EFFECTS OF PHENANTHRENE, A POLYCYCLIC AROMATIC HYDROCARBON ON ZEBRAFISH (*Danio rerio)* EARLY DEVELOPMENT

by

Rana Kalmoni

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Dr. Murali Pillai, Chair

Dr. James Christmann

Dr. Michael Cohen

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Date

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EFFECTS OF PHENANTHRENE, A POLYCYCLIC AROMATIC HYDROCARBON ON ZEBRAFISH (*Danio rerio*) DEVELOPMENT

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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAH) are ubiquitous environmental contaminants released from different sources such as forest fires and ocean oil seeps, as well as land based runoff and creosote. These sources of PAHs are both natural and anthropogenic. Some PAHs have been known to be carcinogenic and mutagenic. In this project, the effects of phenanthrene, a PAH compound, on early embryogenesis involving somitogenesis (development of somites) were studied using zebrafish (*Danio rerio*) early embryos as an experimental system. The developmental stage relevant to this project was the segmentation stage, during which time somites develop. In early embryos, somite patterning is under the influence of Wnt signaling. Although the process of somitogenesis remains to be fully understood, the "clock and wavefront" model is widely accepted and the process involves a variety of cellular and molecular interactions.

This research shows the developmental stage specific effects of phenanthrene on somitogenesis. Other developmental abnormalities due to phenanthrene exposure were also noticed and documented; these abnormalities included pericardial/yolk sac edema and scoliosis. Embryos treated at 2 hours post fertilization (hpf) were most sensitive to phenanthrene exposure and displayed abnormal development of somites and showed a decreased total somite number. Embryos treated at 8 and 12 hpf did not show such abnormalities, suggesting there is a critical time in development when somitogenesis is most affected by phenanthrene. Involvement of the Wnt signaling pathway in somitogenesis with an apparent increase in nuclear β-catenin was tested through pharmacological and immunological studies. 1-Azakenpaullone, an inhibitor of Glycogen Synthase Kinase-3β (GSK-3β), was used to target a specific step in the pathway. The results from embryos exposed to this inhibitor were similar to embryos exposed to phenanthrene. Immunostaining of embryos exposed to both compounds reveals nuclear accumulation of β-catenin, suggesting that phenanthrene may be causing abnormalities by disrupting a specific step in the Wnt signaling pathway. Preliminary Western blot analysis using antibodies to phosphorylated forms of GSK-3β (anti-Ser 9-GSK-3β) also supports this notion.

Chair:
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INTRODUCTION

Over the past two decades, the zebrafish (*Danio rerio)* has become a widely used vertebrate model system for studying developmental biology. This is in part due to the transparent nature of its eggs and embryos, rapid embryonic development, quick generation time and ease of maintenance of adults and embryos, as well as the availability of a well established large tool-box of genetic and cell biological methods (Westerfield, 1995; Bryson-Richardson et al., 2007). Spawning of zebrafish can easily be induced by photoperiod manipulation and feeding regime adjustments. Another advantage to using zebrafish as a study system is their high fecundity. Each female can lay as many as 200 eggs, and through proper maintenance, this yield can successfully be achieved every 5 to 7 days (Hill et al., 2005). In addition to studying vertebrate developmental biology, the zebrafish has recently been widely used in toxicology experiments. Both long and short term effects of various environmental toxins, including carcinogenic agents, have been investigated using this system (Lele and Krone, 1996; Gong et al., 2009). For example, Gong et al. (2009) have developed a transgenic zebrafish system to investigate how zebrafish may respond to the presence of different pollutants in its environment. In these studies, microinjection techniques were used to incorporate the gene for either GFP or dsRed into the genome, giving the fish the ability to fluoresce either green or red when exposed to different pollutants. Two gene promoters, either an estrogen inducible promoter or stress responsive promoter, have

been identified by this group; these genes have been used to drive the fluorescent color genes in the transgenic zebrafish.

There are several distinct stages of early zebrafish development, and these occur rather rapidly: cleavage, blastula, gastrula, segmentation (somitogenesis period), pharyngula, and hatching. Under normal culture conditions, all the major organ systems are laid out in just 24 hours (Westerfield, 1995; Kimmel et al., 1996). The transparency of eggs and embryos allows easy developmental staging and visualization of any developmental abnormalities induced by experimental manipulations including toxicant exposure.

A newly fertilized egg is surrounded by a chorion, a thick protective outer covering. This protective envelope is formed as a result of a massive egg cortical reaction (cortical granule exocytosis) and the reorganization of the egg surface induced by fertilization (Becker et al., 1999). Approximately 40 minutes after fertilization, the embryo enters the cleavage stage. Cleavage is meroblastic and occurs about every 15 minutes, and is restricted to the disc of yolk-free cytoplasm at the animal pole (meroblastic discoidal cleavage). At the 128-cell stage, the embryo enters the blastula stage. During the late blastula stage, epiboly begins and continues into the gastrulation period (Kimmel et al., 1996).

The gastrulation period precedes the segmentation stage, and during this time, the embryo undergoes various types of morphogenetic movements to produce the three primary germ layers (endoderm, ectoderm, and mesoderm) and establishes the embryonic axis (Kimmel et al., 1996). The developmental stage specific to this project was the

segmentation stage during which time the somites develop. Somites are highly transient structures derived from the paraxial mesoderm in the developing embryo. During subsequent stages of development, somites differentiate into such structures as skeletal components of the vertebral column, skeletal muscles, and dermis of the back (reviewed by Holley, 2006).

The segmentation period starts at approximately 10 hpf and lasts until about 24 hpf. During this 14 hour period, a new pair of somites forms approximately every 30 minutes. The process of segmentation involves cells of the presomitic mesoderm (PSM) undergoing mesenchymal to epithelial transitions, forming epithelia around loosely packed mesenchymal cells. Somites are formed in this fashion until the end of the segmentation period, at which point about 30 pairs of somites flank the notochord area (Stickney et al., 2000). The process of somitogenesis in zebrafish is similar to that documented for amphibians, birds, and mammals (reviewed by Kimmel et al., 1995).

By the time segmentation is completed, each individual somite has developed into sclerotome which gives rise to the axial skeleton, and myotome, which gives rise to the skeletal muscles of the trunk. The zebrafish somite is mostly myotome; sclerotome comprises only a small portion (Stickney et al., 2000). This is largely because zebrafish do not need a tough skeleton for support; they are supported by a swim bladder and need larger muscles to help propel them through their environment (Stickney et al., 2000).

The mechanism and regulation of somitogenesis is one of the major unresolved issues in developmental biology (Schnell, 2000). There are several different models that have been proposed to explain the process of somitogenesis, but current hypotheses of

somite development claim the existence of a genetic oscillator called the segmentation clock (Schroter et al., 2008). According to this model, also known as the "clock and wavefront" model, cells in the PSM are able to form somites only at a certain time period in the clock. The wavefront refers to the anterior to posterior patterning of somites in the developing embryo. Furthermore, the wavefront is responsible for the morphogenesis of somites, but its activity is under control of the clock; new somite boundaries can form only when the wavefront reaches cells that are in the appropriate phase of the clock (Holley, 2007). This model was first described by Cooke and Zeeman in 1975 and is still widely accepted. The clock and wavefront model is applicable to other vertebrates as well, such as chick and mouse. During somite formation in zebrafish, expression of at least two genes, *her1* and *her 7,* oscillate on and off in the PSM, with the periodicity equal to the formation of one somite (Stickney et al., 2000; Cinquin, 2007).

A variety of molecular and cellular interactions are thought to be involved in the patterning of somites. These involve signaling molecules from the notochord and neural tube, as well as from the surface ectoderm. Formation of the sclerotome portion of the somite is under influence of the Sonic Hedgehog homologue signal molecules (Shh) which are secreted by the notochord and the floor plate of the neural tube (Capdevila et al., 1998). Patterning of the myotome, which comprises the majority of the somite, involves Wnt signals secreted from the notochord and surface ectoderm. Wnt proteins have been implicated in the formation of the myotome since they are able to induce myogenic differentiation in competent somitic cells in explants of presomitic and somitic mesoderm (Capdevila et al., 1998). In addition to its role in formation of the myotome,

Wnts are responsible in activating the Wnt/β-catenin pathway involved in growth and differentiation of a variety of other embryonic cells and tissues (Komiya et al., 2008).

Wnt/β-catenin Pathway, an Overview

Wnt signaling, a highly conserved signal transduction pathway found in embryonic and adult tissues of all metazoans (Wikramanayake et al., 2003; Nelsen and Nusse, 2004), is clearly involved in a variety of developmental processes including cell fate determination and embryonic patterning in many animal groups (Borello et al., 2006). Defects in this pathway are also strongly linked to the development of various types of cancers (reviewed by Lusting and Beherns, 2003) and teratogenesis, including perturbation of embryonic axis specification (Pillai et al., 2003; Wikramanayake et al., 2003; Lara et al., 2010). β -catenin, historically known for its crucial role in cadherin mediated cell-cell adhesion, has more recently been implicated in cell signaling events within the canonical Wnt signaling pathway. The canonical Wnt signaling pathway involves a complex array of regulators, including multiple kinases and phosphatases (see Fig. 1). It is now known that in an unstimulated cell, the enzyme Glycogen Synthase Kinase 3β (GSK-3β) exists in a complex with an array of other proteins and targets β-catenin for degradation via phosphorylation. If the extracellular Wnt signals (a family of cysteine-rich glycoproteins) are present, they interact with cell surface receptors of the Frizzled family, activate Disheveled (Dv1), and ultimately result in the inhibition of GSK-3β activity. When β-catenin is no longer phopshorylated by GSK-3β, it accumulates in the cytoplasm, some of which moves into the nucleus, and activates target

genes by binding to transcription factors of the LEF/TCF family (reviewed by Ferekey and Kimelman, 2000; Percina-Slaus, 2010).

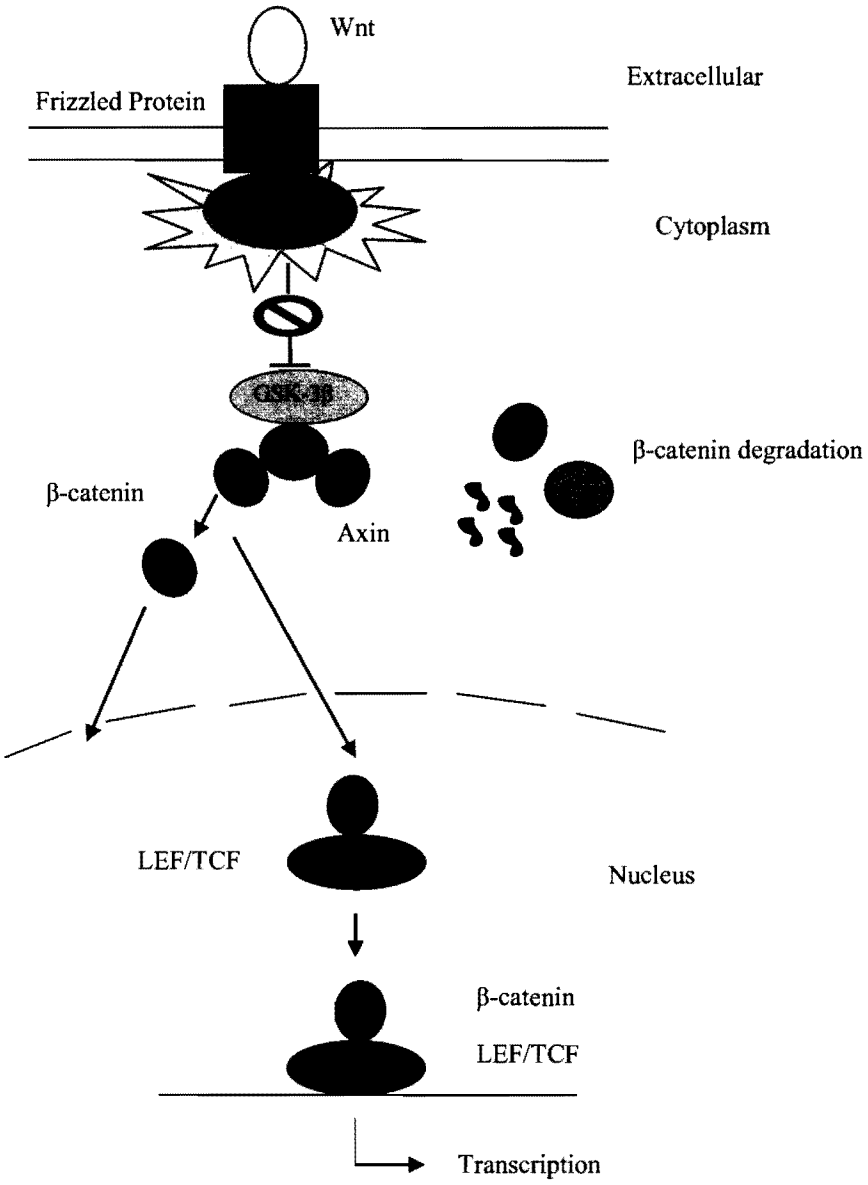

Figure 1. The canonical Wnt signaling pathway (modified from Komiya and Habas, 2008). When present, the extracellular Wnt signals interact with the cell surface receptors of the Frizzled family. This in turn recruits Dishevelled to the cell membrane, where its activity inhibits the function of Glycogen Synthase Kinase-3β (GSK-3β). GSK-3β exists in a complex with Axin, APC, and other cytosolic regulatory proteins. The job of GSK-3β is to mark β-catenin for degradation, therefore not allowing it to move into the nucleus. Because GSK-3β is constitutively active, β-catenin in the cytoplasm is constantly being degraded. Once the activity of GSK-3β is inhibited (as shown in the pathway above), β-catenin is not degraded, rather, it is translocated into the nucleus, where it functions as a co-activator of transcription for the LEF/TCF responsive family of genes.

Chemistry and Toxicology of Polycyclic Aromatic Hydrocarbons

This project investigates the effects of a common class of environmental pollutants, polycyclic aromatic hydrocarbons (PAHs), on the Wnt signal transduction pathway associated with zebrafish somitogenesis. PAHs consist of two or more fused benzene rings (See fig. 2) and are very stable in the environment (Alkio et al., 2005). These organic compounds enter the environment from a number of sources including ocean oil seeps, coal, wood preservatives (creosote), petroleum and fossil fuels, engine exhaust, and cigarette smoke (Gmeiner et al., 1997; Baek and Jenkins, 2004; Billiard et al., 2006). Human exposure to PAHs usually occurs by breathing air that has been contaminated by tobacco smoke, residential wood burning, etc. and eating foods that have been charbroiled. Since PAHs are generally insoluble in water, they tend to adhere to solid particles and settle to the bottom of rivers and lakes. Coal tar creosote, which is largely used as a wood preservative, is comprised of over 300 chemical constituents; the majority of these are PAHs such as phenols, creosols, xylenols, phenanthrene and benzene (Carlsten et al., 2005). The PAH constituents of creosote have been shown to be cytotoxic, genotoxic, and carcinogenic to humans and other organisms (reviewed by Pillai, et al., 2003). Previous studies have shown that PAHs cause increased incidences

of lung, liver, bladder, and gastrointestinal carcinomas in humans (reviewed by Varanasi, et al., 1989; Pillai et al., 2003). Recent studies have also shown that occupational exposure to PAHs may lead to an increased risk of developing skin, prostate, lung, and bladder cancers as well (Moretti et al., 2007).

Because of their hydrophobic nature, PAHs are able to permeate biological membranes and accumulate in cells and tissues. Studies using laboratory animals such as mice have shown increased incidences of skin cancers due to exposure of creosote which contains various PAH compounds (Carlsten et al., 2005). Although toxicological effects of PAHs are generally mediated by binding to the aryl hydrocarbon receptors (AhR) in target cells and their subsequent activation into reactive compounds (Tanguay et al., 1999), studies have shown a possible link between PAH exposure and teratogenesis in developing embryos through its effect on the Wnt signal transduction pathway. In a study by Pillai et al. (2003), following exposure to a variety of PAH compounds including phenanthrene, early sea urchin embryos displayed developmental defects such as exogastrulation. This study also showed that disruption of axial development caused embryos to develop with evaginated archentra via increased nuclear β-catenin, a crucial mediator of signaling and transcriptional changes regulated by the canonical Wnt signaling pathway.

Earlier studies of zebrafish exposure to PAHs have shown a number of developmental perturbations. These included reduced growth, cranial-facial malformations, yolk sac and pericardial edema, and subcutaneous hemorrhage (Wassenberg and Di Guilio, 2004). These morphological and developmental defects are

consistent with the findings of Incardona et al. (2003), in which zebrafish embryos treated with several PAHs, including phenanthrene, showed severe yolk sac and pericardial edema. Embryos were exposed to PAHs or DMSO, using static renewal exposure conditions, at 4-8 hpf. Phenanthrene treated embryos showed severe pericardial and yolk-sac edema. Whether any of these observed effects of phenanthrene on these critical stages of development are mediated through disruption of one or more components of the Wnt signaling pathway is unknown.

This project examined the effects of phenanthrene, one of the more abundant PAH constituents found in the environment, on zebrafish early embryos. Significant levels of phenanthrene have been found in the air, food, and in many aquatic environments (Kang, et al., 2007).

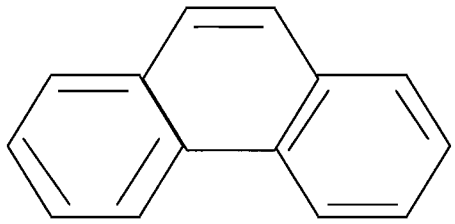

Figure 2. Structure of phenanthrene.

I aimed to study the effects of this compound on somitogenesis in the developing zebrafish embryo. I documented the developmental stages and concentration at which embryos were most sensitive to phenanthrene exposure, as well as morphological defects seen in somites from the experimental group. Changes in somite number were also investigated; this involved comparing somite numbers in experimental groups to somite numbers in the control groups. Other developmental abnormalities induced by

phenanthrene in embryos were noted as well. To examine whether a certain step in the Wnt signaling pathway was directly being affected by phenanthrene, studies were conducted using several commercially available GSK-3β inhibitors. In addition, preliminary studies were conducted to examine if phenanthrene exposure would cause a decrease in GSK-3β activity with a concomitant increase in nuclear β-catenin accumulation.

Western Blot analysis of embryo extracts using antibodies against phosphorylated forms of GSK-3β was also performed to investigate the possible mechanism by which phenanthrene may induce increase of nuclear β-catenin accumulation.

MATERIALS AND METHODS

Animal Maintenance and Embryo collection

Stocks of adult zebrafish (*Danio rerio)* were purchased from Aquatica Tropicals Inc. (Plant City, FL) or Carolina Biological Supply (Burlington, NC) and maintained under standard laboratory conditions at 28°C with a 14 hour light/10 hour dark cycle. Fish were induced to spawn through photoperiod manipulation and alteration of feeding regime, as described by Westerfield (1995). Fish were fed flake food (Tretramin) twice a day; the night before induction of spawning, frozen brine shrimp were incorporated into the diet. Clean marbles were placed in the bottom of the tank the night before spawning, and embryos were siphoned the following morning. After collection, embryos were washed in embryo medium (EM) containing 0.14 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM K_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4 \cdot 7H_2O$, and 4.2 mM $NaHCO_3$ (Westerfield, 1995) at least three times to remove any debris.

Dechorionation of Early Embryos

Prior to toxicant exposure, embryos were dechorionated using a mild solution of pronase (2 mg/ml, Sigma Aldrich, St. Louis, MO) in EM. Removal of the chorion allowed for ease of visualizing development of somites and other morphological abnormalities (Westerfield, 1995) as well as better penetration of toxins. For dechorionation, approximately 100 embryos were placed in sterile glass fingerbowls containing 10 ml of pronase (2mg/ml) solution for three to five minutes while being

gently agitated at room temperature. Following pronase treatment, embryos were washed in several changes of fresh EM. This caused the chorions to fall off. Such dechorionated embryos were used in experiments described below.

Chemicals and Solutions

Phenanthrene (purity 98%) was purchased from Sigma Aldrich (St. Louis, MO). GSK-3β inhibitors Indirubin and [4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8)] were obtained from Alexis Pharmaceuticals (Farmingdale, NY) while 1-Azakenpaullone was from Calbiochem (San Diego, CA). Unless stated, all other chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO). Phenanthrene and GSK-3β inhibitors were initially dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions at 5-10 mg/ml. Appropriate volumes of the stock solutions were then added to the cultures as described below to obtain desired final concentrations of the drugs. Final DMSO concentration in any of the treatments did not exceed 0.1%.

Exposure to Phenanthrene/GSK-3β Inhibitors

Figure 3 summarizes the general experimental strategy used to expose embryos to phenanthrene and GSK-3β inhibitors. Embryos were collected and dechorionated as described above. To test for stage specific effects of phenanthrene and GSK-3β inhibitors, embryos were allowed to develop until the appropriate time post fertilization:

2 hpf, 8 hpf, and 12 hpf. After embryos had reached the desired time period in development, they were exposed to various concentrations of either phenanthrene or GSK-3β inhibitors.

For phenanthrene exposure studies, embryos were pulsed with sublethal concentrations ranging from [2-32 μM] for 2 hours, after which embryos were washed 3 times and allowed to develop in drug-free EM until the completion of somitogenesis in the control group. Culture dishes were gently agitated upon addition of phenanthrene to prevent its precipitation.

Embryos were pulsed with sublethal concentrations of GSK-3β inhibitors ranging from [0.25-1.5 μM] for 2 hours. After the exposure, embryos were washed and allowed to develop in drug-free EM as described above until the completion of somitogenesis in the control group.

Embryos in the control group were exposed to the highest concentration of DMSO used, but never more than 0.1%. Control embryos were also pulsed for 2 hours, after which time embryos were washed and allowed to develop in EM without DMSO. For exposure to phenanthrene, GSK-3β inhibitors or DMSO, embryos were cultured in 40 ml sterile glass fingerbowls containing 10 ml of EM. For each concentration of either phenanthrene or GSK-3β inhibitors, experiments were run in triplicate, with at least 10 embryos per dish.

At 24 hpf, embryos were examined for abnormalities in overall morphology and abnormal somitogenesis. The guidelines for examining somites are detailed on the following page.

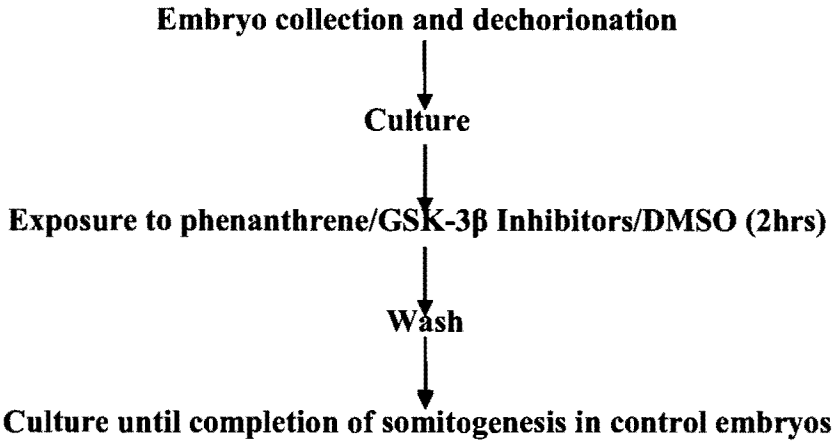

Figure 3. General experimental strategy. Embryos were collected and dechorionated as described earlier. Embryos were then cultured until the desired stage, after which they were exposed to either DMSO, phenanthrene or GSK-3β inhibitor 1-Azakenpaullone. After a two hour pulse, embryos were washed 3 times in EM, and cultured until the completion of somitogenesis in the control group.

Examination of Somites

Upon the completion of somitogenesis in control cultures, generally 24 hpf, somites were examined by using an Olympus CK2 inverted light microscope or an Olympus BH2 upright microscope. Because live embryos tended to twitch during examination, tricane was used as an anesthetic (0.4% tricane in embryo medium) as described by Westerfield (1995). The effect of tricane was reversible once embryos were placed in tricane-free EM. Somites in the experimental groups were examined for abnormalities in shape, for irregular borders, and also to see if there were indications of fusion between neighboring somites. Control embryos were examined first to allow for distinction between normal and abnormal somitogenesis. If somites in the experimental groups did not have the typical "chevron shape" as apparent in the control group, were rounded, or appeared fused, they were considered morphologically abnormal. In addition

to examination of morphological abnormalities, the total number somites were counted in both the control and experimental groups. The somite number in the experimental group was compared to the full set of somites developed in the control groups, typically a set of 30 somites (Kimmel et al., 1995). In addition to examination for somite abnormalities and number, other morphological abnormalities such as pericardial and yolk sac edema, and scoliosis were noted.

Deyolking of Embryos

Before exposure to phenanthrene or DMSO (control), embryos were dechorionated. Embryos were then deyolked prior to analysis using SDS-PAGE, because the yolk contains many proteins that may interfere with Western blotting (WB) and immunostaining. Deyolking of embryos was performed using a deyolking buffer (pH 7.5) containing 55 mM NaCl, 1.25 mM $NaHCO_3$, and 1.8 mM KCl. Embryos were placed in deyolking buffer and gently pipetted up and down several times followed by agitating for 5 minutes using an adjustable vortex. Embryos were then centrifuged at 5000 rpm for 30 seconds. The supernatant was discarded and replaced with a wash buffer (pH 7.5) containing 110 mM NaCl, 3.5 mM KCl, 2.7 mM $CaCl_2 \cdot 2H_2O$ and 10 mMTris/HCl. Embryos in the wash buffer were agitated as described above, followed by centrifugation for 2 minutes at 5000 rpm. The supernatant was discarded and deyolked embryos were harvested in the wash buffer (Link et al., 2006). Samples were then prepared for SDS-PAGE by treating with 2X sample buffer containing 0.5M Tris-HCl,

pH 6.8, glycerol, 10% (w/v) SDS, bromophenol blue, β-mercaptoehanol, and heated for 5 minutes (Laemmli, 1970).

Immunostaining of Embryos

To observe whether phenanthrene exposure caused an enhanced nuclear accumulation of β-catenin in somitic cells, embryos were stained with anti-β-catenin polyclonal antibodies purchased from Sana Cruz Biotechnology Inc. (Santa Cruz, CA). Embryos in both the experimental and control groups were fixed with 5% paraformaldehyde in EM at room temperature (RT) for two hours. Fixed embryos were then washed in phosphate buffered saline (PBS) 3 times, 15 minutes each. To block non-specific antibody binding sites, embryos were treated with 5% BSA in PBS for one hour at RT. After a quick rinse in PBS, embryos were treated with 100% cold methanol for five minutes. The primary antibody, rabbit polyclonal anti-β-catenin, was applied at a 1:250 dilution in PBS. Embryos were incubated in primary antibody for at least one hour at RT followed by overnight incubation at 4°C. Following incubation in the primary antibody, embryos were washed 3 times, 15 minutes each. The secondary antibody, Alexa Flour-488 cojnugated goat anti-rabbit antibody purchased from Invitrogen (Carlsbad, CA), was applied at a 1:500 dilution in PBS for two hours at RT. Embryos were washed as described above and mounted in 75% glycerol containing 0.02% sodium azide. Immunostained embryos were examined using an Olympus FV 300 Confocal Laser Scanning Microscope or an epifluorescence microscope attached to an Olympus BX-61 upright microscope.

Immunoblotting

Lysates of embryos containing soluble proteins were solubilized in Laemmli's buffer and subjected to SDS-PAGE followed by WB analysis, using antibodies to phosphorylated serine or tyrosine residues on GSK-3β, purchased from either Santa Cruz Biotechnology (Santa Cruz, CA) or Abcam Inc. (Cambridge, MA). Briefly, proteins from deyolked embryo lysates after SDS-PAGE were transferred to 0.45μm-pore-size nitrocellulose membrane at 10 mA for 1hr (semi-dry method), as described by Towbin et al. (1979). The membrane containing transferred proteins was blocked for 1 hr at RT with 4% BSA in 0.1M PBS supplemented with 0.1% Tween-20 (PBS-T) and probed with anti-phospho-tyrosine or anti-phospho-serine antibodies for 1 hr at RT. Washed blots were then incubated with secondary antibody (goat-anti-rabbit antibodies conjugated to horse-radish peroxidase, purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Probed blots were developed and visualized by the enhanced chemi-luminescence method (Piece Chemicals).

RESULTS

Chronicle of Zebrafish Development

Figure 4 below shows the chronicle of early development in zebrafish embryos observed in our laboratory under controlled conditions and it is consistent with what has been documented by Kimmel et al. (1995).

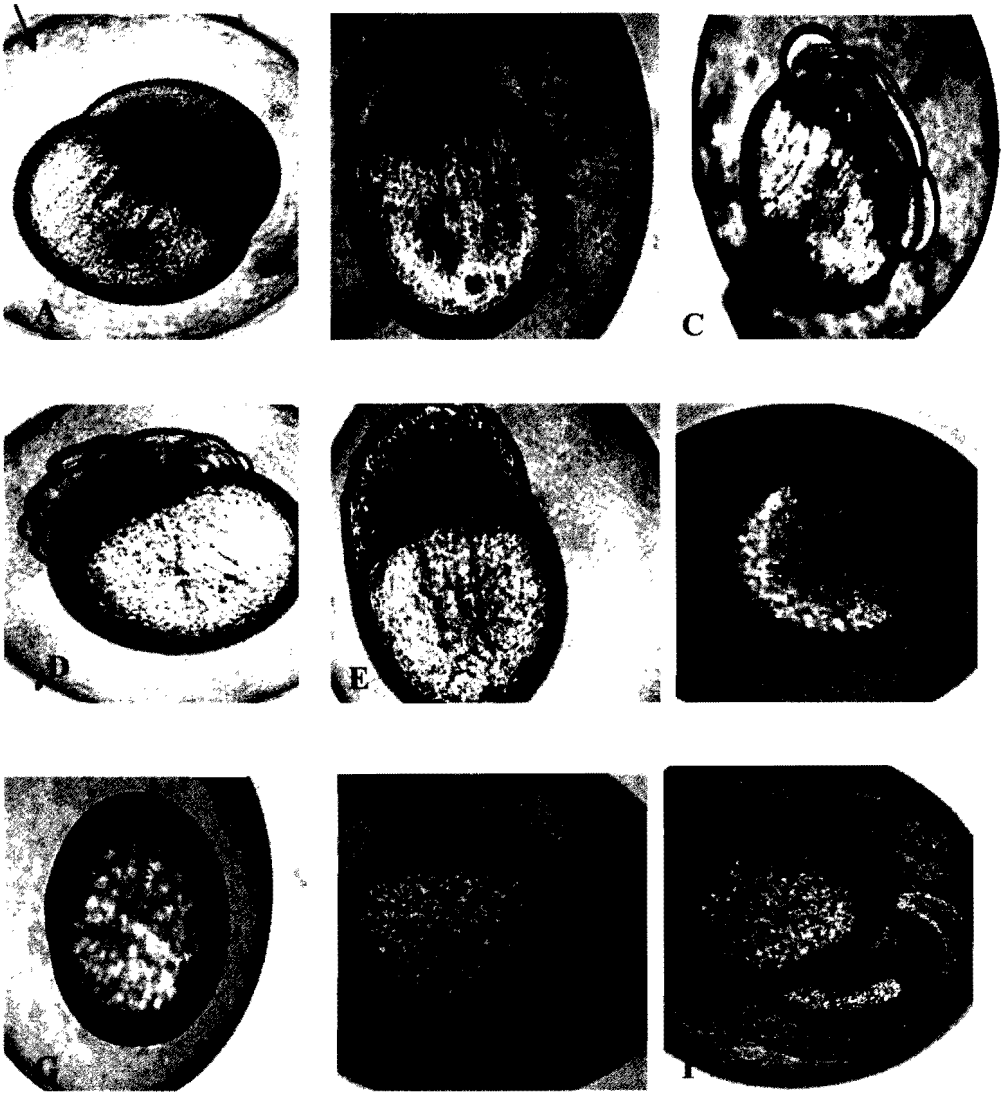

Figure 4. Micrographs showing the chronicle of zebrafish development. A) A newly fertilized egg; B-E) Embryos undergoing various stages of early cleavage; F-G) Embryos undergoing epiboly; H) An embryo at the two somite stage (somites indicated by arrow); I) An embryo that has completed somitogenesis (24 hpf), possessing about 30 fully developed somites. **Magnification: X100**

A recently fertilized egg at the 1 cell cell stage (Fig. 4A), 0-1 hour post fertilization (hpf), is surrounded by a thick chorion (arrow). Figures B-E are micrographs of embryos undergoing a series of cleavage divisions. Cleavage occurs about every 15 minutes and is meroblastic discoidal type. After cleavage, the embryo undergoes epiboly (Figs. 4F-4G). At this time, the epithelial cells migrate down to cover the yolk. Epiboly continues into the gastrulation period, during which time the embryo forms the three germ layers: endoderm, ectoderm, and mesoderm.

At 10 hpf, after gastrulation has been completed, the segmentation period begins and lasts until approximately 24 hpf (Fig. 4H). During this period, somites form from the mesoderm in an anterior-posterior direction approximately every 30 minutes, along the trunk and the tail regions of the developing embryo. The embryo in figure 4H has begun somitogenesis and is at the two somite stage (arrow). Figure 4I is a micrograph of an embryo that has completed somitogenesis at 24 hpf. The embryo has developed a full complement of chevron shaped somites, approximately 30 pairs.

Effect of Phenanthrene on Overall Morphology

Developmental abnormalities were noticed when embryos were exposed to sublethal concentrations of phenanthrene at 2 hpf. Figure 5 shows examples of some abnormalities

observed. These included pericardial and yolk sac edema, irregular heartbeat (not shown), delayed hatching from the chorion [control embryos generally hatched out of

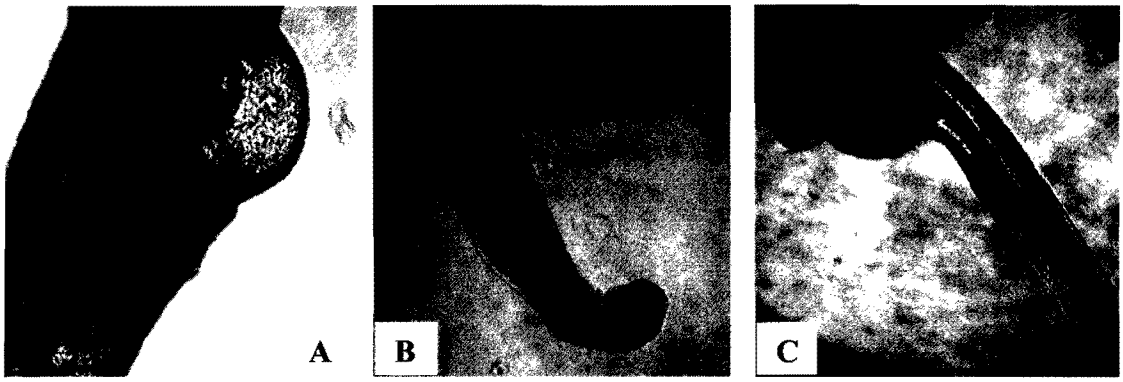

Figure 5. Micrographs showing morphological abnormalities in embryos treated with 8 μM phenanthrene. A) Embryo showing pericardial and yolk sac edema; B) Embryo showing scoliosis; C) A control embryo cultured in DMSO, the solvent used for phenanthrene. Scoliosis and edema were later effects and were noticed 3 dpf. **Magnification: X200 (A); X100 (B); X40 (C).**

their chorions 3 days post fertilization (dpf) while treated embryos at the same developmental stage had not yet hatched], and scoliosis. Figure 5A shows an embryo with pericardial and yolk sac edema, characterized by an enlarged yolk sac. Although not recorded, embryos with this characteristic also had an irregular heartbeat. Compared to embryos is the control group, treated embryos had tachycardia; these embryos had heartbeats much faster than control embryos at the same stage in development. Figure 5B is an embryo with clear signs of scoliosis, which often included a curved tail. Scoliosis was a later effect and was noticed anywhere from 3 to 5 dpf. Figure 5C is a control embryo that has completed somitogenesis, with approximately 30 somites with the characteristic chevron shape, and possessing normal morphological characteristics.

Effect of GSK-3β Inhibitor 1-Azakenpaullone on Overall Morphology

Of the three GSK-3β inhibitors used in the study, Indirubin, TDZD-8 and1-Azakenpaullone, 1-Azakenpaullone was most effective in inducing developmental abnormalities. Therefore, further inhibitor studies were conducted using this compound. The observed defects were very much like what was observed embryos exposed to phenanthrene.

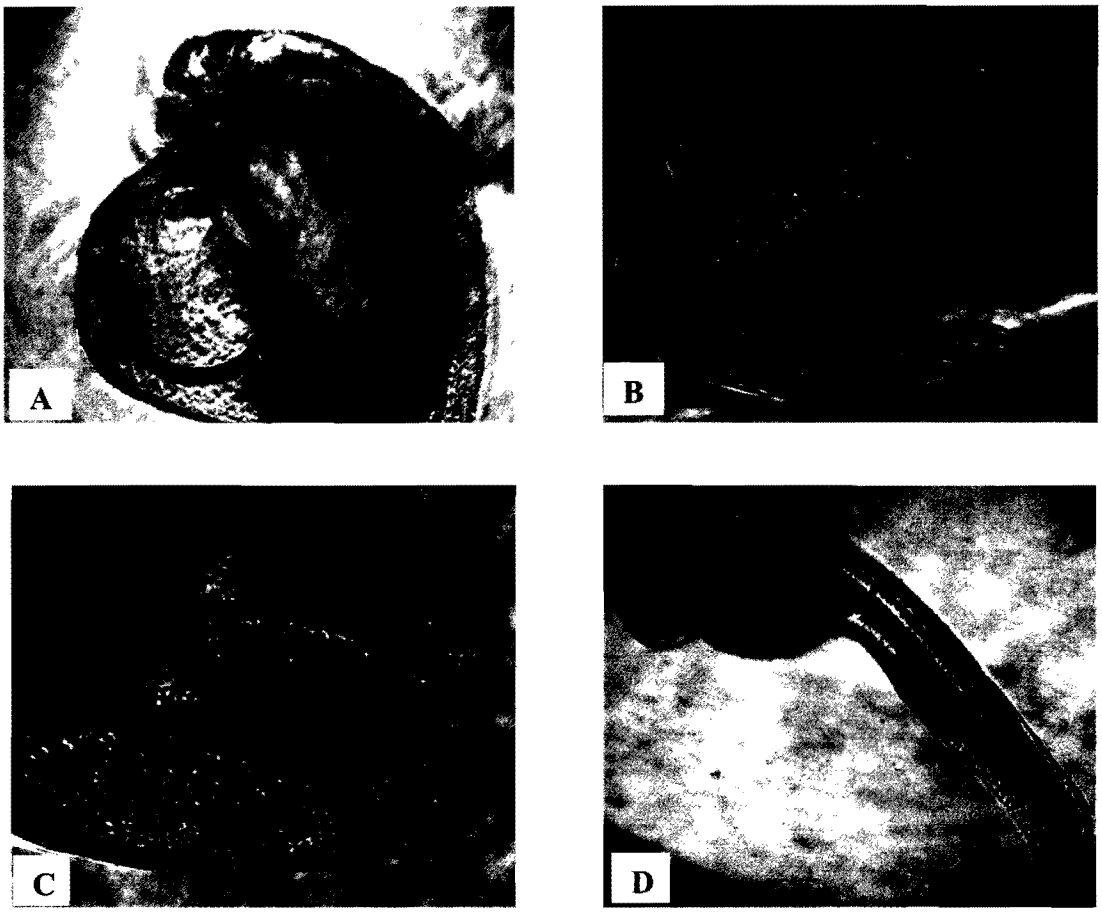

Figure 6. Morphological defects caused by exposure to 1-Azakenpaullone. A) Percardial/yolk sac edema; B) Scoliosis; C) Notochord malformation (arrow) and abnormal somite development; D) A control embryo cultured in DMSO. All embryos were exposed to 1μM 1-Azakenpaullone or 0.1% DMSO (control) at 2 hpf. **Magnification: X100 (A, B, and C); X40 (D).**

Figure 6A shows an embryo with pericardial/yolk sac edema as a result of exposure to 1-Azakenpaullone at 2 hpf. Some treated embryos (Fig. 6B) developed scoliosis, a defect that was observed 3 dpf. These observations are similar to with what was observed in embryos exposed to phenanthrene. Figure 6C shows an embryo with notochord malformation, as indicated by the arrow. The notochord is clearly abnormal, with notable curvature, compared to control embryos. Such embryos also had abnormal somites that appeared round, and had lost the typical chevron shape. Aside from the abnormalities shown in the micrographs above, 1-Azakenpaullone treated embryos did not show normal movement as in the control group (not shown), but this could probably be attributed to abnormal fin development seen in the treated embryos. While control embryos were able to swim short distances, treated embryos remained stationary and exhibited twitching.

Effect of Phenanthrene on Somitogenesis

Embryos were exposed to different concentrations of phenanthrene [2-32 μM] to investigate its effect on somite development. Stage specific effects of phenanthrene exposure were also investigated by exposing embryos to various concentrations of phenanthrene at different time points post fertilization; the data from these studies are presented later. In embryos exposed to phenanthrene at 2 hpf, somite abnormalities were observed. These abnormalities were observed at a range of [5-8 μM] and appeared to be non-lethal. Most severe somite abnormalities, however, were noticed at a concentration of 8 μM. Any concentration above 8 μM used in the experiment was lethal.

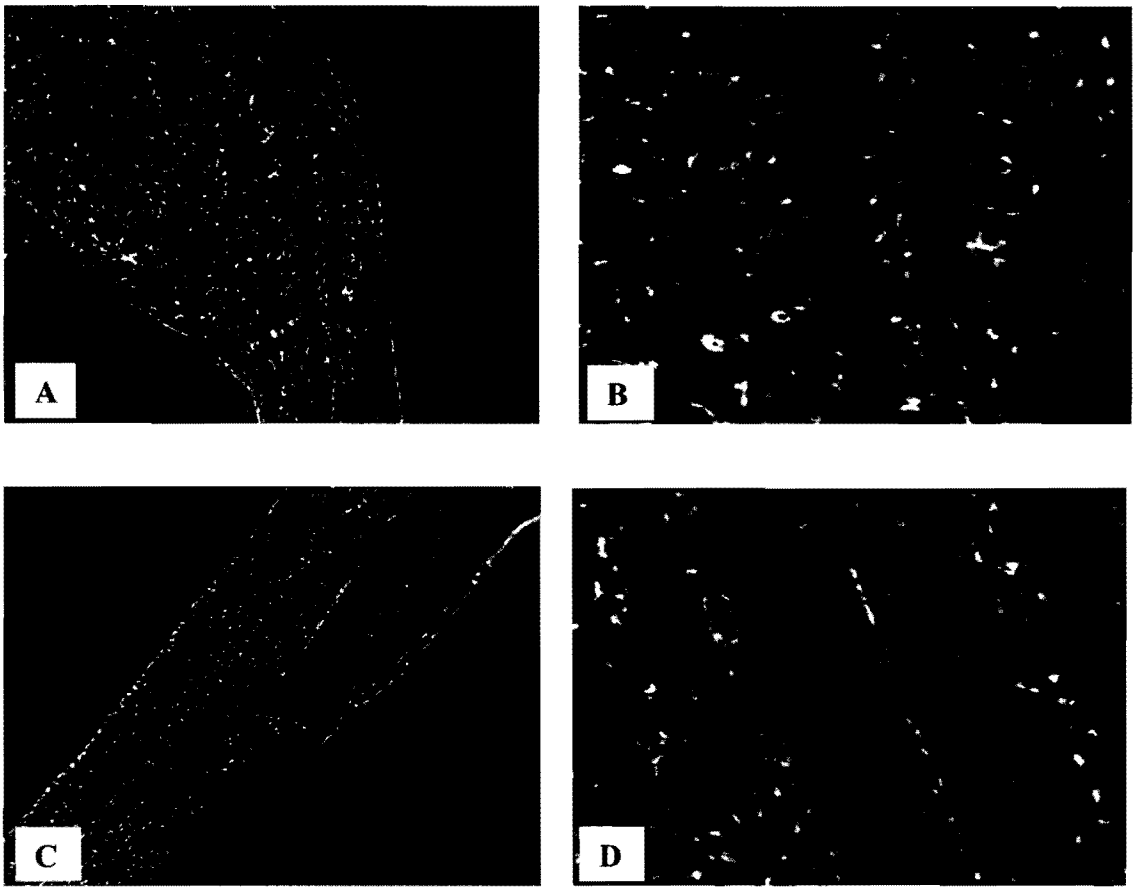

Figure 7. Micrographs showing somite abnormalities induced by phenanthrene exposure. A) Somites from an experimental embryo treated with 8 μM phenanthrene; B) Higher magnification of these somites showing abnormal, round shape; C) A control embryo cultured in EM containing 0.01% DMSO; D) Higher magnification of control somites, possessing the clear boundaries, and a distinct chevron shape. **Magnification: X200 (A, C); X400 (B, D).**

Figures 7A and B show the somites of an embryo treated with 8 μM phenanthrene at 2 hpf. Unlike the typical chevron shaped somites seen in control embryos (Figs. 7C and D), this embryo clearly has developed somites that are not of the distinct shape, but rather are round and almost fused in some cases. Unlike the clear boundaries that separate the somites in control group, somites in the treated embryos do not show such a distinction. The overall somite morphology was disorderly compared to that of the control. Figures

7C and 7D show the somites in a control embryo cultured in DMSO after the completion of somitogenesis at 24 hpf. These somites are of a clear, distinct chevron shape and have clear boundaries between them.

Effect of GSK-3β Inhibitor 1-Azakenpaullone on Somitogenesis

1-Azakenpaullone induced abnormal somitogenesis in embryos. Figure 8A below shows an embryo with full complement of somites that have the typical chevron shape and are separated by clear, distinct boundaries; this embryo was cultured in 0.01% DMSO (control). Figure 8B shows an embryo at the same developmental stage that has been exposed to 1 μM 1-Azakenpaullone. Somites are markedly abnormal, appear round and fused in some cases, and have clearly lost the typical chevron shape (arrows). Boundaries between somites are not apparent as they are in the control embryo. Like phenanthrene induced somite abnormalities, these abnormalities were stage specific; embryos were affected during a narrow window of development (2 hpf).

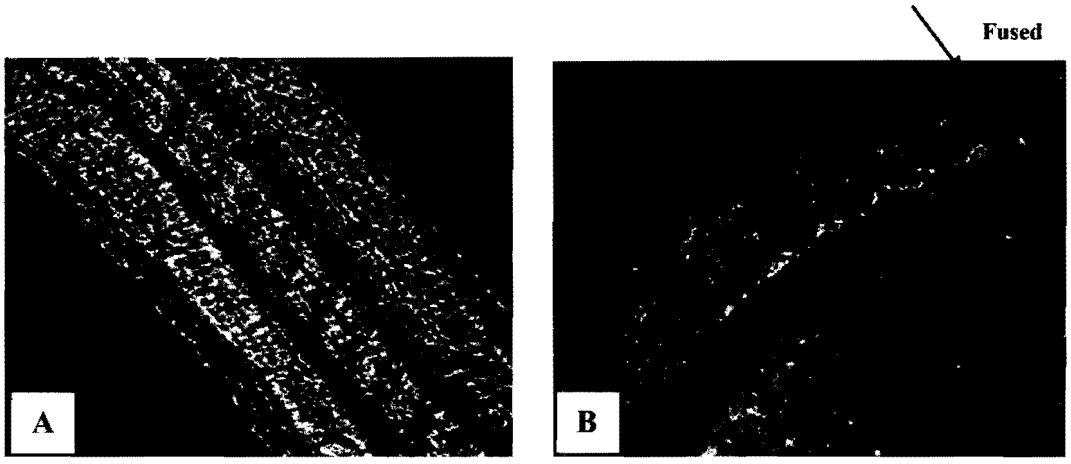

Figure 8. Effect of the GSK-3β Inhibitor 1-Azakenpaullone on somitogenesis. A) Control embryo at 24 hpf, after completion of somitogenesis. Note the clear, chevron shape and the distinct boundaries separating each individual somite; B) Embryo treated with 1μM 1-Azakenpaullone at 2 hpf and examined

at 24 hpf. This embryo has developed somites that have clearly lost the typical chevron shape and appear round, and fused in some cases. **Magnification: X200.**

Stage Specificity of Phenanthrene Induced Somite Abnormalities

In order to investigate the developmental stage specificity of phenanthrene effects on somitogenesis, embryos were exposed to the toxicant at different time points after spawing/fertilization (hours after fertilization; hpf). The table below summarizes the exposure strategies and the results obtained.

Time of Exposure	Concentration of Phenanthrene	Effect on Somitogenesis
Control groups (2, 8, and 12 hpf)	0 μM (0.01% DMSO)	no
2 hpf	[2-32 μM]	yes (lethal above 8 μM)
8 hpf (preceding segmentation)	[2-32 μM]	no (lethal above 8 μM)
12 hpf (6 somite stage)	[2-32 μM]	no (lethal above 8 μM)

Table 1. Table showing stage specific effects of phenanthrene on somitogenesis. Embryos exposed to the toxicant at later stages (8 hpf and 12 hpf) did not exhibit somite abnormalities, while younger embryos (2 hpf) exposed to phenanthrene showed abnormal somites. In each trial, at least 10 embryos were exposed to phenanthrene (at the indicated range of concentrations) or DMSO for 2 hours, washed in embryo medium, and cultured until somitogenesis in the control group had completed (approximately 24 hpf).

Phenanthrene did not appear to have an effect on embryos when exposed at later stages in development (8 hpf and 12 hpf). In such embryos, somites developed normally and had the characteristic chevron shape as seen in the control group treated with 0.01% DMSO. However, embryos exposed to phenanthrene at 2 hpf were affected. In this case, somites

developed abnormally, and appeared round and almost fused in some cases. Figure 9 below shows micrographs that illustrate the stage specific effects of phenanthrene on somitogenesis, and the lack of effect that phenanthrene had on somitogenesis when embryos were exposed at a later time. When compared to the control (Fig. 9A), embryos exposed to phenanthrene at 2 hpf (Fig. 9B) developed somites that did not have clear boundaries between them and in some cases appeared fused (arrow). The embryo is Figure 9C was exposed to 8 μM phenanthrene at 8 hpf, and still developed normal somites.

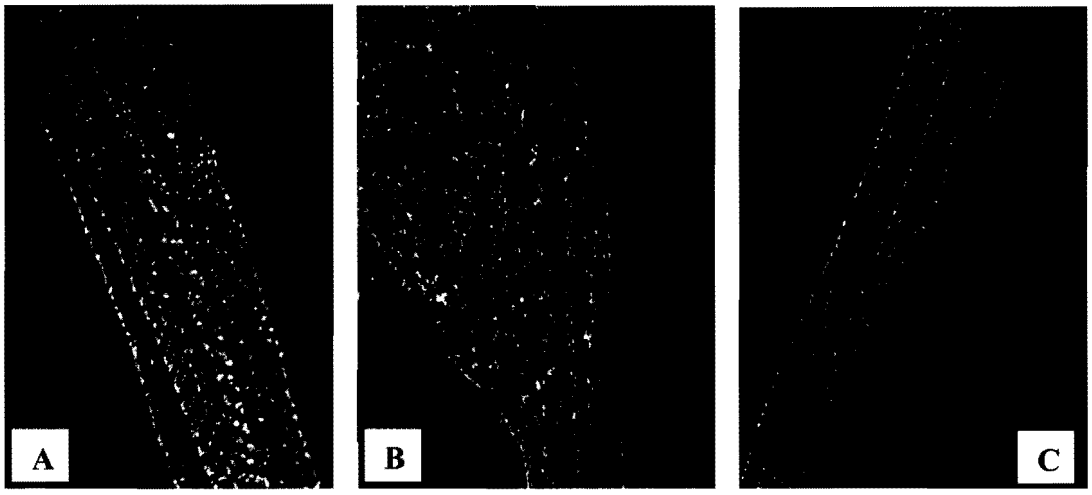

Figure 9. Micrographs showing stage specificity of phenanthrene exposure on somitogenesis. A) A control embryo cultured in DMSO at 24 hpf; B) An experimental embryo treated with 8 μM phenanthrene at 2 hpf. This embryo shows abnormal somites; C) An embryo treated with 8 μM phenanthrene at 8 hpf. The treated embryo does not show somite abnormalities. Both control and treated embryos exhibit somites that are separated by distinct boundaries and are chevron shaped. **Magnification: X200 (A, B); X100 (C).**

Effect of Phenanthrene on Somite Numbers

By the completion of somitogenesis in control embryos, a full complement of approximately 30-32 somites is formed as has been reported in the literature (Holley, 2007). In embryos treated with phenanthrene at 2 hpf, a decrease in somite number was observed. Table 2 shows the effect of different concentrations of phenanthrene on somite number.

Phenanthrene Concentration	Number of Somites [Mean ± SD]; n=12
Control (0.01% DMSO)	32.4 ± 0.54
2μM	29.1 ± 1.22
4 μM	26.1 ± 1.41
8 μM	27.2 ± 1.64
16 μM and above	Lethal (100% mortality)

Table 2. Table showing the effect of various concentrations of phenanthrene on somitogenesis, as determined by the number of somites developed. Twelve trials (n=12) we conducted. In each trial, at least 10 embryos at 2 hpf were pulsed with phenanthrene or DMSO for 2 hours, washed in embryo medium, and cultured until somitogenesis was completed in control groups (24 hpf). Somites were then counted. Each trial was performed in triplicate. Phenanthrene concentrations above 8 μM were lethal to the embryos. For all three concentrations shown here, $p < 0.05$.

Embryos exposed to sublethal concentrations of phenanthrene at 2 hpf and examined upon the completion of somitogenesis showed a significant reduction in somite number. At 4 μM and 8 μM phenanthrene, a reduced number of somites was observed.

Calculated *p*-values were less than 0.05 for each of the concentrations shown in the table. Any concentration of phenanthrene above 8 μM was lethal to the embryos.

Effect of 1-Azakenpaullone on Somite Numbers

To examine whether 1-Azakenpaullone interfered with somite formation, embryos were treated with several different concentrations of the inhibitor. A change in somite number was observed in embryos treated with the various concentrations of the inhibitor shown here. The table below summarizes the findings.

1-Azakenpaullone Concentration	Number of Somites [Mean ±SD];n=4
Control (0.01% DMSO)	28.2 ± 0.2
0.25μM	27.3 ± 0.35
0.5 μM	27.4 ± 0.35
0.75 μM	26 ± 0
1 μM	21.2 ± 0.21
1.25 μM and above	Lethal (100% mortality)

Table 3. Table showing the effect of different concentrations of 1-Azakenpaullone on somite number in embryos exposed at 2 hpf as compared to the control. Four trials were conducted (n=4). In each trial, at least 10 embryos were pulsed to 1-Azakenpaullone for or DMSO 2 hours, washed in EM, and cultured until the completion of somitogenesis in the control group (24 hpf). Somites were then counted. Each trial was performed in triplicate. Concentrations of 1-Azakenpaulline above 1 μM were lethal.

Lower concentrations of 1-Azakenpaullone [0.25 μM -0.5 μM] did not appear to cause much of a reduction in somite number, however, somite number was significantly reduced when embryos were exposed to 1 μM of the inhibitor. Concentrations above 1 μM were investigated (1.25 μM and 1.5 μM) but were lethal.

Immunolabeling of Embryos

Embryos exposed to DMSO, phenanthrene, or 1-Azakenpaullone as previously described were fixed at 24 hpf and stained with anti-β-catenin antibody to observe for an increased accumulation of β-catenin in the nuclei of somitic cells. Figure 10 shows embryos that were exposed at 2 hpf to either phenanthrene (A), 1-Azakenpaullone (B), or DMSO (C). Treated embryos (Figs. 10A and 10B) showed some nuclear accumulation of β-catenin, although this labeling is present in only a few cells. The control embryo does not show such labeling; it appears that most of the labeling is prominent at the cell-cell junctions.

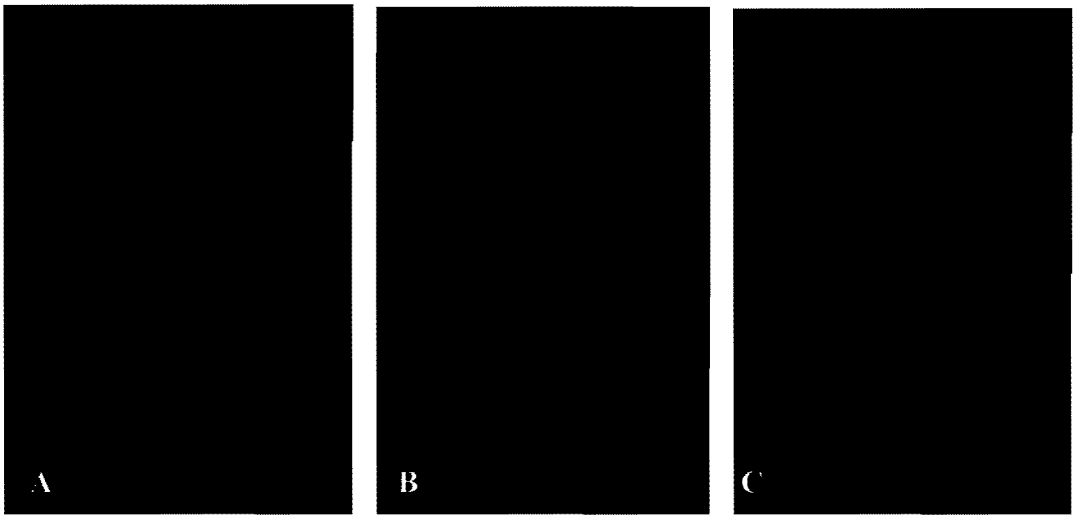

Figure 10. Staining of embryos with anti-β-catenin antibody. A) An embryo treated with 8μM phenanthrene and fixed at 24 hpf, followed by immunostaining; B) An embryo treated with 1μM 1-Azakenpaullone; C) A control embryo cultured in DMSO. Figures A and B show some accumulation of β-catenin in the nuclei of somite cells, although not all cells have this accumulation. The control embryo does not show such staining. β-catenin is concentrated in the cell-cell junctions, as it is historically known for its role in cell-cell adhesion. **Magnification: X200.**

Immunoblotting

The activity of GSK-3β depends on its phosphorylation status. When GSK-3β is phosphorylated on Serine 9, it is inactive. Phosphorylation on Tyrosine 216 activates GSK-3β. Figure 11 is a WB showing the effect of phenanthrene on the phosphorylation status of GSK-3β. Figure 11A shows the effect of phenanthrene on Serine 9 GSK-3β phosphorylation using sea urchins as a comparison. Compared to the control, there is an increase in the signal of Serine 9 GSK-3β. Figure 11B is preliminary data of the effect of phenanthrene on the phophorylation status of GSK-3β in zebrafish embryos, at 2 hpf.

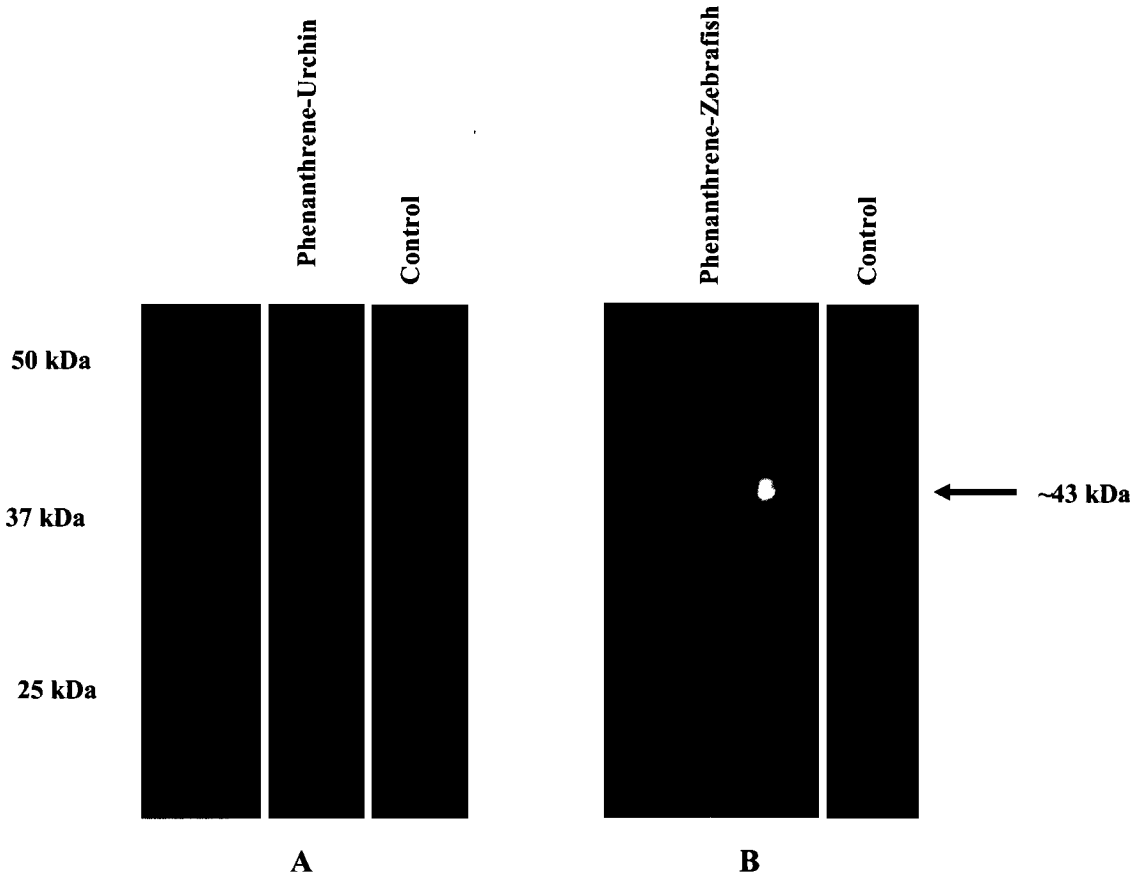

Figure 11. Immunoblotting to detect Serine 9 GSK-3β activity in treated embryos. Embryos were treated with phenanthrene or DMSO and prepared for WB. A) An immunoblot showing an increase in the level of Serine 9 GSK-3β in a sea urchin embryo exposed to phenanthrene for comparison. B) An immunoblot showing an increase in the level of Serine 9 GSK-3β in zebrafish embryos treated with phenanthrene. Note the marked signal in the middle panel. The arrow corresponds to the known molecular weight of GSK-3β. The control does not show such a signal.

Embryos were treated with phenanthrene and prepared for WB as previously described. The first two images in figure 11B are blots showing an increase in the signal of Serine 9 GSK-3β. In the second image, there is a marked increase in the signal, suggesting that there is a correlation of phenanthrene exposure and the phosphorylation status of GSK-3β. This is the distinct band at 43 kDa (arrow). The control does not show such an increase in signal. Control embryos were cultured in DMSO.

DISCUSSION

Somitogenesis is still one of the major unresolved issues in developmental biology, although the "clock and wavefront model" is widely accepted. Through a variety of cellular and molecular interactions, somites form from the presomitic mesoderm (PSM) in an anterior to posterior fashion (Capdevilla et al., 1998). In zebrafish, once somitogenesis is completed at 24 hpf, the embryo has developed about 30 somites (Holley, 2000). This study investigated the effects of phenanthrene induced developmental abnormalities in zebrafish early embryos, focusing specifically on somitogenesis.

I tested the hypotheses that: 1) phenanthrene disrupts somitogenesis and its effect is developmental stage specific; 2) phenanthrene targets a step in the canonical Wnt signaling pathway, and that its effects are associated with enhanced nuclear β-catenin accumulation through perturbation of GSK-3β activity. Previous studies have shown that early sea urchin embryos exposed to a variety of PAH compounds, including phenanthrene, resulted in morphological abnormalities such as exogastrulation and an increased amount of β-catenin in the nucleus (Pillai, et al., 2003). Ung et al. (2010) showed that mercury induced hepatotoxicity in zebrafish. Among many observations, it was found that mercury caused hepatotoxicity by deregulation of kinases such as GSK, which is an important component of the Wnt signaling pathway.

This study shows several morphological defects caused by phenanthrene in zebrafish early embryos, including somitogenesis. In summary, I showed these defects

are observed only when embryos are exposed to phenanthrene at certain time periods post fertilization. Based on the developmental stages chosen for this study, this particular time period was 2 hpf, well before the segmentation stage. Hong et al. (2010) reported that transient embryonic structures are responsible for patterning, nourishing, and protection of embryos. Specifically in the zebrafish, the yolk syncitial layer produces signals that induce formation of the endoderm and mesoderm. Cells in another extraembryonic structure, the enveloping layer, first form in the zebrafish at 2 hpf, and this signifies one of the earliest differentiation steps in development (Hong et al., 2010). With this information, and what is observed in embryos exposed at 2 hpf, it is suggested that this is a critical time period in development, and any disruptions during this time can cause long term developmental effects. Gastrulation, which produces the three germ layers (endoderm, ectoderm, and mesoderm) does not occur in the zebrafish embryo until about 5 hpf and lasts until the onset of the segmentation period (Kimmel et al., 1995). Exposing the embryos to phenanthrene prior to the gastrulation period may have affected the formation of the three germ layers, as this process is highly coordinated and requires proper interaction between cells. Because the somites are derivatives of the mesoderm, their development may be affected due to disruption by phenanthrene.

The patterning of the zebrafish somite, which is mostly comprised of myotome, is under the influence of Wnt signals from both the ectoderm and neural tube (Chow et al., 2003). In this study, embryos treated with phenanthrene developed morphologically abnormal somites. This observation suggests that the patterning of somites was affected via perturbation of the Wnt signaling pathway. Furthermore, studies using the GSK-3β

inhibitor 1-Azakenpaullone may indicate that phenanthrene is directly targeting a certain component of the pathway. Recent studies by Lim et al. (2008) reported enhanced levels of β-catenin in brain endothelial cells when several different GSK-3β inhibitors were used, including 1-Azakenpaullone.

The morphological abnormalities in somitogenesis led me to investigate whether phenanthrene exposure had an effect in decreasing the number of somites formed at the given time periods post fertilization. Through immunological studies using anti-β-catenin antibodies, I was able to observe the possible involvement of the Wnt signaling pathway on somitogenesis, and whether any of the phenanthrene induced somite abnormalities lead to an increase amount of nuclear β-catenin.

Effect of Phenanthrene on Overall Morphology

Phenanthrene induced morphological abnormalities such as scoliosis and pericardial and yolk sac edema. Incardona et al. (2003) reported the same cardiac defects in zebrafish embryos treated with phenanthrene, as well as six other PAHs. Although the embryos used in their studies were exposed to 56 μM phenanthrene at 4-8 hpf, the morphological abnormalities I observed (pericardial and yolk sac edema and scoliosis) are very similar to what was observed by Incardona et al. (2003) but with a much lower concentration (8 μM).

While embryos in the control cultures were able to swim in short distances at 3 dpf, treated embryos at the same stage tended to twitch instead of move around normally. This could probably be attributed to abnormal fin development (data not shown). Scoliosis was observed at a later time point in development, generally 3-5 dpf. Bleichinger et al. (2002) reported scoliosis as one of the predominant morphological defects observed in zebrafish embryos exposed to cadmium. These embryos were exposed to cadmium (0-125 μM) beginning at 3 dpf, and were more sensitive to the toxin at later stages in development, rather than during their first day of development. Pericardial edema was also noted. These two defects induced by cadmium are consistent with my observations, though a different compound was used.

Effect of 1-Azakenpaullone on Overall Morphology

Of the three GSK-3β inhibitors used, all initially caused some morphological defects in embryos exposed at 2 hpf (data not shown) but I focused on 1-Azakenpaullone. This inhibitor acts as a potent ATP competitive inhibitor of GSK-3β (Tighe et al., 2007). Because GSK-3β is phylogenetically most related to cyclin dependent kinases (CDKs), it is not unexpected that this inhibitor would act on both GSK-3β and CDKs (Kunick et al., 2003). Liu et al. (2009) demonstrated that both lithium chloride, a well known inhibitor of GSK-3β, as well as 1-Azakenpaullone, resulted in increased translocation of β-catenin in human islet cells. I used a range of [0.25 μM-1.5 μM] 1-Azakenpaullone to study the effects on somitogenesis while also observing abnormal overall morphology. A

concentration of 1 μM was sublethal and most effective in causing developmental abnormalities such as pericardial and yolk sac edema, as well as scoliosis, and also caused marked abnormalities in somite morphology. It is interesting to observe the abnormal notochord morphology induced by 1-Azakenpaullone, which was not observed in phenanthrene treated embryos. The notochord is a tissue essential to the patterning of the somite (Chow et al., 2003) and is responsible for secreting some of the Wnt signals involved in the pattering of the myotome, the majority of the zebrafish somite. Chow et al. (2003) also observed notchord abnormalities when embryos at the gastrulation stage were exposed to cadmium. Another study by Haendel et al. (2004) observed a severely twisted notochord at 24 hpf in zebrafish exposed to metam sodium.

Effect of Phenanthrene on Somitogenesis

Phenanthrene was effective at disrupting somitogenesis in a developmental stage specific fashion. Embryos at three different time periods post fertilization were exposed to phenanthrene: 2 hpf, 8 hpf (preceding segmentation) and 12 hpf (6 somite stage). Embryos exposed to phenanthrene at 2 hpf were most sensitive to phenanthrene exposure. Compared to the embryos in the control group, experimental somites lacked a clear chevron shape, appeared round, and were almost fused in some cases. Proper segmentation of the paraxial mesoderm is essential in forming normal somites with clear, distinct boundaries. Van eden et al. (1996) observed abnormal somites in a group that

had defects in the somite patterning process. Somites were either fused early on or had irregular boundaries.

In the present study, a range of [2-32 μM] phenanthrene was used to see at which concentration there would be an effect on somitogenesis, while not being lethal to the embryo. In the initial studies, a concentration of 8 μM of phenanthrene was found to be most effective. Not many studies have been done observing the effect of PAHs on somitogenesis in zebrafish, however, Chow et al. (2003) showed that cadmium caused such abnormalities when embryos were exposed at the gastrulation stage (4-10 hpf). Their observations included somites that were packed in a disorderly manner, and loss of typical chevron shape. Abnormal somite morphology as shown in Fig. 3 observed in embryos treated with phenanthrene under our laboratory conditions caused defects similar to those seen by Chow et al. (2003). Results from this study strongly suggest that there is a critical time period during development when embryos are more affected by exposure of phenanthrene.

Apparent morphological abnormalities in somite development led me to investigate further to determine whether phenanthrene was causing a decrease in somite number. As shown in Table 2, somite number had decreased for each increasing concentration of phenanthrene used, although the mean of somites counted for the 4 μM concentration had a slightly lower number of somites than that of the highest concentration (8 μM) shown in the table.

Because of the amount of research regarding the effects of PAHs on zebrafish somitogenesis is scarce, experiments using different PAHs may be useful in studying the segmentation process in vertebrates, as the process of segmentation remains to be fully understood (Schnell, 2000). It would be interesting to observe whether different PAHs would have the same effect on somite morphology and number as phenanthrene did.

Effect of GSK Inhibitor 1-Azakenpaullone on Somitogenesis

The Wnt signaling pathway is a highly conserved molecular pathway found in embryonic and adult tissues of all metazoans, and is central to cell differentiation (Lara et al., 2010). Defects in this pathway leading to an accumulation of β-catenin in the nucleus have been found in cancers of the skin, colon, prostate, liver, endometrium, and ovary (Doble and Wodgett, 2003). In these cases, β-catenin is not being phosphorylated by GSK-3β and is allowed to move, in excess, into the nucleus where it acts as a co-activator of transcription.

To investigate whether phenanthrene was directly targeting a step in the Wnt signaling pathway, leading to a possible accumulation of β-catenin in the nucleus, I focused on inhibition of GSK-3β activity. In canonical Wnt signaling, the role of GSK-3β is to phosphorylate β-catenin, targeting it for ubiquitylation and proteosomal degradation (Doble and Wodgett, 2003). When this does not happen, β-catenin is not marked for degradation, and moves from the cytoplasm into the nucleus.

Somite number was affected by exposure to 1-Azakenpaullone, but a drastic decrease in number compared to the control was apparent at a concentration of 1 μM (See Table 3). For the lower concentrations used, somite number decreased only slightly as compared to the control. Concentrations above 1 μM were lethal and not used in the study.

Immunolabeling of Embryos

Embryos exposed to phenanthrene or 1-Azakenpaullone, and stained with anti-β-catenin antibody, both showed an accumulation of β-catenin in the nuclei of some cells, suggesting that both of these compounds may be disrupting the Wnt signaling pathway by inhibiting the activity of GSK-3β. It is interesting to note that despite the fact that only a few cells in figures 10A and 10B manifest this characteristic, these embryos still developed abnormal somites and an abnormal gross morphology. This could perhaps be due to these affected cells influencing normal surrounding cells, thus leading to the defects that were observed.

Immunoblotting

Preliminary immunoblot analysis of embryos treated with phenanthrene and probed with polyclonal anti-GSK-3β antibodies revealed an increase in the levels of Serine-9 GSK-3β. The activity of GSK-3β is dependent on its phosphorylation status.

When GSK-3β is phosphorylated at Tyrosine 216, it is active; phosphorylation at Serine-9 renders the enzyme inactive (Aguilar-Morante et al., 2010). Originally known as a regulator of glycogen metabolism since its discovery, GSK-3 has been found to have a role in numerous signaling pathways that are involved a variety of cellular processes (Xu et al., 2009). It is a unique kinase in that it is constitutively active and is inactivated in response to cellular signals (Rayasam et al., 2009; Xu et al., 2009).

I hypothesized that phenanthrene was directly acting on a step in the Wnt signaling pathway, leading to an increase in the amount of nuclear β-catenin. Immunostaining of phenanthrene treated embryos (Fig. 10A) showed an excess of β-catenin in the nucleus of some cells. Figure 11B shows an increase in the levels of Serine- 9 GSK-3β in zebrafish embryos treated with phenanthrene. Taken together, these findings strongly suggest that phenanthrene may be affecting the Wnt signaling pathway via perturbation of GSK-3β activity.

It is important to note that this preliminary immunoblot data does not include studies on Tyrosine 216 GSK-3β. However, in treated embryos, I would expect to see a decrease in the level of Tyrosine 216 GSK-3β, since phosphorylation on this site activates the enzyme and leads to the subsequent degradation of β-catenin. Analysis using anti-GSK-3β antibody specific to Tyrosine 216 is needed to determine this.

Phenanthrene and 1-Azakenpaullone both resulted in strikingly similar morphological defects in early zebrafish embryos (pericardial and yolk sac edema, scoliosis), as well as a disruption is somitogenesis. These effects were developmental

stage specific and happened during a narrow window of development. The present study investigated the possible effects of phenanthrene on the Wnt signaling pathway, focusing on a specific step. GSK-3β is just one of the many components of this pathway, but whether this is the only target of phenanthrene remains to be tested. Other components of the Wnt pathway will need to be investigated to address this question.

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