cells.

In general...

cyclin E expression decreased

cyclin A expression tended to stay at levels the same as control

cyclin B expression decreased

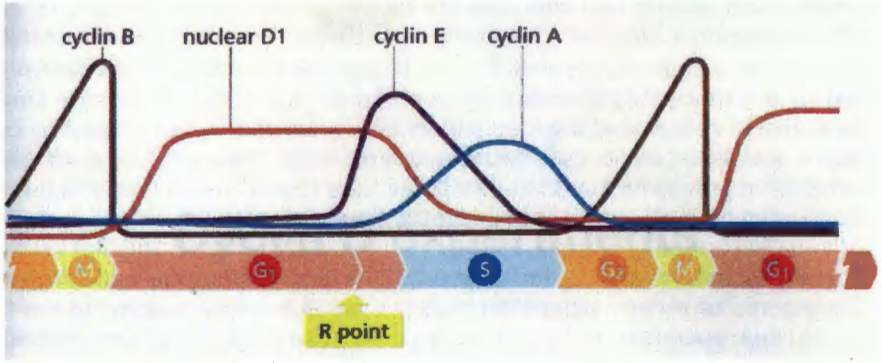
Discussion

G1 block?

or

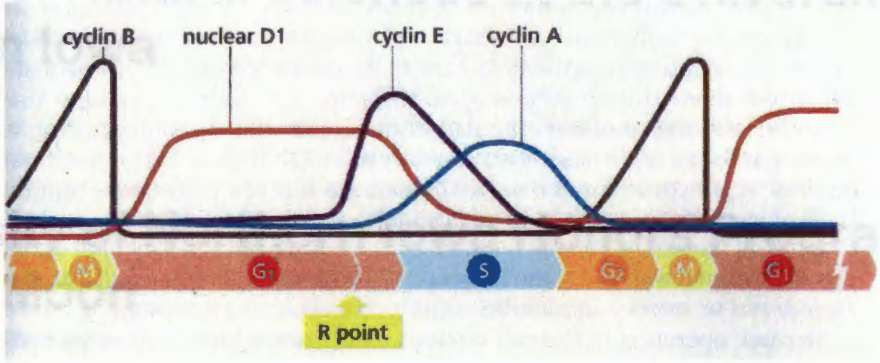
G2 block?

Treatment	Cyclin E	Cyclin A	Cyclin B	Possible result
24 hour atrazine exposure	Decreased at all levels	No change compared to control	No change compared to control	G_1 block and maybe G_2 block
24 hour atrazine exposure with synchronization	Decreased at all levels but 50ppb	No change, but trended to decrease at all but 500ppb	Decreased at 50, 300, and 500 ppb	G_1 block
48 hour atrazine exposure	Trended to decrease at all levels, but not statistically significant	Decrease at 50ppb, but otherwise no change	Decreased at all levels	G_1 block or G_2 block



Future Work

Time Course experiments



Cyclin D experiments

Acknowledgements

-Funds were provided by the SOAR Award from the College of Natural Sciences at the University of Northern Iowa

-University of Northern Iowa Honors Program and Jessica Moon

-Special Thanks to Dr. Kavita Dhanwada, Peter Berendzen, and Dr. Jeff Elbert

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