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Ingrid Albert
Thesis for the degree Master of Pharmacy
Spring 2013

Supervisors:
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May 2013, Ingrid Albert

SUMMARY

The zebrafish is an exciting model organism with a lot to offer and many possible areas of application. Toxicity assays using zebrafish embryos are becoming increasingly popular with the pharmaceutical industry as a tool to bridge the gap between conventional cell based screens and animal testing. The main aim of this project was to explore the zebrafish as a model system for use in safety pharmacology, and to look into some of the different methods that can be used to do so. Dexamethasone and a novel antimicrobial drug lead were tested for toxicity in the zebrafish embryo by visual assessment of mortality and malformations, and by performance of apoptosis assay and western blotting to look at apoptosis and proliferation.

Treatment with relative low concentrations of dexamethasone caused a significant increase in embryo malformation rate, while staining with acridine orange revealed altered apoptotic activity in brain tissues and in the urogenital tract of treated embryos. The antimicrobial peptide, $\beta^{2,2}$-amino acid derivative 161, showed generally low toxicity to zebrafish embryos, as measured by the LC_{50} estimated in this project, compared to previously reported MIC and IC_{50} values for the drug against multi-resistant bacterial strains and cancer cells. However, truncation of tail and damage to tail tissue were seen after treatment with the drug. Staining with acridine orange showed increased apoptotic activity in brain tissues and the urogenital tract of treated embryos. Western blots of whole embryo extracts of embryos treated with either drug gave conflicting result, and a refinement of the method is suggested for future experiments.

In parallel with the zebrafish experiments, studies using mammalian cell cultures were carried out. Both excess glucocorticoids and decreased levels of the transcription factor PAX6 have been associated with development of cataract and glaucoma. Glucocorticoids and PAX6 also influences glioblastoma cancer patient prognosis. We wanted to explore if treatment with dexamethasone would influence expression levels of PAX6 in human lens epithelial cells and human glioblastoma cells. We were also interested in looking at proliferation and apoptosis in these cell lines following dexamethasone treatment. To investigate this, western blots were done. However, results were inconclusive, and further studies are needed to answer this.

ABBREVIATIONS

ADME	Absorption, distribution, metabolism, excretion
AMP	Antimicrobial peptide
BrdU	Bromodeoxyuridine
Dex	Dexamethasone
DMEM	Dulbecco´s modified eagles medium
DTT	Dithiothreitol
EC	Effective concentration
FCS	Fetal calf serum
FE-SEM	Field emission scanning electron microscope
GBM	Glioblastoma multiforme
GC	Glucocorticoid
GR	Glucocorticoid receptor
Hpf	Hours post fertilization
IC	Inhibitory concentration
IHC	Immunohistochemistry
IOP	Intraocular pressure
ISH	*In situ* hybridization
LC	Lethal concentration
LD	Lethal dose
MEM	Minimum essential medium eagle
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
MRSE	Methicillin-resistant *S. epidermis*
NEAA	Non-essential amino acids
PBS	Phosphate buffered saline
PH3	Phospho-histone H3
PTU	N-Phenylthiourea
qPCR	Quantitative real-time polymerase chain reaction
RBC	Red blood cells
SDS	Sodium dodecyl sulfate
SGK	Serum- and glucocorticoid-inducible kinase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

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1. INTRODUCTION

1.1 Drug discovery and safety pharmacology

The development of novel drugs is a laborious and costly process, where many lead compounds never make it to the finish line (1). The usual course of testing for a drug lead starts with *in vitro* cell based toxicity assays, followed by *in vivo* testing in animals, most commonly mice, rats and dogs (2, 3). Then, if all goes well, human clinical trials can finally start up many years after the initial testing begun, and it will typically take between 12-15 years before the drug potentially reaches the market (2). However, less than 1 % of novel compounds actually get this far, due to organ toxicity or poor *in vivo* administration, distribution, metabolism or excretion (ADME) profiles, usually discovered during animal testing (2, 4). Needless to say, by this time there have already been vast amounts of money invested in the project. Being able to identify and discard poor candidates early on in the process would therefore be of immense value, since the expenses associated with novel drug development increases the longer a lead is allowed to proceed through the hierarchy of preclinical studies or even enter clinical phase 1 trials (4, 5).

Although the early *in vitro* studies are both time- and cost-effective, they have limited predictive and translational value to the human situation (6). Cell based assays are restricted in their capacity to model multi-cellular processes, as well as being limited in their ability to reflect the metabolism of a whole organism (4, 7). Drug exposure may therefore affect organ function in ways that can be difficult to uncover by means of standard toxicity tests on specific cell types (8). For this reason, many lead compounds meet their faith during animal trials. The International Conference on Harmonisation (ICH) S7A guidelines requires that all clinical drug candidates be evaluated with regard to basic vital functions such as the cardiac, central nervous and respiratory systems before first exposure to humans (9). This is when the *in vivo* ADME characteristics and possible off-target effects of the drug is first put to the test. When so many candidates fall through during this step in the process, why not screen for some of these things earlier, thereby allowing selection of the leads that show the most promise and may be worth investing in at an earlier point in time?

While traditional *in vivo* test methods where toxicity studies of the compound is carried out in mammals are paramount at some point in the process, this is both expensive and time-

consuming, and is poorly suited for rapid screening using small amounts of compound (5, 10). Meanwhile, the pressure from both regulatory authorities and animal welfare organizations to develop alternatives to animal testing and to minimize the number of mammals used in toxicity assays increases (4, 10, 11). There is clearly a gap existing between the inexpensive and high-throughput *in vitro* toxicity assays and the costly and slower *in vivo* assays conducted later on in the drug discovery process, which could benefit from novel screening tools (7, 12). Making it possible to introduce organ toxicity and safety pharmacology assays earlier in the process, using a high-throughput and affordable model system, may make a great difference when it comes to lead selection and could reduce attrition rates later on (4). With this in mind, numerous ideas for alternative model systems have been proposed. These include advanced *in vitro* cell- or tissue-based assays, but however advanced; these will always lack the biological complexity of *in vivo* models (4). Another option, already in use by several pharmaceutical companies, is the zebrafish.

1.2 The zebrafish

Originating from freshwater pools and streams in South Asia, the tropical zebrafish (*Danio rerio*) has been a popular inhabitant in home aquariums for many years (13, 14). This small fish of the carpe family grow to be about 3 cm in length, and gets its name from the dark blue and silvery vertical stripes flanking its body (Figure 1-1) (15). Male fish display darker blue stripes with a pink tint in between and are more streamline shaped than females, whose abdomen is often whiter and more protruded, especially before laying eggs (16). Adult zebrafish are relatively tough, and can tolerate large fluctuations in water pH, temperature and electrolyte concentrations quite well (17). These factors will however be of importance for the quality of eggs laid by the females, and fish held for research purposes should therefore be kept at optimal conditions (17). The temperature is usually kept between 26-28.5 °C, while the pH should be monitored regularly and lay in the range of 6.8 to 7.5 (18). As light stimulates the fish to mate, zebrafish in the wild usually spawn shortly following daybreak (17).

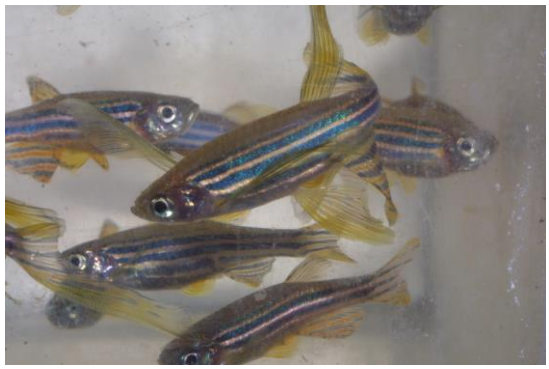


Figure 1-1: Adult zebrafish

The zebrafish embryogenesis is quite rapid, with embryos turning into larvae swimming about seeking for food within four days after fertilization (19). Kimmel and co-workers have divided the zebrafish´ embryogenesis into seven periods, all occurring during the first 72 hours post fertilization (hpf) (19). Organogenesis starts during the first 24 hpf, and by 96 hpf most organs are fully developed (20, 21). Hatching marks the end of the embryogenesis, and usually takes place throughout the third day of development (19). During the next 24 hours, the early larvae will drain almost all of the yolk sac´s content, the swim bladder will inflate, and the larvae will start to swim actively around (19).

As early as in the 1930s zebrafish was used to study developmental biology and molecular genetics, however many other possible areas of application for the zebrafish have more recently been recognized (22). Some of these include drug discovery and development through high-throughput screens and safety assessments, cancer research and studies of environmental pollution, while mutant zebrafish are used as disease models and in studies of organ development (23-26).

The work to fully sequence the zebrafish genome started in 2001, and is now nearly finished (27). There is relatively high conservation of gene function between zebrafish and humans, and the zebrafish organs share surprisingly many similarities with their human counterparts, both anatomically and physiologically as well as from a molecular point of view (15, 28). Thousands of zebrafish mutants mimicking human genetic diseases have been generated through random mutagenesis and forward genetic screens, and are available commercially (14, 22). Reverse genetic approaches have also been established (15). Embryos of wild type strains can easily be exposed to drugs or chemicals and assessed with regard to survival and development of vital organs in a matter of days (6). Although disease modeling and safety assessments of novel drug leads are most commonly carried out using zebrafish embryos or larvae to benefit from their rapid development, experiments using adult fish are also proving valuable (22, 29). In short, the zebrafish has proven to be a versatile model in which the possibilities are endless.

1.3 Drug toxicity assessment using the zebrafish

1.3.1 The zebrafish offers many advantages to testing in mammals

Due to the small size of the fish and the fact that they are relatively easy to care for, many individuals can be kept together taking up minimum space and maintenance time, thereby making the animal husbandry in itself much less expensive and time consuming than keeping for instance rodents (Figure 1-2). The cost to maintain zebrafish as research animals have been calculated to 1 US cent per fish, as compared to 1 USD per mouse (20).

Figure 1-2: Zebrafish are easy to care for, and does not require much space in a research facility compared to other animals.

Sexual maturation of the fish is reached in about three months (20). A single female zebrafish paired with a male can then lay up to 300 eggs in one morning, about once a week (30). By mating several pairs of fish, thousands of eggs can be obtained every day of the year if desirable. Zebrafish embryos display rapid *ex utero* development, and a toxicity assessment can be carried out in only a week (6). In contrast to mammals, the zebrafish embryos do not undergo a lengthy fetal period where major structures or organ systems are formed, but have functioning vital organs at time of the assay (6). Also, malformed embryos with defects to vital organs can usually survive past the time at which the affected organ would normally start functioning, allowing assessment of defects that would otherwise be difficult (28, 31).
Both the chorion surrounding the embryos until hatching, and the embryos themselves are transparent, making it easy to visually identify specific organ or tissue effects in the live animal under a dissecting microscope. This situation differs greatly from that in rodents, where one would typically have to dispatch of the mother before dissecting the embryo to observe organ development (15, 23).

The zebrafish embryo and young larvae only measures around 1-2 mm in length, and can therefore be arranged in multiwell plates where they can survive in as little as 50 µL of water if a 384-well plate is used (15, 20). Small quantities of test material will therefore be required compared to the amounts that would be necessary for drug administration to bigger animals. Zebrafish are sensitive to chemical exposure during early development, and will absorb compounds dissolved in the incubation media through their skin, gills and mouth (20, 31). Highly hydrophilic drugs may be injected (32).

Although testing in zebrafish can never completely replace mammalian assays, it can provide a bridge between cell-based methods and testing in higher animals. Using the zebrafish as a model system for safety studies early in novel drug discovery not only allows assessment of toxicity and possible off-target effects, but it also permits identification of active compounds with good pharmacokinetic properties as well as making the detection of prodrugs and tissue-selective modifiers possible (20). Thus, many of the advantages of animal testing using higher animals are preserved, while keeping it much more cost-efficient, quicker and all the while reducing the need for testing in mammals.

1.3.2 Determining toxicity

As mentioned, the translucency of the zebrafish embryos make it easy to identify morphological changes to organs and other tissues without having to use advanced equipment or techniques. Dead eggs and embryos are also easy to identify by the change in coloration and loss of translucency that occurs due to coagulation and precipitation of proteins (Figure 1-3) (17).

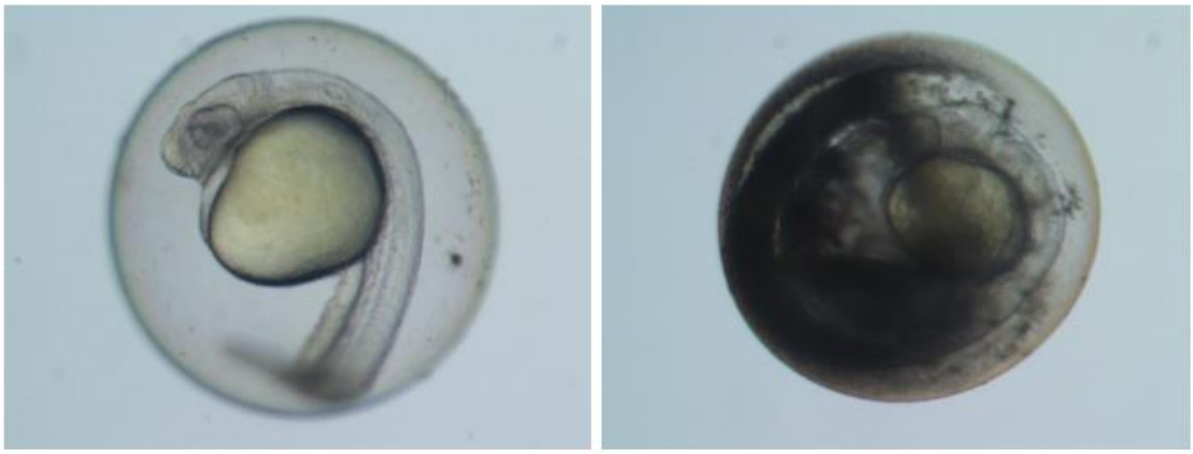


Figure 1-3: The change in coloration makes dead embryos easy to identify. Dying embryo (right) compared to a healthy embryo (left) at 24 hpf.

When doing a toxicity assessment of drugs or other chemicals, the LC_{50} or LD_{50} is often of interest. This represents the compound concentration or dose that would be expected to kill 50 % of the population of a certain species in a given time span, and is commonly used as a measure of toxicity (33). When fish are used as test species, the fish are exposed to certain concentrations of the compound, and thus LC_{50} is the most appropriate parameter. This value can be used to compare toxicity of different compounds or toxicity of a given chemical between different populations; low LC_{50} indicates high toxicity (33). The test species is exposed to several different concentrations of the compound and is assessed with regard to mortality. When plotting mortality rate against the logarithm of concentration, the resulting dose-response curve will typically be sigmoidal (S-shaped) (34). Several methods are available for determining the LC_{50}, but a probit analysis of the data is frequently used (35). By expressing mortality rate as probits (probability units) instead of percent, the sigmoid dose-response curve is transformed into a straight plot that can be analyzed by linear regression (34). To use this method, one would have to assume that the relationship between test objects responding and concentration is normally distributed (34). Abbotts formula should be used to correct for mortality in the control group, so as not to make biased estimates of the LC_{50} (17).

1.3.3 Apoptosis assay as part of toxicity assessment

Apoptosis, or programmed cell death, is an important part of development and pathology in vertebrates, and is involved in normal cell turnover, eliminating unnecessary cells after differentiation, as well as being activated in response to environmental stress (36, 37). There are two main apoptotic signaling pathways in vertebrates, namely the intrinsic and extrinsic one. The intrinsic pathway is triggered by for instance damage to the cell, growth factor withdrawal or cytotoxic drugs, while the extrinsic pathway is involved in controlling immune response and homeostasis (38).

Most of the changes a cell goes through during apoptosis can be ascribed to the activity of caspase enzymes, which belong to a family of proteases (39). Inactive caspase proenzymes exist dormant in the cell, and are activated in a cascade reaction as a response to nuclear, metabolic or an externally activated stimuli, culminating in the activation of effector caspases, primarily caspase-3, which in turn lead to DNA fragmentation, reduction of the cell nucleus and consequent cell death (Figure 1-4) (37, 38).

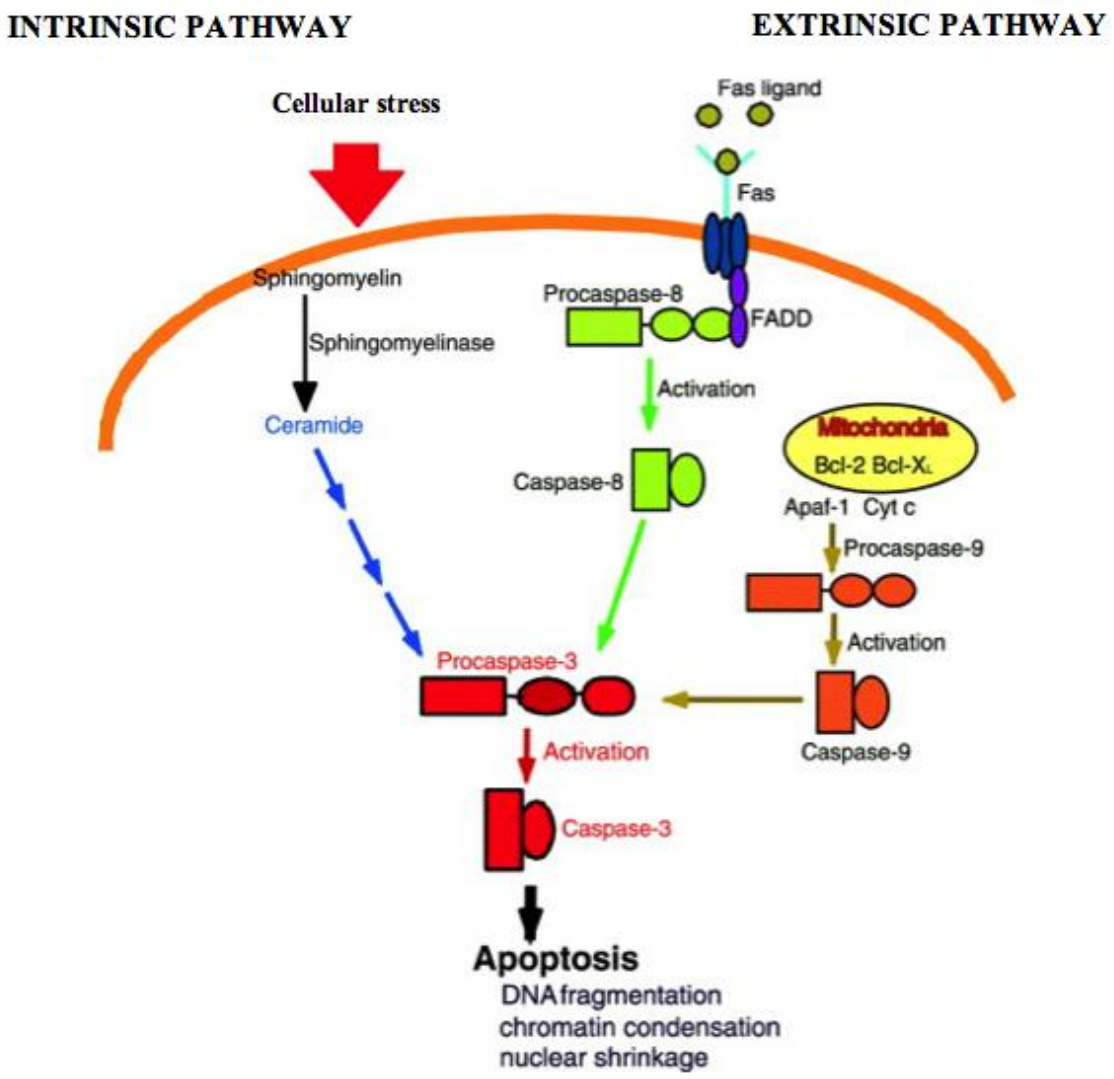


Figure 1-4: The apoptotic machinery. Caspase-3 is one of the primary effector caspases responsible for cell changes leading to apoptosis. Modified from (37).

Stress-induced apoptosis is thought to be a factor in the pathophysiology of malformations during embryogenesis (37). Moreover, the process of apoptosis during development is very well conserved between zebrafish and higher animals, making the zebrafish a suitable model in which to study embryotoxic effects of environmental stimuli (37).

Staining with acridine orange is a useful tool to identify apoptotic cells live. Emitting green fluorescence when bound to dsDNA, and binding especially strongly to fragmented DNA, short incubation of living embryos with this dye will result in selective labeling of apoptotic cells (39). Another method for identifying apoptosis *in situ* is the use of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) (40). Like staining with acridine orange, TUNEL staining is also based on labeling of fragmented DNA.

Fluorescent dUTP is incorporated into DNA breaks in cell nuclei of fixed embryos or tissue samples (40). As the zebrafish embryo remain near-to translucent throughout most of the embryogenesis, cells undergoing apoptosis can be identified through any of these methods when viewed by fluorescence microscopy. However, pigmentation of the embryos could cloud the image, and it is therefore advisable to treat embryos with N-Phenylthiourea (PTU) from the 28 somite stage (approximately 23 hpf), thus inhibiting pigment formation and generating completely transparent embryos, (Figure 1-5), if apoptosis is to be detected in embryos older than 24 hpf (41).

Figure 1-5: Treatment with PTU can be used to generate completely transparent embryos. Control embryo (left) and PTU-treated embryo (right) at 72 hpf.

1.3.4 Proliferation assay as part of toxicity assessment

Correct regulation of cell proliferation, both through regulation of apoptosis and cell division, is vital for organogenesis and normal development in general (42). Failure in this area is the hallmark of cancer, and is also an important factor in the pathology of different birth defects and degenerative diseases (43). Consequently, both increased and decreased proliferation compared to the normal standard can be detrimental to health.

Phospho-Histone H3 (PH3)-staining can be used to visualize cell proliferation in fixated embryos. Histone H3 is one of the proteins that form the histone core of the nucleosome, the important structure of the chromatin fiber. Both meiotic and mitotic chromosome condensation is associated with phosphorylation at serine 10 of H3, which has been shown to be required for processes following metaphase during cell division (44). Immunostaining of phosphorylated H3 will thus label proliferating cells.

Bromodeoxyuridine (BrdU)-incorporation can also be used to identify cell proliferation *in situ*. When living embryos are incubated in BrdU, 5-bromo-2'-deoxyuridine will be incorporated into replicating DNA of cells preparing for cell division (42). Embryos are then fixed and immunolabeling can be used to assess the number of proliferating cells (42).

1.3.5 Detecting changes in gene and protein expression

Methods for detecting changes in gene expression include RNA *in situ* hybridization (ISH) and quantitative real-time polymerase chain reaction (qPCR). In ISH, the presence of a specific strand of RNA is identified through hybridization between a labeled probe and its complementary sequence (45). ISH is done on tissue sections, or in the case of zebrafish embryos, whole mount ISH is commonly performed due to the embryos´ small size. The probe labeling tag is recognized with an antibody, marking the strand of interest with a dye or with fluorescence (46). This then makes identification of specific areas or particular cells expressing the gene of interest possible (45). For instance, using a probe that interacts with Histone H3 mRNA can be used to spot proliferating cells (47). qPCR can be used to quantify the expression of a gene of interest, using RNA as starting material. Reverse transcriptase is applied to generate cDNA, which next is used in a qPCR reaction. The polymerase chain reaction exponentially amplifies the cDNA template, resulting in a quantitative relationship between the amounts of accumulating product and starting material (48). The amount of product is measured by fluorescence, for instance by using a dye that only glows when interlaced in dsDNA (48).

Protein expression can be studied through methods such as immunohistochemistry (IHC) or western blotting, which can be viewed as the ISH and qPCR equivalents for protein, rather than gene, detection. In IHC, antibodies are used to identify the protein of interest in specific tissue. Protein-antibody interaction is detected through fluorescence or coloration of the antibody itself, or by means of a secondary antibody. Like ISH, this allows detection of specific cells expressing the protein in question. For instance, PH3-staining described in Section 1.3.4 is an immunohistochemical approach for identification of proliferating cells. The technique of western blotting comprises three key steps, namely protein separation by size through gel-electrophoresis, transfer to a membrane, and finally marking and visualizing the target protein with fluorescent primary or secondary antibodies (49). The relative expression of the protein can then be quantified based on the intensity of fluorescence. Whereas ISH and IHC are methods that allow detection of the target *in situ*, qPCR and western blotting allow detection and quantification of the target from tissue samples without providing information on exact location of expression.

1.3.6 Zebrafish as a model for human drug development

The zebrafish and human chromosomes share large blocks of synteny, and most human genes have orthologous genes in the zebrafish (50). Although the amino acid identity of zebrafish proteins may be less than 70 % of their human counterparts, the conservation in the functional domains is much greater, approaching 100 % similarity (32). As the substrate-binding region of a particular protein is commonly the targeted area for drugs, the high degree of conservation in this region is of much greater significance than the variable overall protein conservation. Numerous chemicals and drugs have shown analogous effects in zebrafish and humans (15, 32). For instance, the effects of alcohol on embryo development seen after alcohol consumption during mammalian pregnancy, such as delayed development, short stature and altered facial expression, can be mimicked by incubating zebrafish embryos in ethanol, (Figure 1-6) (51).

Figure 1-6: Zebrafish embryos treated with alcohol show delayed and abnormal development. Embryos treated with 2 % EtOH (right) compared to controls (left) at 72 hpf.

LC_{50} values obtained from toxicity assays on zebrafish embryos have been shown to correlate well with rodent LD_{50} values (52). Moreover, several studies on how well zebrafish toxicity assays may predict human toxicity have been conducted, using drugs with known effects in humans (4). These have obtained results that suggest the zebrafish "*can attain acceptable levels of predictivity*", ranging from 65-75 %, termed "*sufficient*", to 75-85 %, which is considered good predictivity (4).

1.4 Glucocorticoid drugs

Endogenous glucocorticoids (GC) are steroid hormones involved in many regulatory mechanisms important for instance for development, bone formation, inflammatory and immune responses, metabolism, circadian cell cycle rhythm and stress response (53-55). Effects of GCs are mediated through binding to the intracellular glucocorticoid receptor (GR). Upon binding, the resulting GC-GR-complex translocate to the cell nucleus where it either works as a transcription factor binding to glucocorticoid response elements, or works together with other transcription factors to stimulate or repress transcription of specific genes (55). The GC-GR complex can also produce more rapid, transcription independent effects, for instance several anti-inflammatory responses (56, 57). Synthetic GCs are an extensively used class of drugs, especially important in treatment of chronic autoimmune, inflammatory and allergic diseases (55).

Due to similarities between glucocorticoid signaling in zebrafish and humans, the zebrafish has emerged as a possible model in which to further study GC and GR function. While most fish species have multiple copies of the glucocorticoid receptor, zebrafish, like humans, have only one well conserved GR-gene (58). Furthermore, splicing of this gene renders two possible isoforms, which is also the case for human GR (59). The human GRβ isoform has been shown to be an inhibitor of the transactivational properties of the GRα isoform *in vitro*, but how this translates to *in vivo* conditions are not fully understood (58). In fact, before the GRβ isoform was discovered in zebrafish, humans were the only species in which the presence of this isoform had been established (58). The similarities between human and zebrafish GR have made the zebrafish an interesting model in which to study GC and GR function, and both embryos and adult zebrafish have been used in various research on the field (29, 59-61).

1.4.1 Effects of excess glucocorticoids on the fetus

As an important part of treatment for several quite common disorders, many women of reproductive age are taking glucocorticoids. This class of drugs is also administered prenatally to women at risk for preterm delivery as a preventive measure against respiratory distress syndrome and intraventricular hemorrhage of the child (62, 63). While the endogenous glucocorticoid cortisol plays an important role in embryogenesis, for instance by

regulating maturation of the lungs during late stages of pregnancy, fetal exposure to elevated levels of GCs may not be beneficial (64). Although somewhat inconclusive results have been obtained, animal experiments and clinical studies suggest that both short- and long-term exposure to excess GCs during pregnancy may be unfortunate for the child (65-67). Potential adverse events may be long-term altered hypothalamic-pituitary-adrenocortical-axis regulation, altered organ development and low birth weight, as well as adverse cognitive effects, hypertension and altered metabolic function later in life (65, 66, 68). Outcomes seem to be dependent on the timing and length of GC exposure, and in some cases also the sex of the child (66, 69).

1.4.2 Effects of excess glucocorticoids on adults

The list of possible adverse effects following systemic glucocorticoid treatment is long. Cushing´s syndrome describes the common symptoms of prolonged high levels of GCs. These include redisposition of adipose tissue, usually to abdomen, face and upper back, excess sweating and easy bruising due to thinning of the skin (70). Mood changes such as lethargy, anxiety, depression and mania can also be a complication (71). Furthermore, hypertension, hyperglycemia, increased susceptibility to infections, and reduction of bone density can develop (70).

Another well-documented consequence of long-term use of GCs is a significantly increased risk of developing glaucoma and cataract (72, 73). The presence of a transcriptionally active GR has been shown to exist in human lens epithelial cells, however the molecular mechanisms behind GC-induced eye maladies remain largely unknown (74, 75). Development of cataract and glaucoma have in some cases been linked to decreased expression of PAX6, a transcription factor important for developmental processes of the eye and central nervous system (76-78). This transcription factor, its molecular function and target genes, is a major project at the Pharmacology research group, Department of Pharmacy, University of Tromsø (79, 80).

1.5 Antibiotic drugs: current problems and new possibilities

1.5.1 Antibiotic resistance

The introduction of penicillin for human use in the 1940s completely revolutionized medicine, and represents a hallmark in medicinal history. The way we practice medicine today could not have been possible without antibiotics, which are essential for instance for patients undergoing major surgery, organ transplantation or chemotherapy (81). Unfortunately, the widespread use and misuse of antibiotics since its introduction has rendered antibiotic resistance a major global issue (81). Today, resistant bacteria strains are spreading much faster than new antibiotics are being developed (82). Modern medicine, and thus public health, will suffer huge setbacks if this problem is not dealt with. Development of novel antimicrobial agents that show activity against multi-resistant bacteria strains such as methicillin-resistant *S. aureus* (MRSA), methicillin-resistant *S. epidermis* (MRSE) and Gram-negative bacteria, who are generally more resistant to antibiotics, is essential (81, 83). Today and during the last couple of decades, new approaches are and have been investigated, but none has yet made it to general clinical use (84).

1.5.2 Antimicrobial peptides

As an important part of the innate immune system in both pro- and eukaryotes, and with remarkably broad activity spectra, antimicrobial peptides (AMPs) have emerged with the potential to form a new generation of antibiotics effective against multi-resistant bacteria (85, 86). Both synthetic and naturally occurring AMPs show activity against Gram-negative and –positive bacteria, as well as fungi, viruses and parasites (85). Some even show anti-cancer properties (86). AMPs vary in length, sequence and structure, but are usually less than 100 amino acids long, and have a net positive charge as well as a significant hydrophobic part, which allows the peptides to interact both with lipophilic surfaces and hydrophilic, negatively charged substances (86). One of the features of antimicrobial activity of the AMPs is their ability to lace themselves into the bacterial cytoplasmic membrane, either forming pores or otherwise disrupting the lipid organization of the membrane, causing leakage of cell contents (82). Some AMPs does not disrupt the bacterial membrane, but simply translocate through it to act on intracellular targets (86). The much higher content of anionic lipids on the surface of bacterial membranes compared to mammalian ones is thought to assure selectivity for microbial cells (87). Although promising as novel antibiotic drugs, high production cost and

poor systemic pharmacokinetic profile due to proteolytic degradation, as well as a potential risk of adverse effects on apoptosis and mast-cell degranulation, is a problem (85, 86).

1.5.3 Antimicrobial $\beta^{2,2}$-amino acid derivatives

In an attempt to overcome the above mentioned problems associated with traditional AMPs, the Natural Product and Medicinal Chemistry research group, Department of Pharmacy, University of Tromsø has developed a new class of short AMPs based on the minimum pharmacophore requirements for antimicrobial activity, namely $\beta^{2,2}$-amino acid derivatives (88, 89). These have been further improved with regard to suitability for oral administration (90). The derivatives have been synthesized from inexpensive starting materials, using methods that would be fit for industrial production (90). One of these compounds is $\beta^{2,2}$-amino acid derivative 161, a lead compound that has proven to be a promising candidate against several multi-resistant bacterial infections, due to low MIC values and high selectivity (90). Anticancer properties against Ramos cells have also been shown (91).

1.6 Objectives

The main aim of this thesis is to explore the zebrafish as a model system for use in safety pharmacology, and look into some of the different methods that can be used to do so. Approaches include visual assessment of mortality and possible malformations, and performance of apoptosis assay and western blotting to look at apoptosis and proliferation. The glucocorticoid drug dexamethasone is tested for teratogenicity in the zebrafish embryo. Also, a novel antimicrobial peptide, $\beta^{2,2}$-amino acid derivative 161, is tested for toxicity, with the aim to determine an LC_{50} for this drug when given to zebrafish embryos.

In parallel with the zebrafish experiments, studies using mammalian cell cultures are carried out. This is done as a side project in case fish egg production ceases during the project period. The biologically relevant cell line B3 (human lens epithelial cells) was chosen because both decreased levels of PAX6 and administration of glucocorticoids are associated with the development of cataracts in humans. Likewise, U251 (human glioblastoma cells) was chosen because both presence of PAX6 and use of glucocorticoids are associated with glioblastoma cancer patient prognosis. The major goal for the cell studies is therefore to see if the level of

expression of PAX6 will change upon dexamethasone treatment, and it is also of interest to look at proliferation and apoptosis.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffers and solutions

Table 2-1: Buffers and solutions used in this thesis

Method	Buffer	Contents
Embryo handling		
	E3	Stock solution 60 x E3: 17.2 g NaCl 0.76 g KCl 29 g $CaCl_2$ x 2 H_2O 4.9 g $MgSO_4$ x 7 H_2O Distilled water ad 1 L pH-adjusted 1 x E3 was used for embryo handling, pH: 6.8 – 6.9
Preparation for protein analysis		
	1 M Dithiothreitol (DTT)	1.54 g dithiothreitol 33.3 µL 3 M NaOAc, pH 5.2 Distilled water ad 10 mL
	2 x Sodium dodecyl sulfate (SDS) gel loading buffer	5 mL 1M TrisHCl, pH 6.8 10 mL 20 % SDS 20 mL 50 % Glycerol 0.1 g Bromophenol blue Distilled water ad 50 mL
Gel electrophoresis		
	Running buffer	Commercial: Invitrogen Life technologies, NuPAGE MES SDS Running buffer (20x) 1 x Running buffer in distilled water was used for gel electrophoresis

Table 2-1: Continued

Method	Buffer	Contents
Western blotting		
	10 x TBS	10 g KCl 400 g NaCl 200 mL 1 M TrisHCl, pH 7.5 Distilled water ad 5 L
	TBS-T buffer	0.5 L 10 x TBS 5 mL Tween-20 Distilled water ad 5 L
	Transfer buffer	29 g Tris base 144 g Glycine 1 L Methanol Distilled water ad 5 L
	Blocking buffer	Commercial: LI-COR Biosciences, Odyssey Blocking Buffer
General washing		
	Phosphate buffered saline (PBS)	8 g NaCl 0.2 g KCl 1.44 g Na_2HPO_4 0.24 g KH_2PO_4 Distilled water ad 1 L pH adjusted: pH 7.4
Preparation for electron microscopy		
	1 M Sørensens phosphate buffer	15.87 g NaH_2PO_4 x H_2O 137.83 g Na_2HPO_4 x 12 H_2O 140 g Sucrose Distilled water ad 5 L Adjustments: pH: 7.4 Osmolarity: 300 – 320 mOsm

2.1.2 Chemicals

Table 2-2: Chemicals used in this thesis

Product name	Manufacturer
Acridine Orange hemi (Zinc Chloride) salt	Sigma Aldrich
Dexamethasone Water-Soluble	Sigma Aldrich
$\beta^{2,2}$-amino acid derivative 161, trifluoroacetic salt	Natural Product and Medicinal Chemistry research group, Department of Pharmacy, University of Tromsø
N-Phenylthiourea (PTU), Grade I	Sigma Aldrich

2.1.3 Antibodies

Table 2-3: Antibodies used in this thesis

Product name	**Manufacturer**
Primary antibodies	
Anti-Actin, rabbit	Sigma Life Science
Anti-Phospho-Histone H3 (Ser10), rabbit	Millipore
Anti-Active caspase-3, rabbit	BD Biosciences, BD Pharmingen
Anti-PAX6, rabbit polyclonal	Millipore
Anti-SGK, rabbit monoclonal	Millipore
Secondary antibody	
Donkey anti-rabbit IRDye, 800 CW	LI-COR biosciences, Odyssey

2.1.4 Protein size markers

To monitor the electrophorese progress and the electrotransfer efficiency when analyzing proteins, SeaBlue Pre-Stained Standard (Life technologies, Invitrogen) was used. MagicMark XP Western Protein Standard, 20-220 kDa (Life Technologies, Invitrogen) was used for protein molecular weight estimations on western blots.

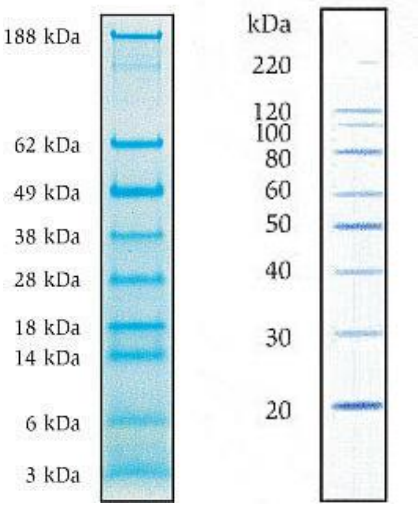


Figure 2-1: SeaBlue Pre-stained Standard (left) and MagicMark XP Western Protein Standard (right) were used as molecular weight markers (92, 93).

2.1.5 Other compounds

Table 2-4: Other compounds used in this thesis; enzymes, cell media components etc.

Name	Manufacturer
Ethyl 3-aminobenzoate, methanesulforic acid salt (Tricaine)	Sigma Aldrich
Pronase from Streptomyces griseus	Fluka, BioChemika
Fetal calf serum (FCS)	Sigma Life Sciences
Sodium Pyruvate 100 mM solution	Sigma Life Sciences
Penicillin-Streptomycin Antibiotics	Sigma Life Sciences
MEM Non-essential amino acids (NEAA)	Invitrogen GIBCO
0.25 % Trypsin-EDTA solution	Sigma Life Sciences
Minimum essential medium eagle (MEM)	Sigma Life Sciences
Dulbecco´s modified eagles medium (DMEM)	Sigma Life Sciences

2.1.6 Cell lines and growth media composition

Human lens epithelial (B3, ATCC: CRL-11421) and human glioblastoma (U251, gift from Dr. Hrvoje Miletic, University of Bergen) cell lines were used in this thesis.

Table 2-5: Composition of growth medium used for B3 and U251 cell lines

Cell line	Components	Volume (%)
B-3		
	MEM	77
	FCS	20
	Pyruvate	1
	NEAA	1
	Antibiotics	1
U251		
	DMEM	87
	FCS	10
	Pyruvate	1
	NEAA	1
	Antibiotics	1

2.2 Methods used for zebrafish experiments

2.2.1 Zebrafish husbandry

2.2.1.1 Living conditions/ fishkeeping

AB/TU TAB-14 wild type zebrafish purchased from the Zebrafish International Resource Center (ZIRC) or bred in the lab were kept in a Zebtec bench-top Aquatic System, in 3.5 L and 8 L plastic aquariums. The system circulates aquatic water and continuously filters and aerates it to keep it fresh and clean. The system also monitors water temperature, conductivity and pH, automatically making adjustments if necessary. These parameters were maintained at 26 – 30 °C, 650 - 750 µS and 6.7 – 7.5, respectively. Lighting conditions were kept at a fixed 14-10 hours light-dark cycle; lights were gradually turned on from 08:45 – 09:00 in the morning, and were turned off from 22:00 – 22:30 at night. The fish were fed three times daily with a varied diet consisting of pellets and live and newly hatched brine shrimp (*Artemia sp*.). Fish over three months of age were considered eligible for spawning.

Due to several weeks of construction work at the zebrafish lab, zebrafish were also held in stand-alone tanks at the ordinary lab facilities for some time. Here, fish were fed as usual with

brine shrimp and pellets. We tried to mimick the conditions of the zebrafish lab with regard to temperature, pH and conductivity of aquarium water. However, adjustments had to be done manually, and were not done more than every other day. Thus, zebrafish held at the ordinary lab were kept under less controlled conditions than those held at the zebrafish lab.

2.2.1.2 Mating

Adult zebrafish were separated by gender in the afternoon the day before intended spawning. Females were told apart from male fish by their bigger and whiter abdomen, while males were identified by their slender, more streamlined appearance, as well as their somewhat darker blue stripes with a pink tint in between (16). As the lights were gradually turned on the following morning, males and females were put together in multi-mating containers. To prevent the zebrafish predating on their embryos, a spawning mesh placed in the middle of the container allowed eggs to drop to the lower basin of the mating tank, thus protecting them from being eaten (94, 95). In order to further promote embryo production, the mating tanks were sometimes tilted to create a depth gradient (96). The fish were left to mate undisturbed for about two hours. After breeding, the zebrafish were returned to their original tanks in the rack. The eggs were collected in a petri dish and brought back to the lab for further handling.

2.2.2 Embryo treatment and drug administration

2.2.2.1 Handling of embryos

The medium used for general handling of the embryos, as well as for dilution of all drug solutions, was 1x E3 (Table 2-1), a medium commonly used when raising zebrafish embryos (5, 97, 98). Newly laid embryos were rinsed extensively in E3. Unfertilized, dead or yeast-infected embryos were removed. This was repeated right before starting drug treatment, as dead embryos generates a poor environment for the remaining embryos, thus counteracting normal growth and development and promoting further embryo death (98). There can be variations in the quality of embryos obtained from different parent fish concerning survival rate, development and sensitivity to chemicals (11). Clutches of embryos laid at approximately the same time were therefore always mixed together before testing of compounds was carried out. Experiments were carried out using 6-well plates (Falcon Tissue Culture Plate, 6 well. Flat bottom with low evaporation lid). Embryos were distributed among

the wells, placing approximately 50 embryos in each. Embryos were never allowed to dry out, and were incubated in E3 at all times until application of drug.

2.2.2.2 Administration of drug and control medium

The desired concentration of the drug to be tested was prepared using E3 as solvent. Solutions of dexamethasone were stored protected from light at 4°C, and were given a shelf life of four weeks. A 0.5 mg/mL stock solution of $\beta^{2,2}$-amino acid derivative 161 was prepared and divided into aliquot parts, before being stored at -20°C. Embryos were kept in the dark in an incubator (Termaks B8058) at 28 °C, and were only taken out for short periods of time for observation of developmental status under a dissecting microscope, or to be prepared for photographing or various testing procedures.

Originally, the intension was to test dexamethasone in concentrations of 10 and 100 μg/mL. However, due to an oversight, dexamethasone concentrations of 0.68 and 6.8 μg/mL were used instead. Treatment was initiated around 3.3 hpf, the "high stage" of embryo development. The developmental stage was determined using Kimmel et al.'s description of the different stages of zebrafish embryo development (19). E3 was removed and 2 mL of the correct drug solution was added to each well where experiments were to be carried out. Simultaneously with all experiments, control embryos were incubated in 2 mL E3. Treatment continued until 24 hpf, 48 hpf or 72 hpf. For experiments with duration past 24 hpf, drug solution or control medium was changed every 24 hours. Only embryos obtained from the zebrafish lab were used when testing dexamethasone.

For testing of $\beta^{2,2}$-amino acid derivative 161, two different experimental designs were used. In method A, embryos were treated with the peptide from 3.3 hpf until the end of the experiment at 24 hpf. In method B, treatment started at 24 hpf and was continued until 48 hpf. Before start of method B, embryos were examined so that only normally developed embryos were included. Many different concentrations of derivative 161 were tested (Table 2-6). Control embryos were simultaneously incubated in E3. 2 mL were used of both control

medium and drug solution. Embryos obtained from both the zebrafish lab and from zebrafish held at the ordinary lab were used.

Table 2-6: Different concentrations of $\beta^{2,2}$-amino acid derivative 161 tested in method A and B

Concentration (mg/mL)	Method A	Method B
0.001	o	
0.01	o/x	
0.02	o/x	
0.03	o/x	
0.04	o/x	
0.05	o/x	o
0.06	o/x	
0.075	o/x	
0.1	o/x	o/x
0.11		o/x
0.12	x	o/x
0.125		o/x
0.13		o/x
0.135		o/x
0.14	x	o/x
0.15		o/x
0.16	x	
0.175		o/x
0.19		x
0.20	x	o/x
0.22	x	x
0.25	x	x
0.30		x
0.40		x
0.50		x

o indicates testing on zebrafish embryos obtained from zebrafish held at the ordinary lab
x indicates testing on zebrafish embryos obtained from the zebrafish lab

2.2.2.3 Observation of embryos

Using a dissecting microscope (Nikon SMZ 1000) zebrafish embryos were counted and assessed with regard to survival and morphological changes every 24 hours from 24-72 hpf, depending on the duration of the experiment. When testing $\beta^{2,2}$-amino acid derivative 161, embryos were also examined one and three hours after starting treatment to check for acute toxicity. Dead embryos were identified by their opaque appearance due to coagulation and by the lack of a heart beat. Malformations observed were recorded and documented through photographing. Abnormally developed embryos were not removed until the end of the experiment, whereas dead embryos were taken out when identified.

2.2.3 Preparing embryos for photographing

Morphological changes were documented through pictures taken with a camera (Nikon digital sight DS-U3, computer program: NIS-elements D 4.00) connected to the lab's dissecting microscope. The software was calibrated for length measurements. Cells undergoing apoptosis in embryos prepared for apoptosis assay (Section 2.2.5) were observed under a microscope (Zeiss Axiovert S100) with a green fluorescence filter with excitation 470/40. This microscope was linked to a Nikon Digital Sight DS-SM camera (computer program: NIS-elements BR 2.30).

2.2.3.1 Dechorionation of embryos

The embryos' chorion was chemically removed using 500 µL 1 mg/ mL pronase in E3. After incubating them in this media for 2-3 minutes, the well plate was lightly stirred until the embryos started to lose their chorions. When the first ones started to drop out, the pronase solution was removed and the embryos were washed in E3 at least three times to neutralize the environment surrounding them and to remove residue. Embryos that were slow in dropping their chorions were helped out manually using small needles to penetrate the chorion and then lifting it off the embryos, or by gently pipetting the embryo in question up and down in a plastic pipette.

2.2.3.2 Anaesthetizing and positioning embryos

1.2 % agarose was prepared by melting agarose in E3 using a microwave oven. While still hot and liquid, 5 mL agarose mixture was added to small petri dishes (5 cm in diameter), and was then allowed to cool off and polymerize. To keep the embryos from moving around when trying to photograph them, they were anaesthetized with 500 µL 1x tricaine in E3. Anaesthetized embryos were then positioned on the agarose gel plate, using micropipette tips to gently shift them around and arrange them as appropriate.

2.2.4 Electron microscopy

Embryos obtained from the zebrafish lab were incubated in 0.1 and 0.2 mg/mL $\beta^{2,2}$-amino acid derivative 161 from 24-25 hpf. Embryos were dechorionated as previously described before peptide treatment started. After one hour of treatment, medium was removed and treated embryos and controls were fixed in 8 % paraformaldehyde in 200 nM HEPES over night at 4°C. After this, samples were further prepared by Dominik Ausbacher and employees of the Electron microscopy department at the University of Tromsø. The methods used will only be described briefly.

Fixed embryos were washed two times during 15 minutes in Sørensens phosphate buffer (Table 2-1), before being incubated in osmium tetroxide in water for 1.5 hours. Embryos were again washed two times during 15 minutes in Sørensens phosphate buffer. Embryos were dehydrated by incubation in increasing concentrations of ethanol, before being chemically dried with hexamethyldiciliazane. Next, embryos were mounted on aluminum stubs with carbon tape, before carbon vapour-deposit. Embryos were looked at and photographed with a field emission scanning electron microscope (Zeiss, Sigma FE-SEM, computer program: SmartSEM).

2.2.5 Apoptosis assay

2.2.5.1 Inhibiting melanogenesis

To improve detection of apoptotic cells, N-Phenylthiourea (PTU) was used to inhibit melanogenesis and thereby generating completely transparent embryos. Embryos intended for apoptosis assay at 48 or 72 hpf were incubated in PTU from the 28 somite stage, about 23 hpf. A solution of 75 µM was prepared fresh in E3, either the same day or the day before intended use, and was kept protected from light at 4°C (41). The solution was heated to 65°C prior to use to ensure that the compound was properly dissolved, and was then cooled down again before addition of dexamethasone or $\beta^{2,2}$-amino acid derivative 161.

2.2.5.2 Staining with Acridine orange

Dechorionated embryos were incubated in 1x acridine orange in E3 for 30 minutes. Embryos were then washed three times in E3 during 10 minutes, before they were anaesthetized with 1x tricaine. Stained embryos were protected from light at all times, and visualization and photographing of apoptotic cells were conducted in a dark room.

2.2.6 Western blotting

The expression of Pax6, pro-caspase-3, active caspase-3 and phospho-histone H3 was evaluated from crushed whole embryo tissue samples by western blotting. Subsequent staining with anti-actin was used to correct for loading of samples.

2.2.6.1 Preparation of tissue samples

Embryos intended for protein analysis were dechorionated if necessary and transferred to eppendorf tubes in segments of ten. This was done for embryos incubated in both concentrations of dexamethasone and selected concentrations of derivative 161, as well as for control embryos, at 24, 48 and 72 hpf (only dexamethasone treated embryos). The yolk sac was removed on embryos taken out for testing at 24 hpf due to its protein richness and the possibility that this could cloud the image. This was done by first anaesthetizing embryos with 1x tricaine or dulling them on ice for about 30 seconds, before gently pricking out the yolk sac with a micropipette tip. Embryos isolated at 48 – 72 hpf were allowed to keep their yolk sac, as the size of it at this point was deemed acceptably small. Incubation medium was removed and embryos were stored at -70°C immediately.

When set to start the analysis, embryos were thawed on ice, before 50 µL 2 x SDS with 20 % 1 M DTT (Table 2-1) was added to each of the eppendorf tubes. The samples were then put in a heating block at 100°C for 8 minutes, before being sonicated (Bioruptor UCD-200) in a 4°C ice- and waterbath for 2.5 minutes at a cycle of 30 second impulses. Finally, the samples were centrifuged (Biofuge Fresco, Heraeus instruments) at 4°C for 2 minutes, 13 000 rpm.

2.2.6.2 Gel-electrophoresis

Two gels were run simultaneously. The gels to be used (Novex, PROTEIN NuPAGE 4 – 12% Bis-tris-gel, 1 mm x 12 well) were assembled in the buffer core and placed in the electrophoresis container (Life sciences, Invitrogen XCell SureLock Mini-Cell). The wells were flushed with running buffer (Table 2-1). About 200 mL running buffer was added to the upper buffer chamber, covering the gels completely (Figure 2-2).

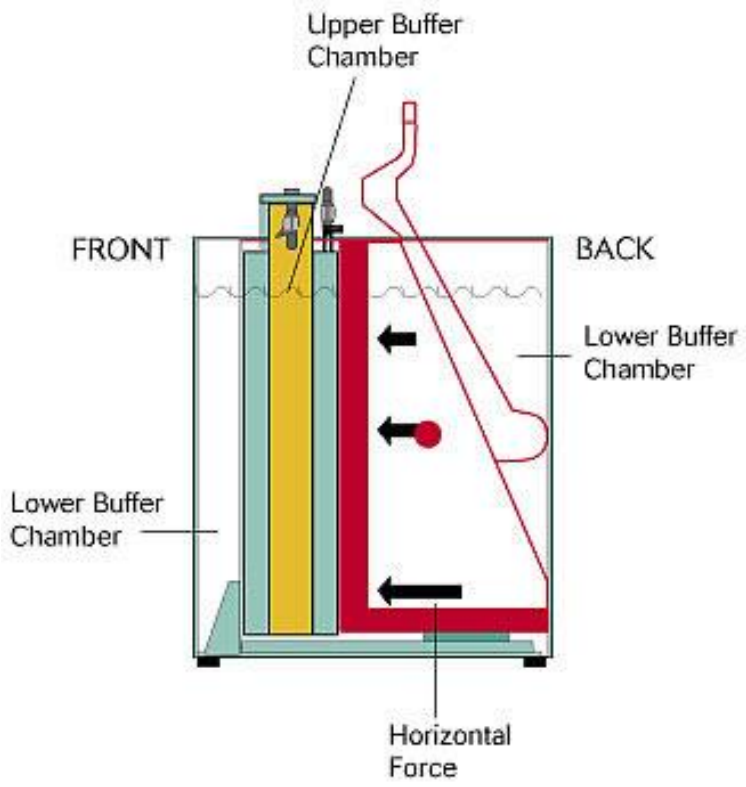


Figure 2-2: XCell SureLock Mini Cell viewed from the side (99)

Ladders and samples were loaded on to the gels. The ladders used, 3 µL SeaBlue and 0.5 µL MagicMarker (Figure 2-1) were mixed together before loading. 10 µL of each sample was applied, as well as 10 µL 3T3-PAX6 as positive control. The lower buffer chamber was filled with approximately 600 mL running buffer, enough to allow access to the gels. Finally, the gels were run at 200 V for 45 minutes (Life sciences, Invitrogen Power Ease 500).

2.2.6.3 Electrotransfer/ Blotting

Two filter sheets and one membrane (LI-COR Biosciences, Odyssey Nitrocellulose membrane) for each gel were cut to fit the gel´s dimensions (7.5 cm x 7.0 cm), and were then soaked in transfer buffer (Table 2-1) along with sponges of approximately the same size. The gels were retrieved from their plastic container, and were sandwiched in the blotting chamber (Invitrogen XCell II Blot Module) as follows:

2 sponges
Filter sheet
Gel A
Membrane A
Filter sheet
Sponge
Filter sheet
Gel B
Membrane B
Filter sheet
2-3 sponges

Air bubbles were removed by using a small rolling pin and the blotting chamber was placed in the electrophoresis cell. Transfer buffer was filled to the top of the upper buffer chamber, approximately 50 mL. Distilled water was added to the lower chamber, almost all the way to the top, but making sure it did not spill over to the blotting compartment. Blotting program at 25 V were started, and were allowed to run for two hours.

2.2.6.4 Blocking and antibody incubation

After electrotransfer, membranes were collected and incubated with about 5 mL blocking buffer (Table 2-1) in a 50 mL centrifuge tube for one hour in room temperature, or at 4°C over night. The membranes were then incubated with primary antibody for one hour. With the exception of anti-caspase-3, which was diluted 1:1200, all primary antibodies (Table 2-3) were diluted 1:1000 in 3 mL blocking buffer (Table 2-1). 0.1 % Tween-20 was added to the mixture before incubation. Membranes were washed in TBS-T (Table 2-1) for 3x5 minutes, before being incubated with 5 mL secondary antibody (Table 2-3) for one hour in room temperature, or at 4°C over night. Secondary antibody was diluted 10 000 times in TBS-T. After incubation with secondary antibody, the membranes were once more washed in TBS-T for 3x5 minutes. During this whole process, care was taken to never let the membranes dry out. All incubations described above were carried out in a test tube rotator, thus continuously

circulating fluid over the membranes. The blots were developed (LI-COR, Odyssesy Sa Infrared Imagin System) and were stored covered in TBS-T at 4°C for later use.

2.2.6.5 Quantification of bands

Background staining was subtracted and the relative fluorescence of each band present on the blot was determined using the Li-Cor Odyssey Sa 4.0 software, yielding "integrated intensities" for each band. For the most part, background subtraction was set to take into consideration the local background on all four sides surrounding each band. However, for bands that had smeared in the longitudinal direction, background subtraction was calculated only with regard to the background immediately to the right and left sides of the band. Integrated intensity of bands of interest compared to integrated intensity of actin in the same lane was used as a measure of protein expression corrected for loading. For each blot, the corrected band intensity for the control from the earliest time point (i.e 24 hpf), was set to 1, and each successive band was corrected for loading and expressed relative to the this, yielding estimates of relative protein expression.

2.2.7 Treatment of data

Experiments were mortality rate in the control group was greater than 20 % were excluded from all calculations. Where appropriate, mean values were compared by independent-samples two-tailed t-test, carried out using SPSS Statistics version 21, to decide if the frequency of lethal or sub-lethal endpoints observed were significantly different in treatment groups compared to the control group. An F-test of equality of variances was first performed, and a suitable t-test was then chosen based on the result. Significance level (α) was set to 0.05.

Fischer exact test was performed to test differences in the incidence of apoptosis in certain tissues of drug treated embryos compared to controls. The software used was R 3.0.0. Significance level (α) was set to 0.05.

Embryo mortality rate after treatment with the different concentrations of $\beta^{2,2}$-amino acid derivative 161 was corrected for death in the control group using Abbotts formula (17):

$$\text{Corrected \% mortality} =$$

$$\frac{\text{\% Mortality test group} - \text{\% Mortality control group}}{100 - \text{\% Mortality control group}} \text{ x } 100$$

LC_{50} for $\beta^{2,2}$-amino acid derivative 161 was estimated separately for method A and B, and for embryos obtained from the zebrafish lab contra the ordinary lab, by probit analysis. Using a table for transformation of percentages to probits, probits corresponding to the nearest whole number of corrected mortality rate for mortality greater than 0% and less than 100% were plotted against log concentration of derivative 161. Linear regression was used, generating an expression of probits of mortality as a function of concentration, making it possible to calculate the LC_{50}. LC_{16} and LC_{84} were also calculated from the linear function expression, and 95 % confidence limits of the LC_{50} were determined by means of the Litchfield-Wilcoxon method (100):

$$S = 0.5(\frac{LC84}{LC50} + \frac{LC50}{LC16})$$

$$\text{Log f} = \frac{2.77 \text{ x Log S}}{\sqrt{n}}$$

Where n is the total number of embryos incubated in the range of concentrations between LC_{16} and LC_{84}.

$$\text{Upper confidence limit} = LC50 \text{ x f}$$

$$\text{Lower confidence limit} = LC50/f$$

2.3 Methods used for cell culture experiments

2.3.1 General cell care

Cells were incubated in 75 cm^2 cell culture flasks (Nunc, cell culture flask with filtered cap) at 37 °C and 5 % CO_2 (Thermo Scientific, HERA CELL 150i CO_2 incubator). All cell work was done in a laminar air flow (LAF)-bench. Aseptic working techniques were practiced.

Cells were split when about 80 % confluence of the flask surface was reached. Fresh medium was heated to approximately 37 °C. Old medium was removed from the cells by suction, and cells were washed with about 10 mL PBS (Table 2-1). PBS was removed and 1 mL Trypsin-EDTA was added to the flask. Cells were incubated at 37 °C and 5 % CO_2 until all cells had detached from the flask surface, usually after about 2 minutes. Microscopy (Zeiss Axiovert S100) was used to confirm detachment. If some cells were slow in coming loose, the cell culture flask was gently knocked against a hard surface to facilitate detachment. When cells had detached, the trypsin was neutralized by adding 9 mL of fresh medium, making the total volume in the flask 10 mL. Cells were dispersed by pipetting medium up and down. Then, depending on how much the cells needed to be split, a certain percentage of the cells were removed, and fresh medium was added. Usually, cells were split 1:10. If cells grew slowly and splitting was done less frequent than every third day, growth medium was exchanged every third day.

2.3.2 Administration of drug

Cells were plated out in 6-well plates (Falcon Tissue Culture Plate, 6 well. Flat bottom with low evaporation lid) before administration of dexamethasone. About 60 000 cells in 2 mL of medium was added to each well. To count cells, a small part of the trypsinized cells was added to a Bürker chamber covered with a glass slide. The number of cells in three big squares was counted. The average of this number was multiplied by 10^4 to yield the number of cells per 1 mL cell suspension. The dilution yielding 30 000 cells/mL was calculated and prepared, and 2 mL of this was added to each well. Cells in culture plates were incubated over night so that they would attach to the plastic surface. The following day, medium was removed by suction and 2 mL drug solution or control medium was added. A fresh 1.0 mM stock solution of dexamethasone was prepared in PBS. This was diluted in growth medium to

yield the test solutions of 1.0 μM and 0.1 μM dexamethasone. Controls were incubated in 2 mL growth medium with a corresponding volume of PBS added.

2.3.3 Western blotting

Cell extracts were collected 4, 20 and 72 hours following initiation of drug treatment. Medium was removed from each of the wells, and cells were rinsed twice with 2 mL PBS. Wells were sucked dry and 100 μL 2xSDS with 20 % 1 M DTT was added to each well. A rubber cell scrape (BD Falcon Cell Scraper, 1.8 cm blade) was used to detach cells from the well surface, and extracts were pipetted to eppendorf tubes. Collected samples were immediately heated for five minutes at 100 °C on a heating block and cooled on ice, before they were stored at -80 °C for protein analysis. When needed, thawed samples were sonicated, and gel-electrophoresis and western blotting was carried out as described in Section 2.2.6. The same antibodies that were used for analysis of zebrafish extracts were used on the cell extracts (Table 2-3), with the addition of Anti-SGK, diluted 1:200 in blocking buffer.

3. RESULTS

3.1 Zebrafish experiments

3.1.1 Dexamethasone

3.1.1.1 Mortality and visible morphological changes

We wanted to investigate the teratogenic potential of the glucocorticoid drug dexamethasone (dex) by simple visual assessment of treated zebrafish embryos compared to controls at 24, 48 and 72 hpf. Figure 3-1 gives a general idea of the experimental set-up at the two latest time points.

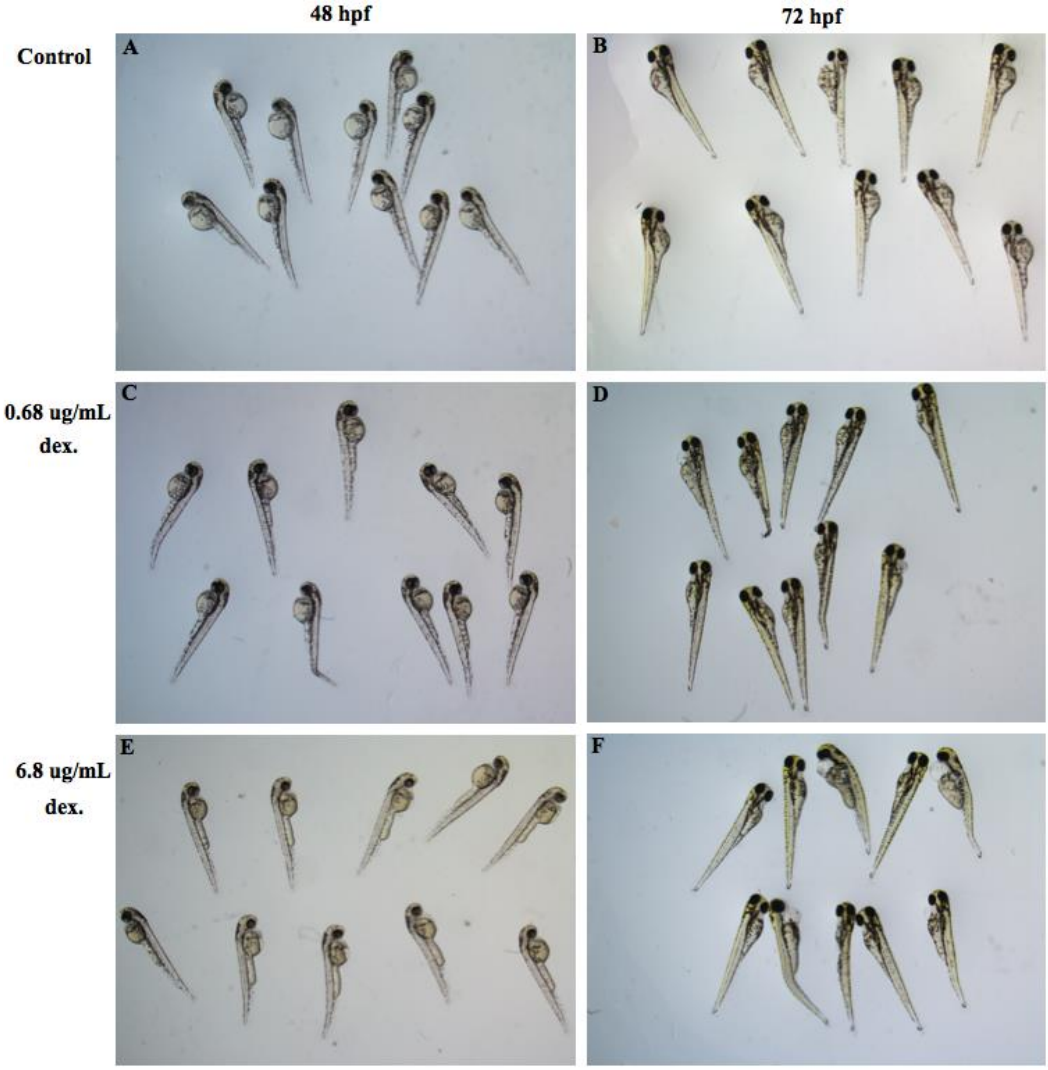


Figure 3-1: Experimental set-up at 48 and 72 hpf. Embryos were incubated in control medium and two different concentrations of dexamethasone. A-B: Control embryos at 48 and 72 hpf, respectively. C-D: Embryos treated with 0.68 µg/mL dex. at 48 and 72 hpf, respectively. E-F: Embryos treated with 6.8 µg/mL dex. at 48 and 72 hpf, respectively.

Embryos were assessed with regard to survival and morphological changes every 24 hours until the end of the experiment. Table 3-1 and Figure 3-2 summarizes the findings. Data from the individual experiments are shown in Appendix (Table A1-1 to Table A1-3). Mortality was assessed within each time interval, whereas the incidence of malformations was evaluated as cumulative rates. There were no differences in mean mortality rate between the groups at any time during the experiments (Table 3-1). Mortality was highest the first 24 hours in all groups. After this, very few deaths were observed. Malformations were only occasionally observed at 24 hpf, but there seemed to be a small increase in the incidence of abnormal development among drug treated embryos. However, the malformation rate was not significantly increased compared to controls at this point. At the two later time points, the occurrence of malformations increased markedly with increasing concentrations of dexamethasone. This increase was significant between treated embryos and controls both at 48 hpf and 72 hpf.

Table 3-1: Overview of dexamethasone experiments

µg/mL dex	Number of experiments*	Total n	Mean mortality (%) ± STD	p-value	Mean a.d (%) ± STD	p-value
3.3-24 hpf						
0	7	889	13.25 ± 2.70		0.15 ± 0.39	
0.68	7	940	18.73 ± 10.39	n.s	0.76 ± 1.07	n.s
6.8	6	909	14.49 ± 8.00	n.s	1.06 ± 1.12	n.s
24-48 hpf						
0	8	690	0.10 ± 0.28		2.48 ± 1.55	
0.68	8	676	0.33 ± 0.66	n.s	10.94 ± 9.03	< 0.05
6.8	7	582	0.22 ± 0.58	n.s	12.67 ± 6.56	< 0.01
48-72 hpf						
0	8	357	0.43 ± 1.22		5.81 ± 4.29	
0.68	8	359	0.38 ± 0.72	n.s	15.04 ± 8.11	< 0.05
6.8	7	332	1.62 ± 2.44	n.s	23.25 ± 6.06	< 0.01

A.d: Malformed or otherwise abnormally developed embryos, expressed as cumulative percentage of survivors
STD: Estimated standard deviation
Total n reflects the total of included embryos throughout the experiments.
Probability values given are for a two-tailed t-test
n.s: difference between treated group and control is not significant (α=0.05).
*Experiments were the control group displayed a mortality rate >20 % were excluded from all calculations. See Appendix Table A1-1 to Table A1-3 for full details on the individual experiments.

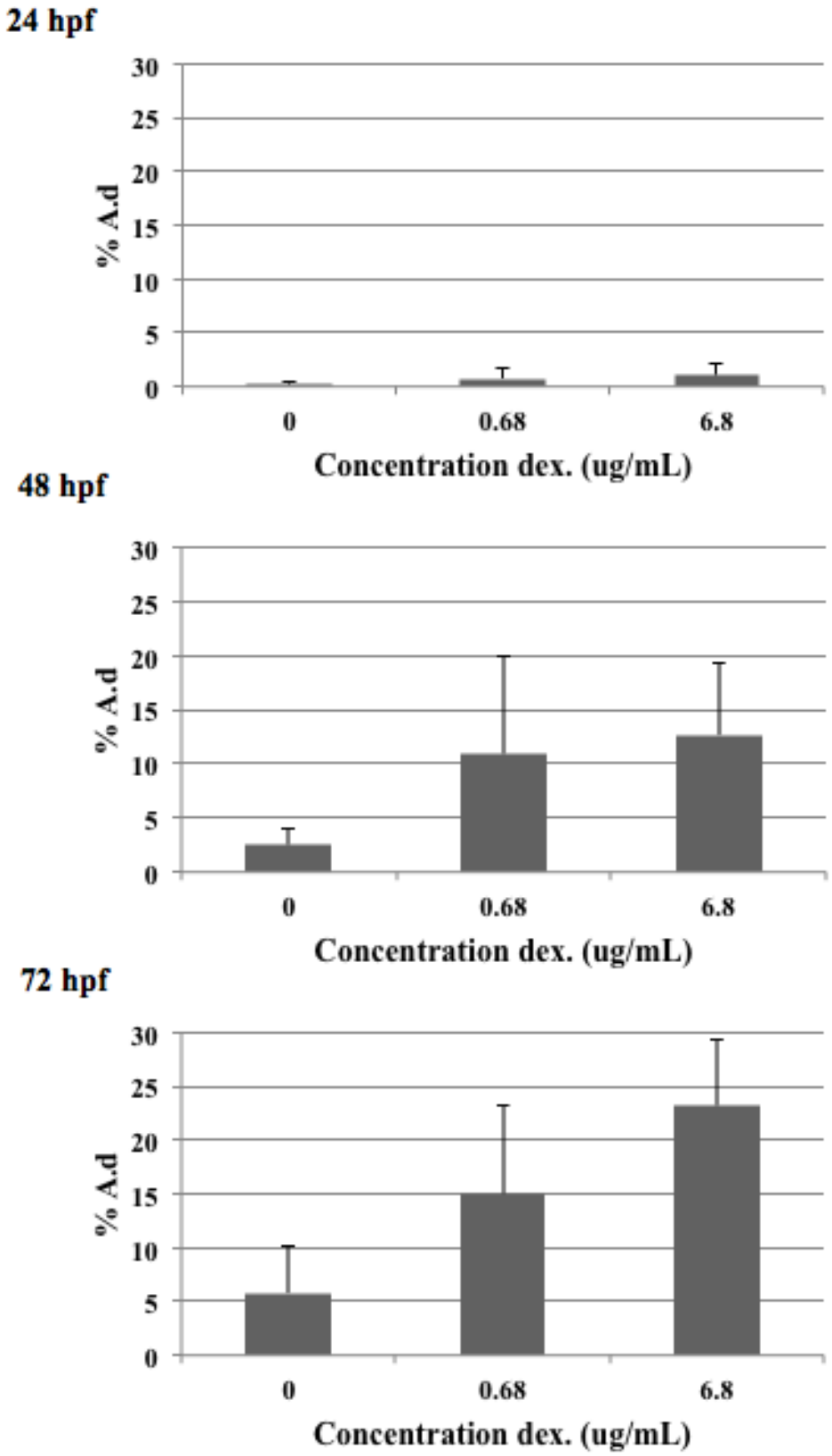


Figure 3-2: Graphic presentation of mean malformation rates and estimated standard deviations after treatment with dexamethasone, as presented in Table 3-1. Malformations or otherwise delayed or unusual development was generally observed more often with increasing drug concentrations, but were only significantly more occurring in treated groups at 48 hpf and 72 hpf. A.d: Malformed or otherwise abnormally developed embryos, expressed as percentage of survivors. Malformation rates are cumulative.

The most commonly observed malformations were pericardial edema and yolk sac edema (Figure 3-3, B and D), or a combination of both. Typically, yolk sac edema could be seen around 48 hpf, whereas pericardial edema became more evident at 72 hpf. Other malformations occasionally observed were kinked tail and abnormal body axis curvature (Figure 3-3, F-H).

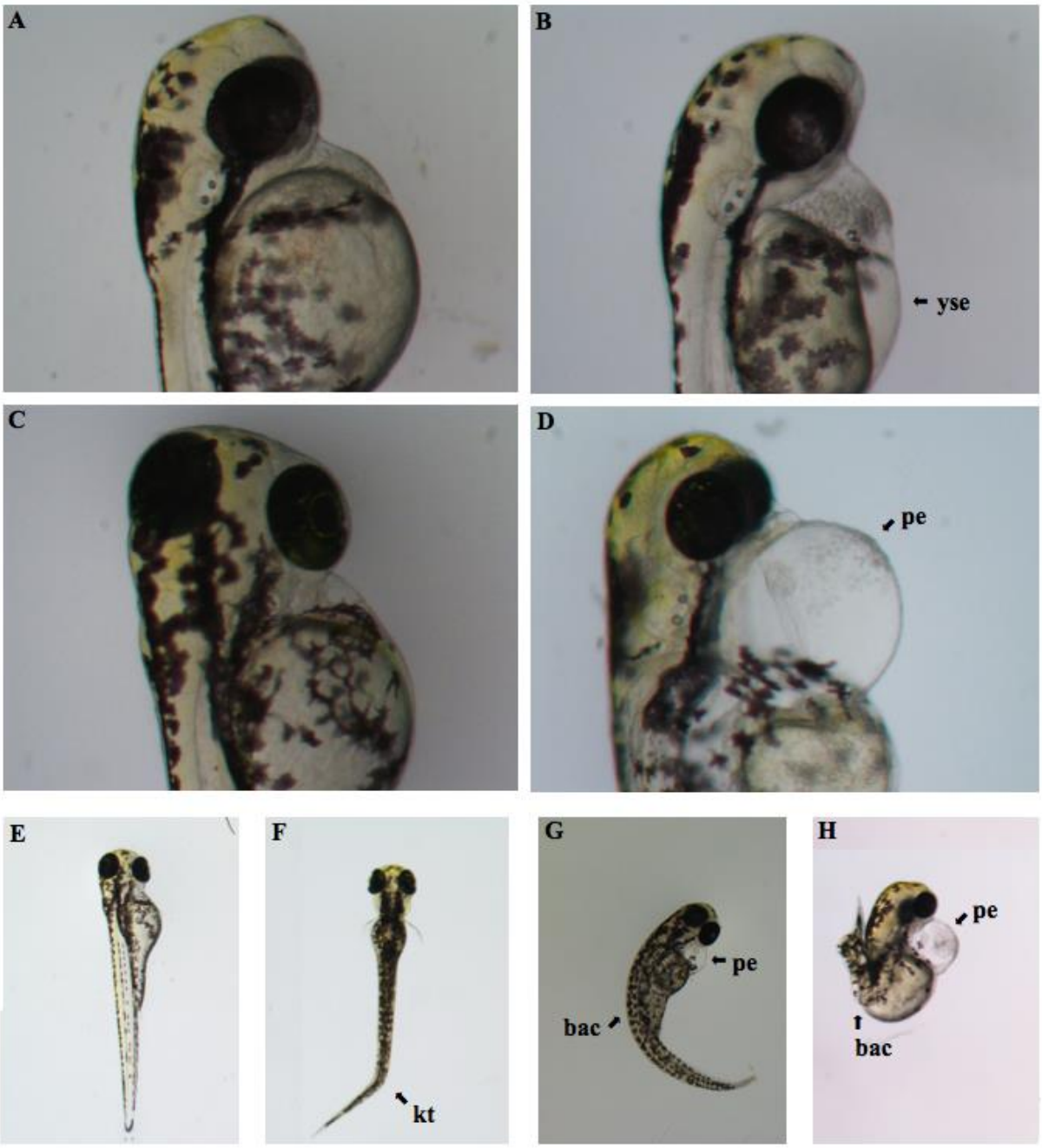


Figure 3-3: Zebrafish embryos treated with dexamethasone showed frequent malformations such as yolk sac edema and pericardial edema, but kinked tail or abnormal body-axis curvature were also observed.
A: Control embryo at 48 hpf. B: Yolk sac edema at 48 hpf. Embryo treated with 6.8 μg/mL dex. C: Control embryo at 72 hpf. D: Pericardial edema at 72 hpf. Embryo treated with 6.8 μg/mL dex. E: Whole control embryo at 72 hpf. F-H: Embryos at 72 hpf suffering from kinked tail and abnormal body-axis curvature in addition to pericardial edema, respectively. Embryos treated with 6.8 μg/mL dex.
yse: yolk sac edema, pe: pericardial edema, kt: kinked tail, bac: abnormal body-axis curvature

Embryos were not measured routinely, but length differences between treatment and control groups were evaluated in retrospect from the images taken. 10 randomly selected embryos in each group were measured from highest point on the head to the tip of the tail, using the measurement application of NIS elements D4 2.00. Measurements done at 72 hpf does not suggest any difference in length between the treatment groups and controls (Table 3-2).

Table 3-2: Average body length of dexamethasone treated embryos and controls at 72 hpf

µg/mL dex	Number of embryos measured	Mean length (µm) ± STD	p-value
0	10	3236.3 ± 132.2	
0.68	10	3274.8 ± 203.1	n.s
6.8	10	3251.3 ± 170.3	n.s

STD: Estimated standard deviation
Probability values given are for a two-tailed t-test
n.s: Difference between treated group and controls is not significant ($\alpha = 0.05$)

3.1.1.2 Apoptosis assay

In order to investigate if dexamethasone treatment would affect apoptosis in zebrafish embryos, staining with acridine orange was used. The dye emits green fluorescence when bound to dsDNA and binds particularly strongly to fragmented DNA, which is a result of the apoptosis machinery at work (39). Thus, selective labeling of cells undergoing apoptosis is achieved. 10 embryos from each treatment group were assessed at the three time points.

Overall, very few apoptotic cells were identified at 24 hpf. The only striking difference observed between the control group and the treatment groups was a significant increase in the number of embryos showing apoptosis in the epiphysis (Figure 3-4, yellow arrows). All embryos incubated in the highest concentration of dex displayed apoptotic activity in the epiphysis, whereas only three of the observed control embryos did (Appendix Table A2-1).

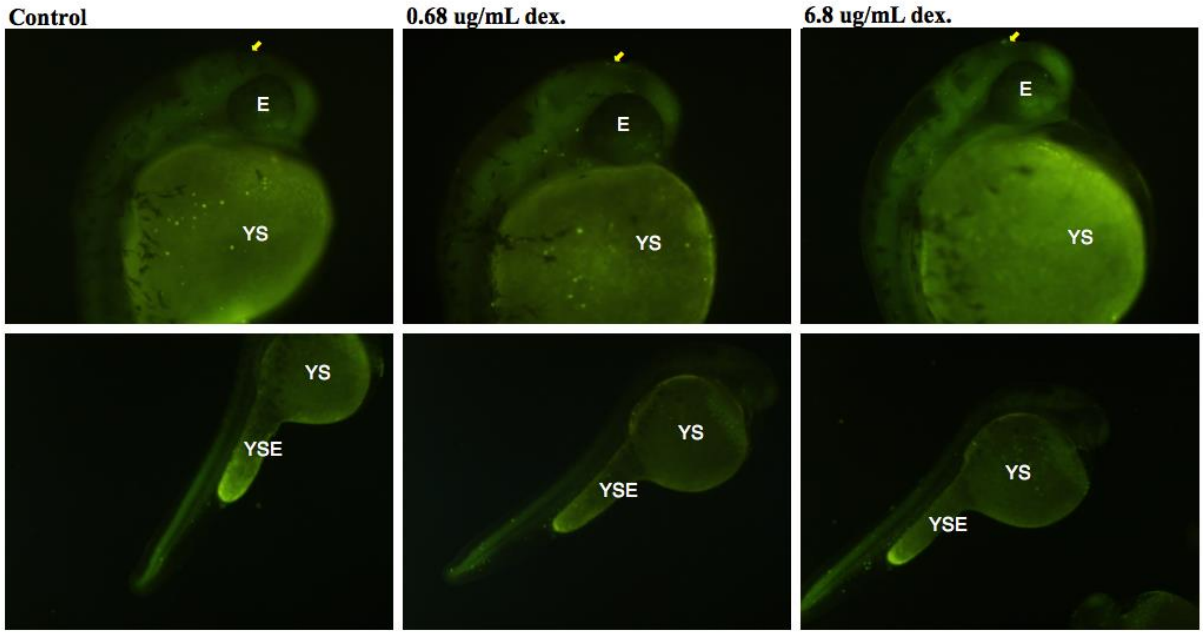


Figure 3-4: There was an increased incidence of apoptosis in the epiphysis of dexamethasone treated embryos compared to controls at 24 hpf. The overall apoptotic activity was low at this time point. A significant larger number of treated embryos than controls showed signs of apoptotic activity in the epiphysis (yellow arrows). Otherwise, no differences were seen between treated embryos and controls (bottom panel). E: Eye, YS: Yolk sac, YSE: Yolk sac extension

Generally, there was a peak in apoptotic activity at 48 hpf compared to 24 and 72 hpf, especially along the yolk sac. Apoptotic activity was seen around the eyes of embryos in both the control group and treatment group, but the incidence of embryos showing apoptosis in this area was significantly higher for drug treated embryos than for controls (Appendix Table A2-2). Furthermore, in most dex treated embryos, the level of apoptotic activity around the eyes appeared greater than it did in controls showing apoptosis in the same place (Figure 3-5, orange arrows). The level of apoptosis seemed to increase with increasing concentrations of dex. All observed embryos showed apoptosis in the olfactory placodes (Figure 3-5, pink arrows), but the level of apoptotic activity was clearly reduced in many of the dex treated embryos compared to controls (Appendix Table A2-2). The urogenital tract was also an area where dex treated embryos showed altered apoptotic activity compared to controls (Figure 3-5, blue arrows). While 10 of 10 control embryos displayed apoptosis in the urogenital tract, none of the embryos treated with dex did (Appendix Table A2-2).

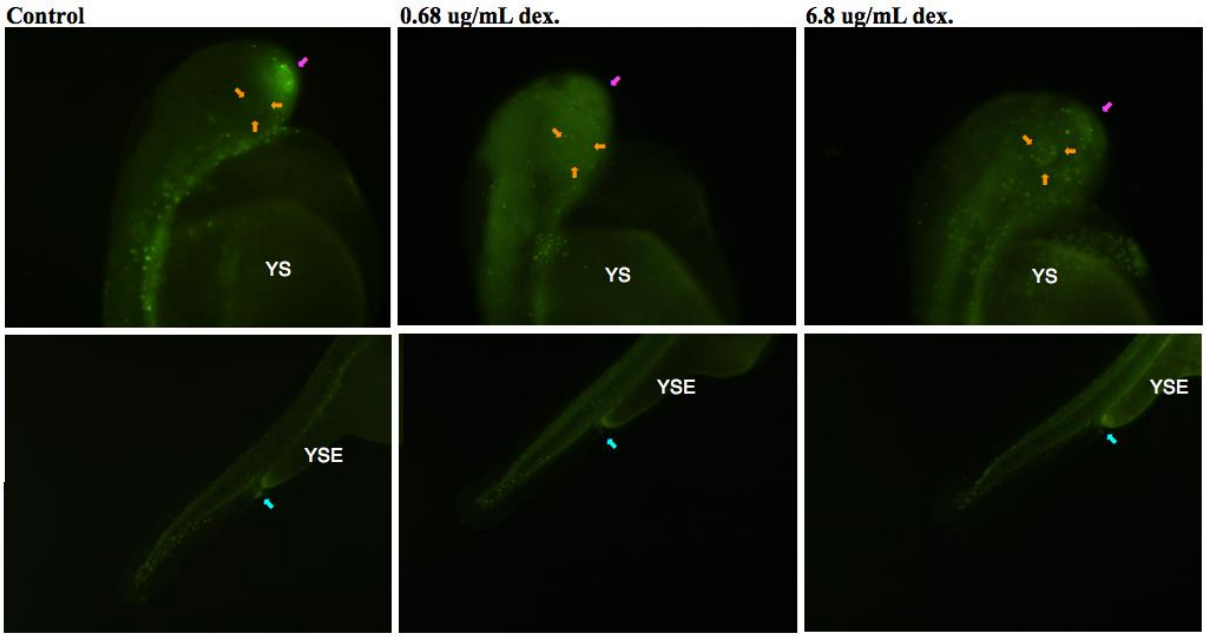


Figure 3-5: Treatment with dexamethasone caused altered apoptotic activity in the brain and in the urogenital tract of embryos at 48 hpf. Acridine orange staining revealed that a significantly larger number of embryos treated with dex showed apoptotic activity around the eyes (orange arrows) compared to the control group. Also, the level of apoptosis in this area seemed to be dose-dependent. Dex treated embryos showed decreased apoptotic activity in the olfactory placodes compared to controls (pink arrows). No treated embryos showed any sign of apoptosis in the urogenital tract (blue arrows), whereas all controls did.
YS: Yolk sac, YSE: Yolk sac extension

At 72 hpf, very little apoptosis was observed around the eyes of embryos, and differences in apoptosis in this area could no longer be seen between controls and dexamethasone treated embryos. Overall, apoptotic activity in the olfactory placodes was more prominent at this time point than at 48 hpf for all embryos (Figure 3-6, pink arrows). Most of the dexamethasone treated embryos showed decreased levels of apoptosis in this area compared to controls (Appendix Table A2-3), and appeared to do so in a dose-dependent fashion; further decreasing with increased concentration of dex. Apoptotic activity in the urogenital tract was seen in significantly fewer embryos treated with dexamethasone than controls (Figure 3-6, blue arrows). While 10 of 10 control embryos showed apoptosis in this area, not one of the 10 embryos observed that were treated with 6.8 μg/mL dexamethasone showed any signs of apoptosis in the urogenital tract (Appendix Table A2-3).

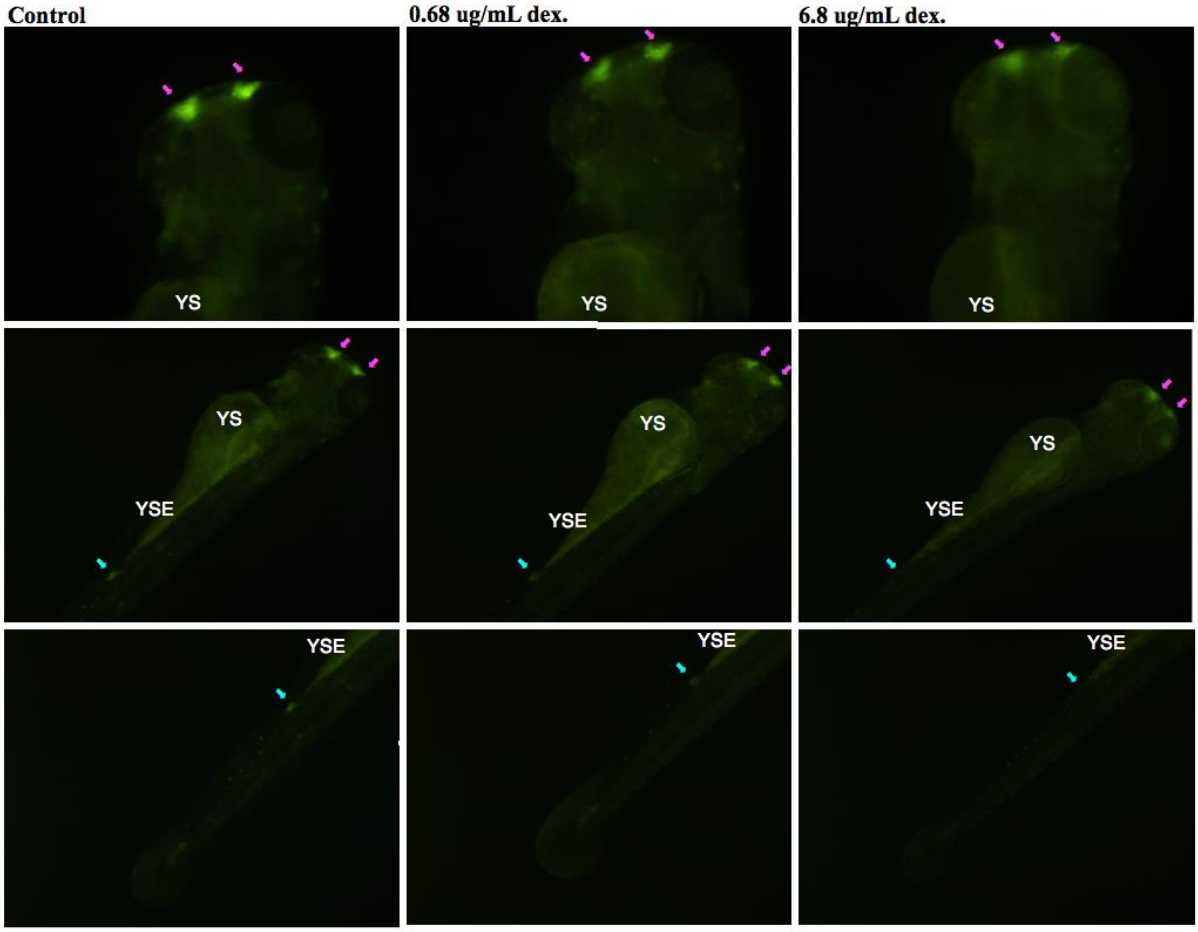


Figure 3-6: Treatment with dexamethasone caused decreased apoptosis in the olfactory placodes and in the urogenital tract at 72 hpf. Drug treated embryos stained with acridine orange showed decreased level of apoptosis in the olfactory placodes (pink arrows), in a dose-dependent fashion. Few treated embryos showed any sign of apoptosis in the urogenital tract (blue arrows), whereas all controls did. No differences in apoptotic activity could be seen around the eyes of controls and dexamethasone treated embryos.
YS: Yolk sac, YSE: Yolk sac extension

3.1.1.3 Western blotting

To compare protein expression between treated embryos and controls, western blotting was used with antibodies directed against the proteins of interest. Tissue samples consisting of 10 whole embryos were crushed and prepared for analysis. Extracts were loaded on a gel where proteins were separated by size trough gel electrophoresis, before being blotted to a membrane. Primary and secondary antibodies were then used to mark the protein of interest with fluorescence. The intensity of the staining depends on the amount of protein present, and can therefore be used as a measure of relative protein expression. To adjust for loading of samples, staining with anti-actin antibodies was done. Actin is a housekeeping-protein present in all eukaryotic cells, not believed to be influenced by the incubated drug (101). The relative

amount of actin present can therefore be used as an indication of the amount of sample loaded onto the gel.

We wanted to use western blotting as a method to look for potential differences in proliferation and apoptosis in the dexamethasone treated embryos harvested at 24, 48 and 72 hpf. In addition, we wanted to look for possible changes in the expression of the transcription factor Pax6. Phosphorylated Histone H3 (PH3) is associated with chromosome condensation prior to cell division, and the expression of PH3 can therefore be seen as a measure of proliferating cells (44). Apoptotic activity can be measured as expression of caspase-3, one of the effector caspases involved in apoptotic activity (38). Here, an antibody that recognizes both the inactive pro-caspase-3 and the active caspase-3 was used. Pax6 is a transcription factor important for embryo development, especially for eye and brain maturation. It is well conserved between species, and works high up in the regulatory ladder in humans, mice and zebrafish (76). The Pharmacology research group, Department of Pharmacy, University of Tromsø has worked extensively with this transcription factor, and since there are indications that Pax6 and glucocorticoids have effects on the same target genes, it was of interest to look for potential changes in Pax6 expression following dex treatment of zebrafish embryos (54, 102).

Western blotting was done on embryo extracts collected from two different experiments, and proliferation (expression of PH3), apoptosis (expression of caspase-3) and expression of Pax6 was evaluated. The two experiments yielded quite different results (Figure 3-7 and Figure 3-8). Dexamethasone in the concentrations used had little influence on the level of expression of any of the proteins in treated embryos compared to controls. Overall, the expression of PH3, pro-caspase-3 and active caspase-3 seemed to decrease over time (Figure 3-7, A-D and Figure 3-8, A-F), indicating a decline in both proliferation and apoptotic activity from 24 to 72 hpf. In the first experiment, this was true also for expression of Pax 6 (Figure 3-7, E and Figure 3-8, G), but in the second experiment the expression pattern looked quite different (Figure 3-7, F and Figure 3-8, H). However, it still seems that Pax6 protein expression is inhibited by the highest dose of dex at 72 hpf in both experiments.

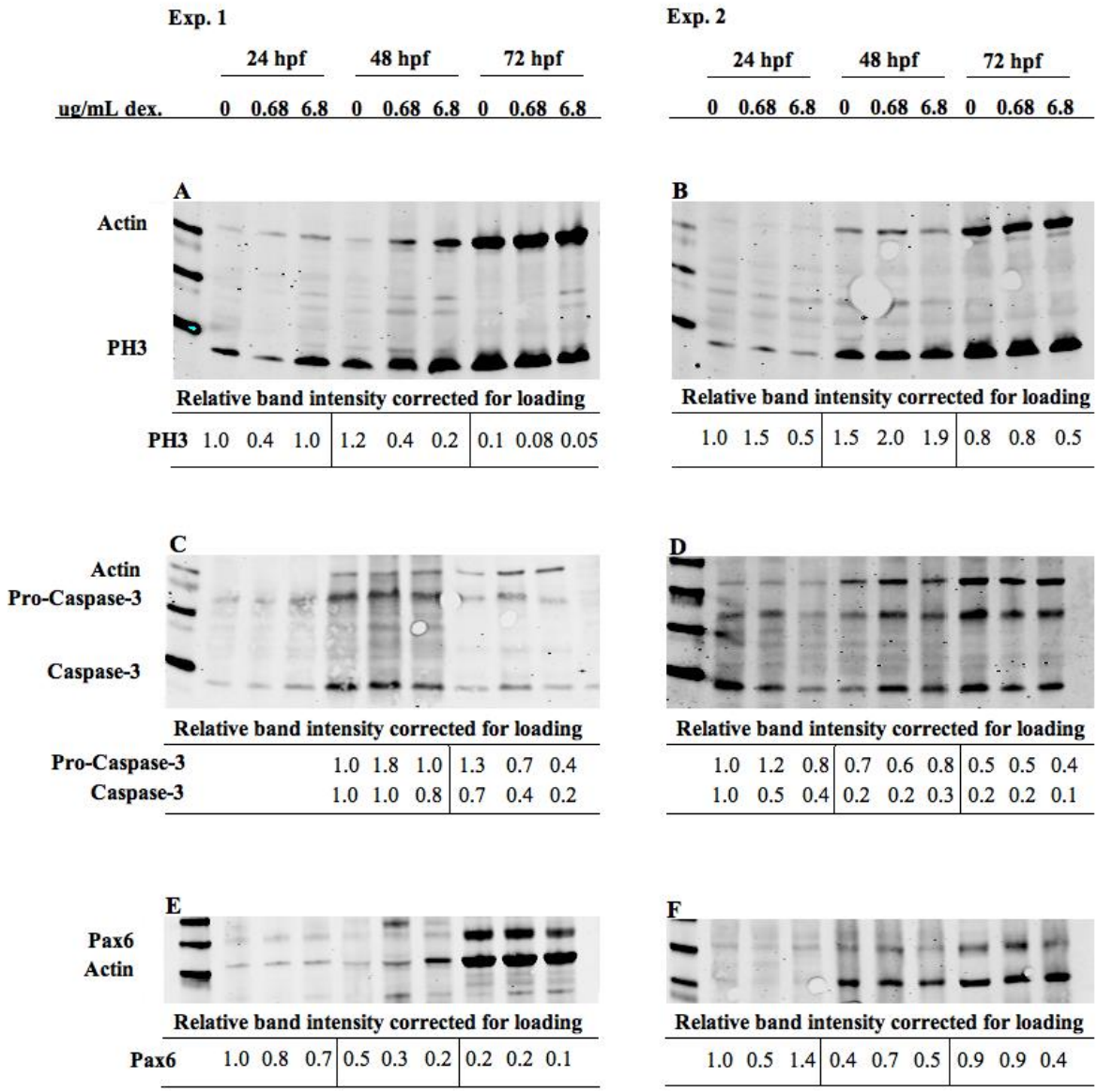


Figure 3-7: Western blots assessing expression of PH3, pro-caspase-3, caspase-3 and Pax6 in zebrafish embryos harvested at 24, 48 and 72 hpf, following treatment with dexamethasone. Possible differences in amount of sample put on the gel were adjusted for by using actin as a loading control. Band intensities were measured with Li-Cor Odyssey Sa 4.0 software (Section 2.2.6.5). Protein levels corrected for loading are here expressed relative to the control value at 24 hpf. For pro-caspase-3 and caspase-3 in experiment 1, actin did not give rise to a band for 24 hpf samples (C). Protein expression is therefore expressed relative to the control value at 48 hpf. Expected molecular weight of target proteins: PH3: 17 kDa, Pro-caspase-3: 32 kDa, Caspase-3: 17-20 kDa, Pax6: 48 kDa, Actin: 42 kDa.

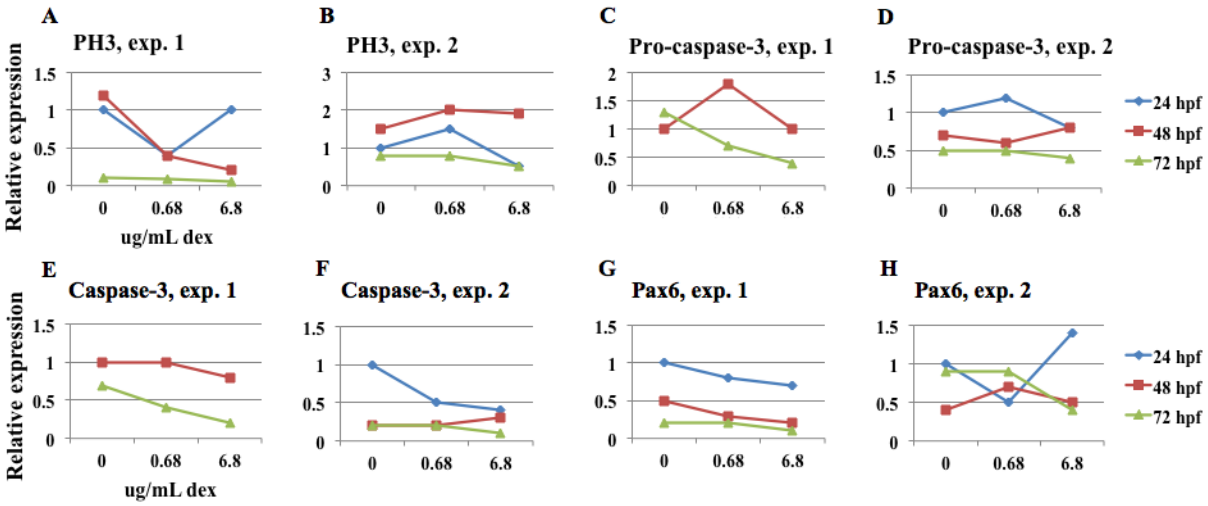


Figure 3-8: Illustration of relative expression of PH3, pro-caspase-3, caspase-3 and Pax6 in zebrafish embryos harvested at 24, 48 and 72 hpf, following treatment with dexamethasone. Visual presentation of relative band intensities calculated for western blots presented in **Figure 3-7**. Band intensities were measured with Li-Cor Odyssey Sa 4.0 software (Section 2.2.6.5). Protein expression has been corrected for loading, and is here expressed relative to the control value at 24 hpf. For pro-caspase-3 and caspase-3 in experiment 1 (C and E), actin did not give rise to a band for 24 hpf samples. Protein expression is therefore expressed relative to the control value at 48 hpf.

3.1.2 $\beta^{2,2}$-amino acid derivative 161

3.1.2.1 Mortality and visible morphological changes

The novel antimicrobial $\beta^{2,2}$-amino acid derivative 161 was tested in zebrafish embryos with the goal to assess toxicity and to estimate the concentration expected to kill 50 % of the test subjects (LC_{50}). Two different experimental designs were used. Embryos were either treated with derivative 161 from 3.3 to 24 hpf (method A) or from 24 to 48 hpf (method B), and were then assessed with regard to survival and morphological changes compared to controls. In both method A and B, embryos were also observed at intervals for 3 hours after drug treatment was initiated to check for acute toxicity of the drug.

3.1.2.1.1 Embryos obtained from zebrafish facility

Method A

No embryos died during the first 30 minutes of treatment. Control embryos never died during the first 3 hours following initiation of treatment. For embryos incubated in concentrations below 0.12 mg/mL, there were seldom observed more than a couple of deaths during the first 3 hours after addition of derivative 161. With regard to acute toxicity, concentrations of 0.2 mg/mL or above were critical. Embryos incubated in 0.2 mg/mL or higher concentrations of

the drug started to die approximately 1 hour following initiation of treatment (Figure 3-9, B). Then, about half of the embryos would perish during 10 minutes. After 3 hours of treatment, close to all embryos incubated in these high concentrations had died (Figure 3-9, C).

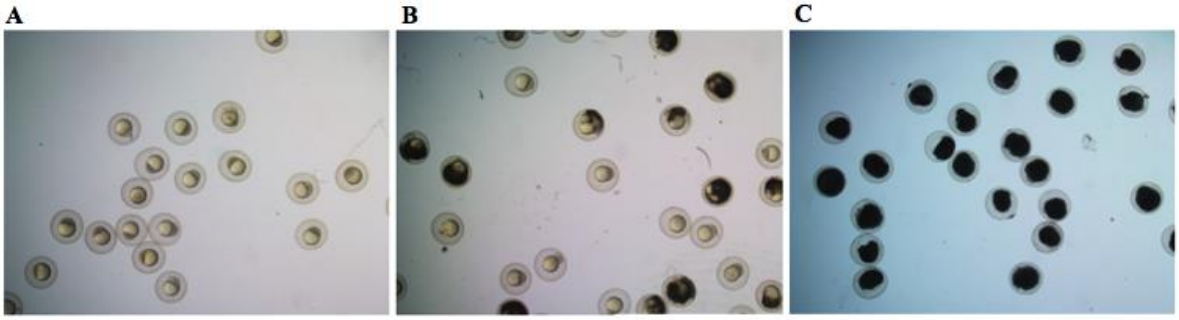


Figure 3-9: Acute toxicity was observed approximately 1 hour after addition of derivative 161 in concentrations of 0.2 mg/mL or above. Approximately 50 % of embryos incubated in concentrations of derivative 161 equal to or higher than 0.2 mg/mL would die during a period of 10 minutes, approximately 1 hour following initiation of treatment (B). After 3 hours, close to all embryos would have died (C). No embryos in the control group ever died during the first 3 hours of treatment. A: Control after 1 hour of treatment. B-C: Embryos incubated in 0.25 mg/mL derivative 161 after 1 and 3 hours of treatment, respectively.

Table 3-3 and Figure 3-10 summarizes method A experiments and mean mortality rates at 24 hpf for the different concentrations of derivative 161 tested on embryos obtained from the zebrafish lab. Mortality was corrected for death in the control group using Abbott's formula. Mortality rates varied quite a lot from experiment to experiment, especially for the middle concentrations of 161. Full details of the individual experiments can be seen in Appendix Table A1-4. Apart from a few irregular values, mortality as a function of drug concentration seems to be following the S-shaped curve typical for dose-response relationships (Figure 3-10).

Table 3-3: Overview of number of embryos treated and mean mortality rates for embryos incubated from 3.3 to 24 hpf (method A) with increasing concentrations of derivative 161

mg/mL 161	Number of experiments*	Total n	Mean Mortality (%)	Corrected mean mortality (%) ± STD
0	5	450	7.20	
0.01	2	100	7.00	-1.11 ± 1.57
0.02	2	100	9.00	0.97 ± 7.66
0.03	2	100	14.00	6.38 ± 9.03
0.04	2	100	15.00	7.54 ± 4.38
0.05	5	450	11.54	4.64 ± 5.02
0.06	3	200	13.33	7.16 ± 8.83
0.075	5	450	26.60	20.84 ± 7.35
0.1	3	350	36.23	31.80 ± 22.53
0.12	3	350	74.90	73.14 ± 2.00
0.14	1	100	42.00	39.58
0.16	3	350	78.67	77.34 ± 11.07
0.20	3	350	85.43	84.49 ± 9.90
0.22	2	250	100.00	100.00 ± 0.00
0.25	2	250	99.65	99.61 ± 0.55

STD: Estimated standard deviation
Total n reflects the total of included embryos throughout the experiments.
Mortality rates have been corrected for death in the control group using Abbotts formula
*Experiments with mortality rate >20 % among controls were excluded from all calculations. See Appendix Table A1-4 for full details on each experiment.

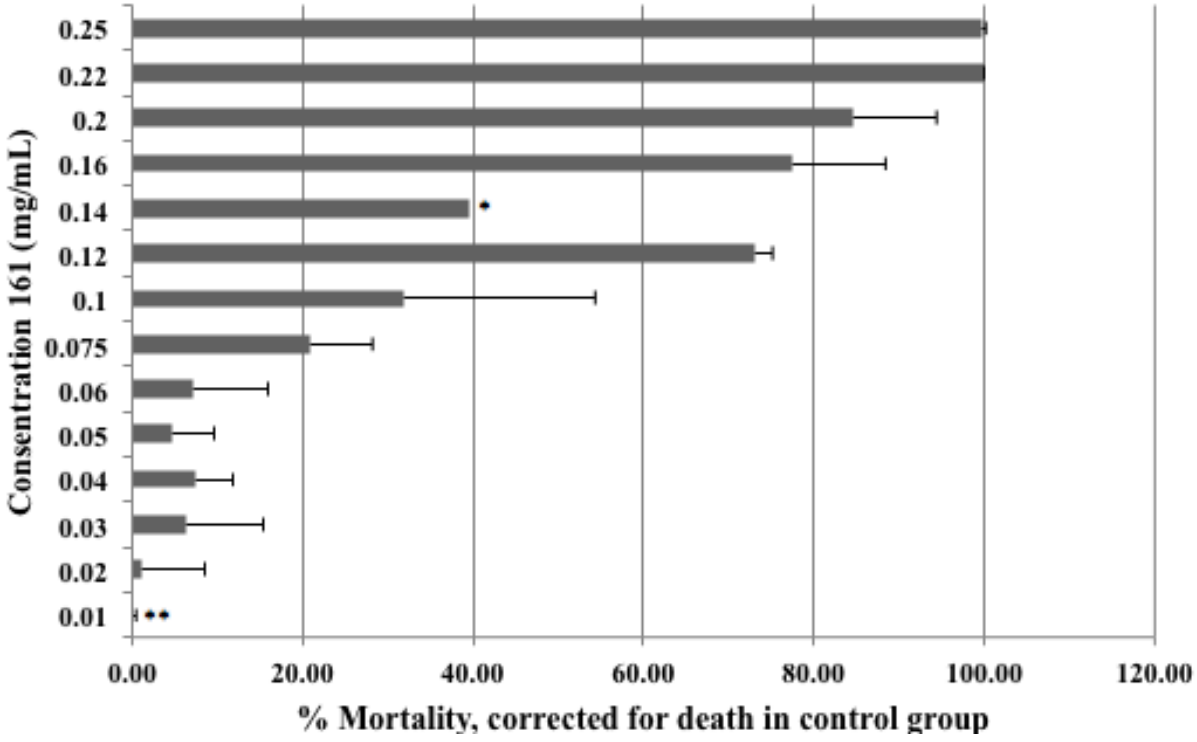


Figure 3-10: Graphic presentation of mean mortality rates and estimated standard deviations after treatment with derivative 161 from 3.3 to 24 hpf, as presented in Table 3-3. Except from a few deviant values, mortality as a function of concentration seems to be following the typical sigmoid curve.
* Based on one experiment only , ** Negative value due to lower mortality than in control group

Based on mortality data corrected for control mortality from each individual experiment, a probit analysis was carried out (Figure 3-11). Probits of mortality were plotted against log concentration of derivative 161 (μg/mL), and linear regression was used to generate a function expression. A probit of 5 corresponds to 50 % mortality, and so the log LC_{50} was derived from the linear function (Figure 3-11, blue dashed line). The LC_{50} was in this manner estimated to 0.109 mg/mL, with 95 % confidence limits of 0.105 – 0.112 mg/mL. This corresponds quite well with mean mortality data presented in **Figure 3-10**; an LC_{50} between 0.1 and 0.12 mg/mL might be expected. Derivative 161 was weighed in as its trifluoroacetic salt when preparing drug solutions, and so the LC_{50} expressed as the molar concentration more correctly shows the toxic properties of the compound. The LC_{50} calculated for the salt when administered to zebrafish embryos from 3.3 to 24 hpf corresponds to 161 μM of $\beta^{2,2}$-amino acid derivative 161, with 95 % confidence limits from 155 to 181 μM.

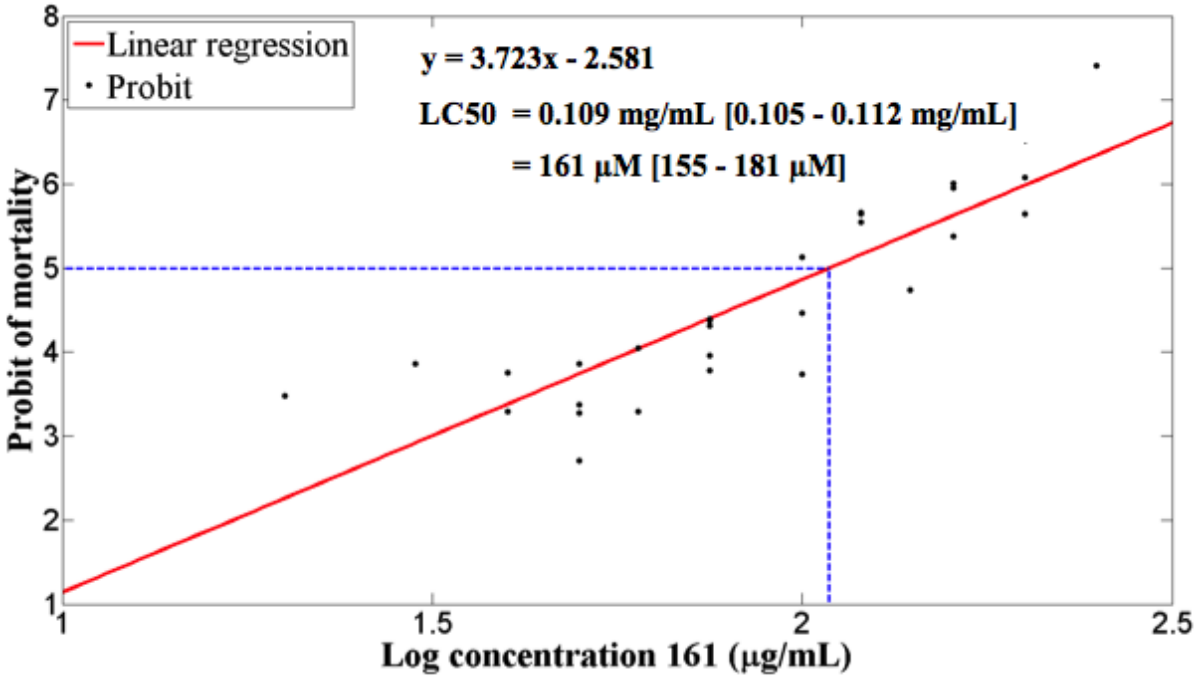


Figure 3-11: Probit analysis on mortality data from experiments where embryos were treated with derivative 161 from 3.3 to 24 hpf. A probit of 5 corresponds to 50% mortality, and solving for y=5, the log LC_{50} was found (blue dashed line). This was then transformed to yield an LC_{50} of 0.109 mg/mL, or 161 µM. 95 % confidence limits are shown in parenthesis**.**

No visible morphological changes were identified in treated embryos compared to controls, (Figure 3-12). There were sporadic incidences of abnormal development, but this was not observed more often for treated embryos than for controls (Appendix Table A1-4). Electron microscopy of method B embryos (Section 3.1.2.2) revealed severe changes to the tail tissue of embryos treated with derivative 161. This region was therefore especially carefully examined, but no differences could be seen between drug treated embryos and controls through the dissecting microscope. Moreover, the length from the end of the yolk sac extension to the tip of the tail was measured on 10 randomly selected control embryos and embryos treated with 0.1 mg/mL 161, however no differences in tail length were found (Table 3-4).

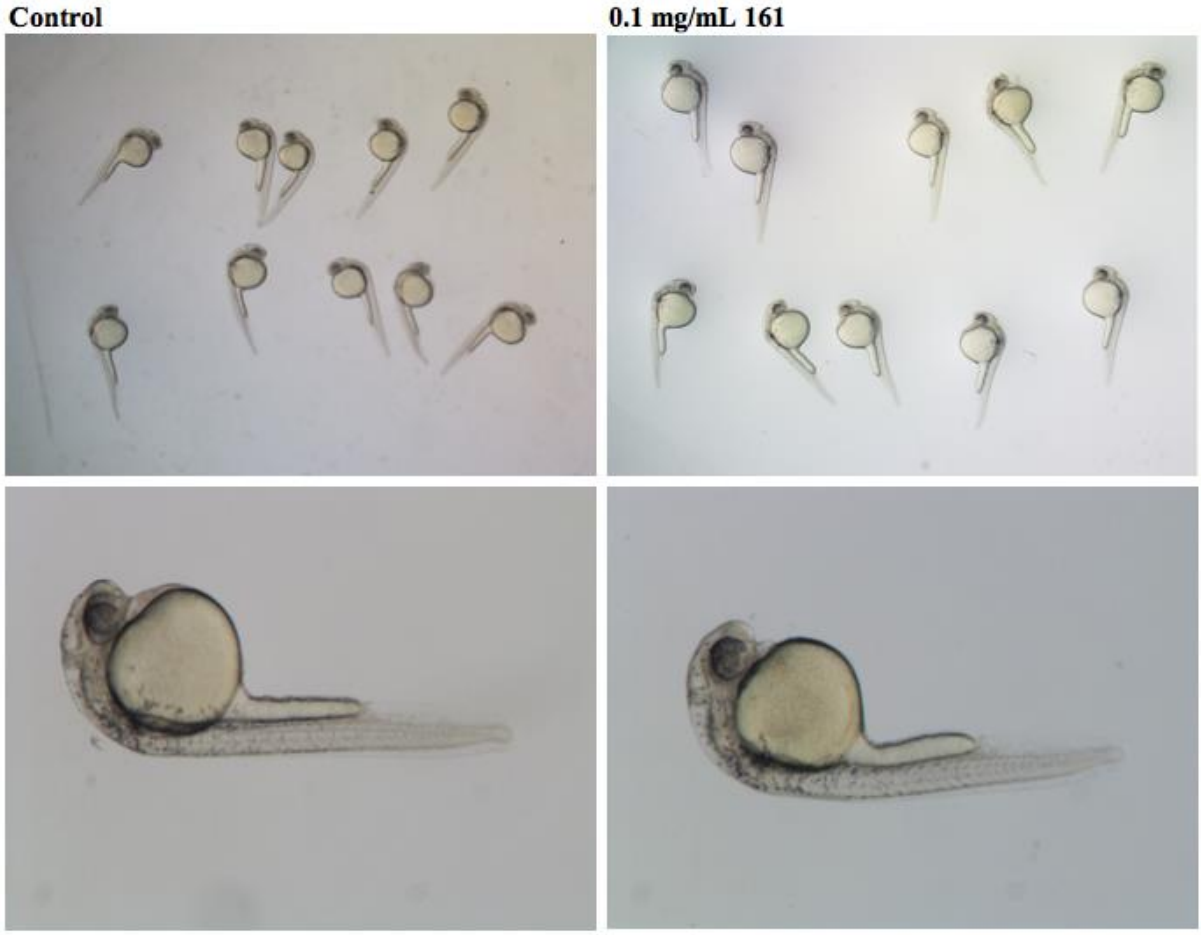


Figure 3-12: No visible morphological changes could be seen in embryos treated with derivative 161 from 3.3 hpf compared to controls at 24 hpf. Controls are shown in photos to the left, treated embryos are to the right.

Table 3-4: Average tail length at 24 hpf of controls and embryos treated with derivative 161 from 3.3 hpf, measured from the end of the yolk sac extension to the tip of the tail

mg/mL 161	Number of embryos measured	Mean length (μm) ± STD	p-value
0	10	73.93 ± 4.43	
0.1	10	73.32 ± 5.02	n.s

STD: Estimated standard deviation
Probability values given are for a two-tailed t-test
n.s: Difference between treated group and controls is not significant ($\alpha = 0.05$)

Method B

In method B, drug treatment of embryos was initiated at 24 hpf and stopped at 48 hpf. With regard to acute toxicity, concentrations equal to or above 0.3 mg/mL were critical. No embryos died during the first two hours of treatment. At concentrations from 0.3 to 0.5 mg/mL, 25-50 % of embryos died during the third hour following treatment start. The

embryos´ tail tissue seemed to be most vulnerable to the drug, with tissue death occurring in this area prior to embryo death (Figure 3-13). Embryos would usually die within an hour of development of necrotic tails. All embryos incubated in concentrations equal to or below 0.25 mg/mL were alive 3 hours following initiation of treatment. However, after 3 hours of treatment with derivative 161 in concentrations ranging from 0.175 to 0.25 mg/mL, there were typically a few (2-5) embryos showing tail tissue death. At concentrations below 0.175 mg/mL, no changes could be seen between treated embryos and controls during the first 3 hours of treatment.

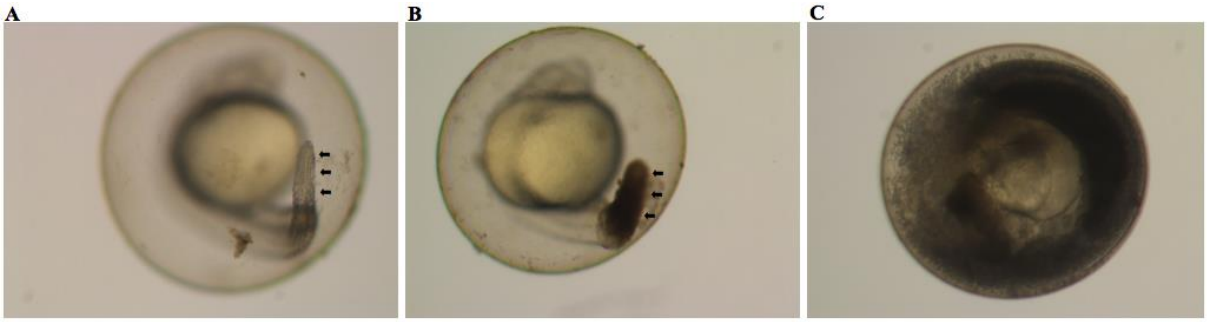


Figure 3-13: When treatment with high concentrations of derivative 161 was started at 24 hpf, the tail tissue seemed most vulnerable to the drug, and embryo tails typically became necrotic after 2-3 hours of treatment. Development of necrotic tail tissue (B) was usually followed by embryo death within an hour (C). Arrows indicate the tail of embryos.
A: Control embryo 3 hours after initiation on drug treatment. B: Embryo treated with 0.2 mg/mL of derivative 161 showing necrotic tail tissue, 3 hours after initiation of treatment. C: Embryo B 4 hours after initiation of treatment. The embryo is now dead.

Table 3-5 and Figure 3-14 summarizes method B experiments done on embryos obtained from the zebrafish facility. Mean mortality rates for death occurring from start of experiment at 24 hpf to the end at 48 hpf are shown. As no control embryos died during the course of the experiments, it was not necessary to correct mortality rates using Abbott's formula. Full details on the individual experiments can be seen in Appendix Table A1-5. As was the case for method A experiments, mortality rates as a function of drug concentration follows a sigmoid curve (Figure 3-14). Embryos treated from 24 to 48 hpf were less sensitive to derivative 161 than embryos treated from 3.3 to 24 hpf, requiring higher drug concentrations to see effects. A drug concentration of 0.1 mg/mL, close to method A LC_{50} (0.109 mg/mL), produced no lethal effects in embryos when treatment was started at 24 hpf. This demonstrates that timing of drug exposure is critical for drug toxicity.

Table 3-5: Overview of number of embryos treated and mean mortality rates for embryos incubated from 24 to 48 hpf (method B) with increasing concentrations of derivative 161

mg/mL 161	Number of experiments	Total n	Mean mortality (%) ± STD
0	6	550	0.00 ± 0.00
0.1	2	100	0.00 ± 0.00
0.11	2	100	4.00 ± 0.00
0.12	2	100	1.00 ± 1.41
0.125	2	100	5.00 ± 7.07
0.13	2	100	6.00 ± 0.00
0.135	4	250	9.75 ± 7.14
0.14	2	100	13.00 ± 1.41
0.15	6	550	19.72 ± 11.09
0.175	4	450	27.83 ± 24.08
0.19	1	50	62.00
0.20	4	450	49.10 ± 8.27
0.22	2	250	55.50 ± 0.71
0.25	2	250	89.50 ± 0.71
0.30	1	100	100.00
0.40	1	100	100.00
0.50	1	100	100.00

STD: Estimated standard deviation
Total n reflects the total of included embryos throughout the experiments
See Appendix Table A1-5 for full details on each experiment

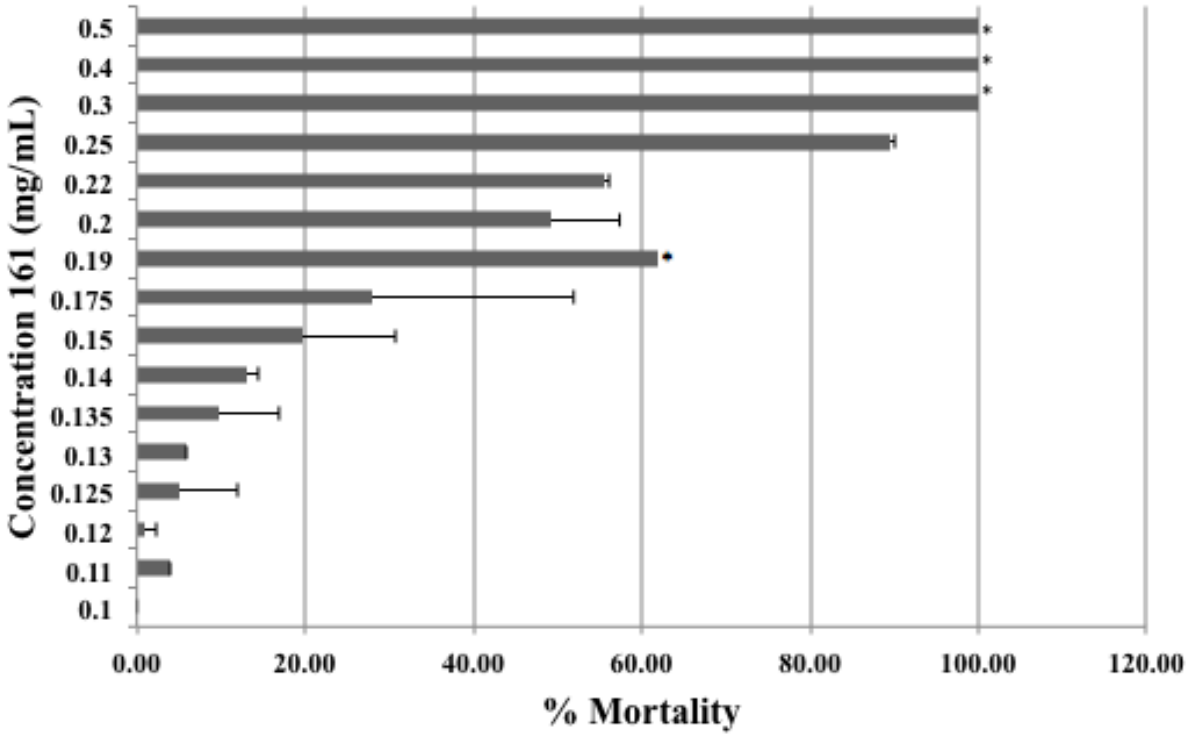


Figure 3-14: Graphic presentation of mean mortality rates and estimated standard deviations after treatment with derivative 161 from 24 to 48 hpf, as presented in Table 3-5.
* Based on only one experiment

An LC_{50} of 0.197 mg/mL with 95 % confidence limits from 0.194 to 0.201 mg/mL was estimated through probit analysis (Figure 3-15). Looking at the mortality data presented in Table 3-5 and Figure 3-14, we see that the mortality rate for embryos incubated in 0.19 mg/mL 161 extends past 60 %, however this was based on only one experiment. Considering the other entries, the estimated LC_{50} looks reasonable. Expressed as the molar concentration of derivative 161, the estimated LC_{50} is equivalent to 292 µM, with 95 % confidence limits from 287 to 298 µM.

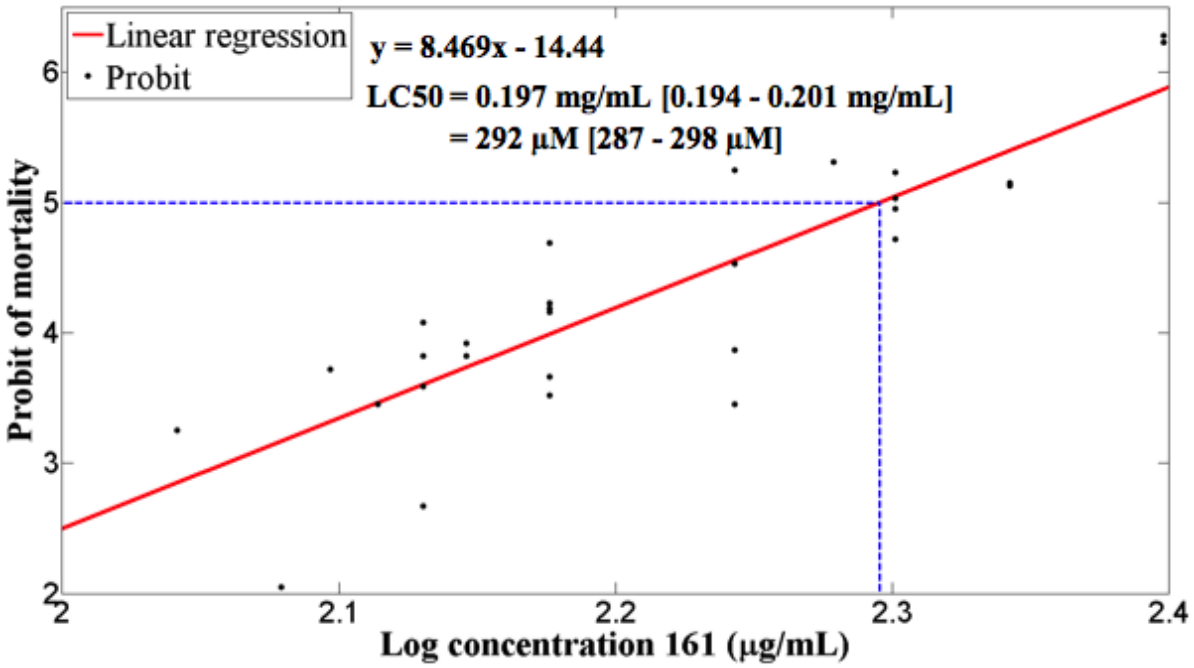


Figure 3-15: Probit analysis on mortality data from experiments where embryos were treated with derivative 161 from 24 to 48 hpf. A probit of 5 corresponds to 50% mortality, and solving for y=5, the log LC_{50} was found (blue dashed line). This was then transformed to yield an LC_{50} of 0.197 mg/mL, or 292 µM. 95 % confidence limits are shown in parentheses.

No visible morphological changes could be identified in embryos treated with derivative 161 from 24 to 48 hpf (Figure 3-16). Only embryos that showed normal development at 24 hpf were eligible for inclusion in method B experiments, and no occurrence of malformed embryos were ever recorded (Appendix Table A1-5). Length measurements from the end of the yolk sac extension to the tip of the tail indicated truncation of the tail of embryos treated with high drug concentrations compared to controls (Table 3-6). Embryos incubated in 0.2 mg/mL 161 generally showed shorter tails than controls. This however was not significant, possibly due to the small number of measured embryos. Embryos incubated in 0.25 mg/mL 161 displayed significantly shorter tails than controls.

Table 3-6: Average tail length at 48 hpf of controls and embryos treated with derivative 161 from 24 hpf, measured from the end of the yolk sac extension to the tip of the tail

mg/mL 161	Number of embryos measured	Mean length (μm) ± STD	p-value
0	10	118.60 ± 7.85	
0.15	10	118.56 ± 3.28	n.s
0.2	10	115.06 ± 6.55	n.s
0.25	10	112.13 ± 5.64	< 0.05

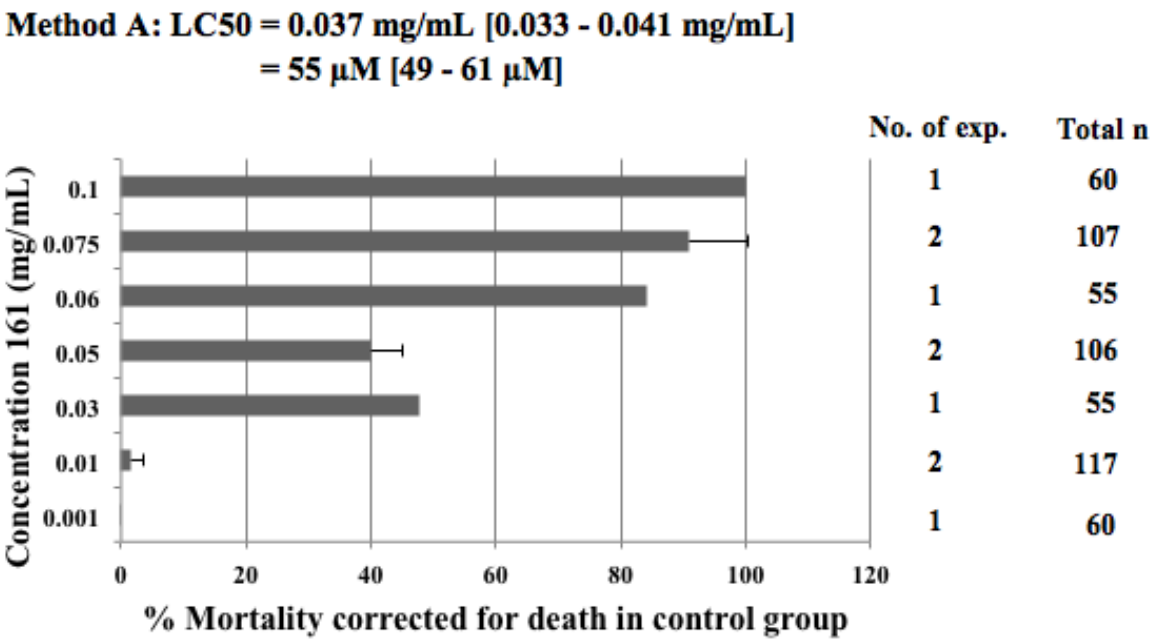
STD: Estimated standard deviation
Probability values given are for a two-tailed t-test
n.s: Difference between treated group and controls is not significant ($\alpha = 0.05$)

Figure 3-16: No visible morphological differences could be seen between embryos treated with derivative 161 from 24 hpf to 48 hpf compared to untreated controls. Controls are shown in photos to the left, treated embryos are to the right.

3.1.2.1.2 Embryos obtained from fish held at ordinary lab

Due to construction work at the zebrafish facilities, experiments were also conducted on embryos obtained from zebrafish kept in stand-alone tanks at the regular lab, under less controlled conditions than fish held at the zebrafish lab. Interestingly, quite different mortality data was obtained from these experiments, indicating that fish care is of importance when conducting embryo toxicity tests and assessing outcomes. Embryos treated with derivative 161 both from 3.3 hpt to 24 hpf, and from 24 hpf to 48 hpf showed higher sensitivity to the drug under these conditions. Results were therefore analyzed separately from results obtained from testing on embryos from the zebrafish lab.

Mortality rates for embryos incubated in various concentrations of derivative 161 are shown in Figure 3-17. Control embryos included in method A experiments showed a higher mean mortality rate than embryos from the zebrafish facility. Based on 3 experiments, the mean control mortality rate was 17.85 % with an estimated standard deviation of 3.72. Moreover, derivative 161 seemed to be more toxic to these embryos. Probit analysis yielded an LC_{50} of 0.037 mg/mL, with a 95 % confidence interval of 0.033 – 0.041 mg/mL for embryos incubated in the drug from 3.3 to 24 hpf. Expressed as the molar concentration of derivative 161, the estimated LC_{50} is equivalent to 55 μM, with 95 % confidence limits from 49 to 61 μM. No control embryos died from 24 to 48 hpf. However, embryos did appear more sensitive to the drug also when treatment started at 24 hpf. Method B LC_{50} was estimated to 0.126 mg/mL, with 95 % confidence limits of 0.122 – 0.131 mg/mL. This is equivalent to 187 μM of derivative 161, with a 95 % confidence interval from 181 to 194 μM.



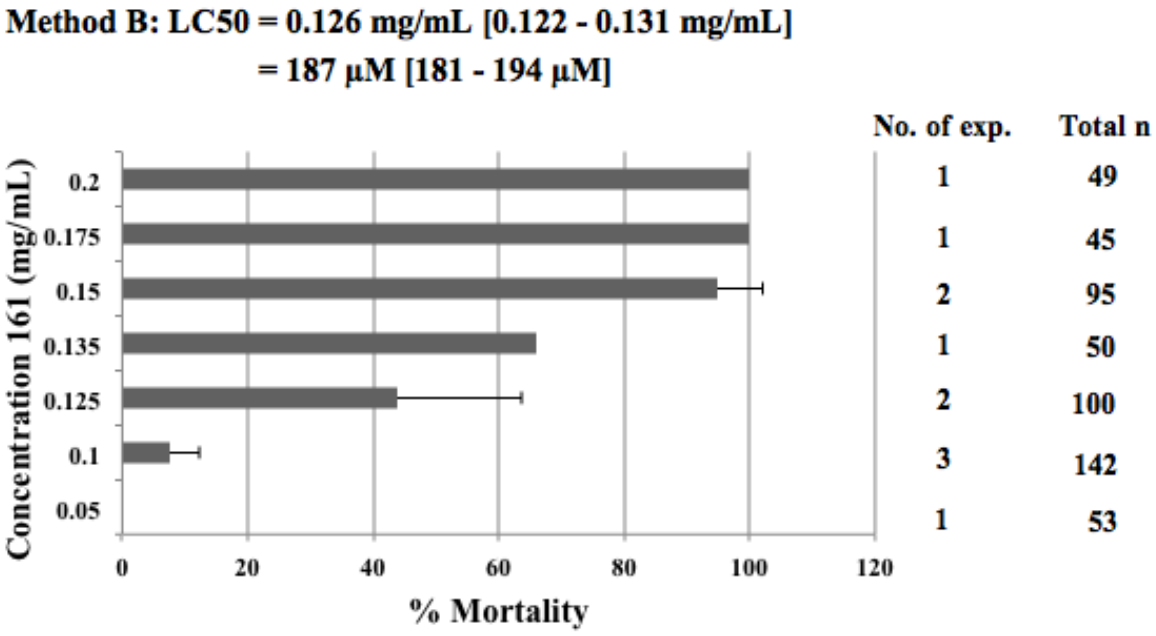


Figure 3-17: Mean mortality rates and estimated standard deviations for method A and B experiments conducted on embryos obtained from fish held in stand-alone tanks at the ordinary lab. These embryos showed higher sensitivity to 161 than embryos obtained from fish kept at the zebrafish facility. LC_{50} for method A was estimated to be 0.037 mg/mL, or 55 µM, while LC_{50} for method B was estimated to 0.126 mg/mL, or 187 µM. 95 % confidence limits are shown in parenthesis.
No. of exp.: Number of experiments conducted using the given drug concentration.
Total n reflects the total number of embryos included throughout the experiments with the given drug concentration.

No other analysis such as western blotting or apoptosis assay was done on embryos obtained from fish held in stand-alone tanks at the ordinary lab. From here on out, only results from experiments conducted on embryos obtained from the zebrafish facilities will be discussed, unless otherwise specified.

3.1.2.2 Electron microscopy

To assess antimicrobial and anticancer effects of $\beta^{2,2}$-amino acid derivatives similar to derivative 161, electron microscopy of drug treated bacterial and cancer cells has previously been conducted with success (91). We wanted to apply this method to zebrafish embryos as well, to further assess toxic effects of derivative 161. Field emission scanning electron microscopy (FE-SEM) was therefore used to take a closer look at embryo tissue morphology following treatment with derivative 161. Drug concentrations were selected based on the mortality data from method B experiments, and embryos were incubated in E3 or the elected concentrations from 24 to 25 hpf so that early effects could be detected. A drug concentration of 0.2 mg/mL, close to the estimated LC_{50} value for embryos incubated in derivative 161 from 24 to 48 hpf, was chosen to look at acute effects of the drug prior to embryo death. In order to detect potential effects on embryos incubated in low concentrations of the drug that could not be detected through the dissecting microscope, 0.1 mg/mL, a concentration were no lethal effects or altered morphology compared to controls had been identified, was also elected. Embryos were incubated in the elected concentrations of derivative 161 for one hour before being fixated and prepared for FE-SEM. Control embryos were also prepared for analysis. Two embryos form each treatment group were evaluated, and the same effects were seen in both embryos from each group.

Severe changes to tail tissue following treatment with 161 were revealed. The tails of treated embryos looked quite fragile compared to controls (Figure 3-18). Also, treated embryos displayed a more rounded tail tip than controls, as if the drug had caused extensive degradation of the tissue. This observation initiated the measurement of tail lengths of 161 treated embryos (Section 3.1.2.1.1), which confirmed that embryos incubated in derivative 161 from 24 to 48 hpf showed decreasing tail lengths with increasing drug concentrations. This corresponds with the tail rounding and shrinkage that is observed here.

Embryos incubated in 0.1 mg/mL derivative 161 for one hour displayed tails that much more resembled control embryo tails than embryos incubated in the higher drug concentration. However, the outer epithelial layer seemed to detach in sheets (Figure 3-18, orange arrows). Small protrusions were observed on the tissue surface (Figure 3-18, blue arrows). These may be an indication of wound repair, showing damaged cells that have been extruded from the tissue surface in order to protect underlying tissue (103). Embryos incubated in 0.2 mg/mL 161 displayed higher tissue damage with thinner tails than controls. The outer layer of tissue seemed to have already detached completely, and very few blebs suggestive of wound healing were present.

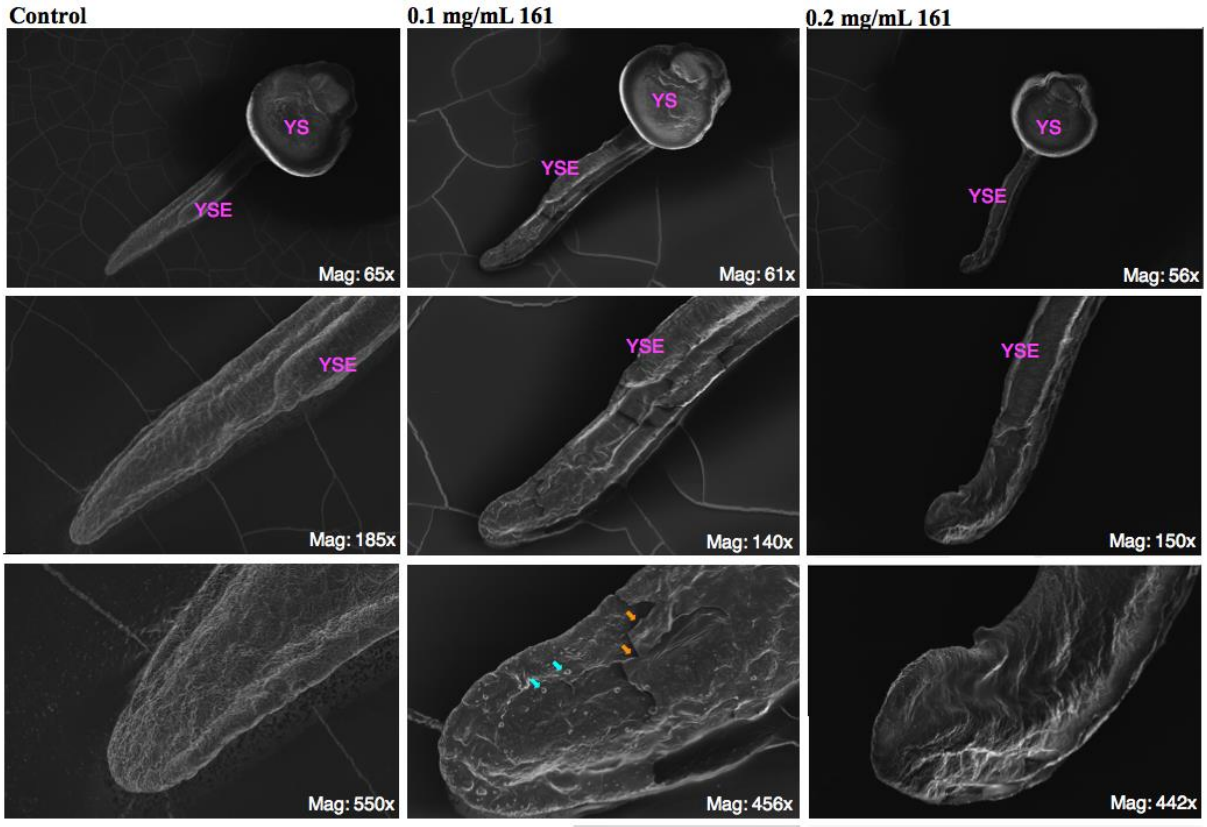


Figure 3-18: Use of FE-SEM revealed severe changes to embryo tail tissue following treatment with derivative 161. The outer tail tissue layer of embryos incubated in 0.1 mg/mL seemed to detach in sheets (orange arrows). Blebs that may suggest wound healing were also observed (blue arrows). Embryos incubated in 0.2 mg/mL 161 displayed thinner tails than controls and less indications of wound repair than embryos incubated in the lower drug concentration. Treated embryos showed shorter, rounded and deformed tail tips.
Exact magnification is given on each photograph. As it is not precisely the same in control pictures and photographs of treated embryos, direct comparison of size is difficult.
YS: yolk sac, YSE: yolk sac extension

3.1.2.3 Apoptosis assay

As for dexamethasone treated embryos, staining with acridine orange was used to assess apoptotic activity following treatment with derivative 161. For embryos treated with the drug from both 3.3 to 24 hpf and from 24 to 48 hpf, concentrations close to and below the estimated LC_{50} was chosen as concentrations at which to look for possible differences in apoptotic activity compared to controls. 10 embryos from each treatment group were evaluated.

Method A

Embryos incubated in 0.075 and 0.1 mg/mL 161 from 3.3 hpf were compared to control embryos with regard to apoptosis at 24 hpf. Apoptotic activity was observed in the epiphysis of a greater number of drug treated embryos compared to controls (Figure 3-19, orange arrows). Whereas only 2 of 10 control embryos showed apoptosis in this area, 8 of 10 embryos treated with 0.1 mg/mL did (Appendix Table A2-4). Embryos in all treatment groups showed apoptotic cells in the olfactory placodes (Figure 3-19, pink arrows). However, most embryos treated with derivative 161 showed increased apoptotic activity in this area compared to controls (Appendix Table A2-4). Furthermore, a significant increase in apoptotic activity was also seen in the urogenital tract of treated embryos compared to controls (Figure 3-21, blue arrows). Although all embryos, regardless of treatment group, showed apoptotic cells in this area, 9 of 10 embryos treated with 0.1 mg/mL of derivative 161 displayed a marked increase in apoptosis in the urogenital tract compared to controls (Appendix Table A2-4). The level of apoptosis in all of the affected tissues appeared to be dose-dependent, increasing in intensity with increasing concentrations of 161. No differences in apoptosis, apart from those in the urogenital tract, could be seen in the tail.

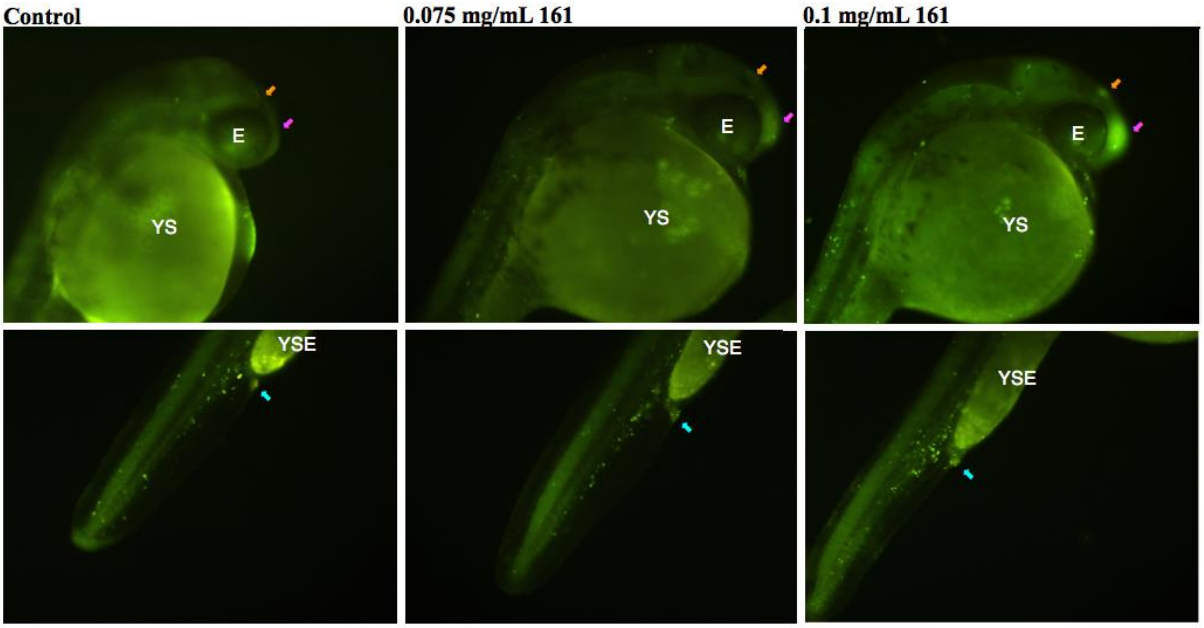


Figure 3-19: Early treatment with derivative 161 caused increased apoptosis in brain tissues and the urogenital tract at 24 hpf. Acridine orange staining showed that a greater number of embryos treated with derivative 161 from 3.3 to 24 hpf showed apoptosis in the epiphysis compared to the control group (orange arrows). Increased level of apoptosis was seen in the olfactory placodes (pink arrows) and in the urogenital tract (blue arrows) of treated embryos compared to controls**.** Differences in apoptosis seemed to be dose-dependent. E: eye, YS: yolk sac, YSE: yolk sac extension

Method B

Embryos incubated in 0.15 and 0.2 mg/mL of derivative 161 from 24 hpf were assessed with regard to apoptotic activity compared to controls at 48 hpf. No apoptosis was observed in the epiphysis at this time point. The increase in apoptotic activity in the olfactory placodes (Figure 3-22, pink arrows), and in the urogenital tract (Figure 3-22, blue arrows) observed for embryos treated with the drug from 3.3 to 24 hpf were seen here as well. Significantly more embryos treated with either concentration of derivative 161 showed increased level of apoptosis in these areas compared to controls (Appendix Table A2-5). When it comes to apoptotic activity in the tail, generally little, and no differences between treatment groups, were seen, which does not immediately correspond with FE-SEM results.

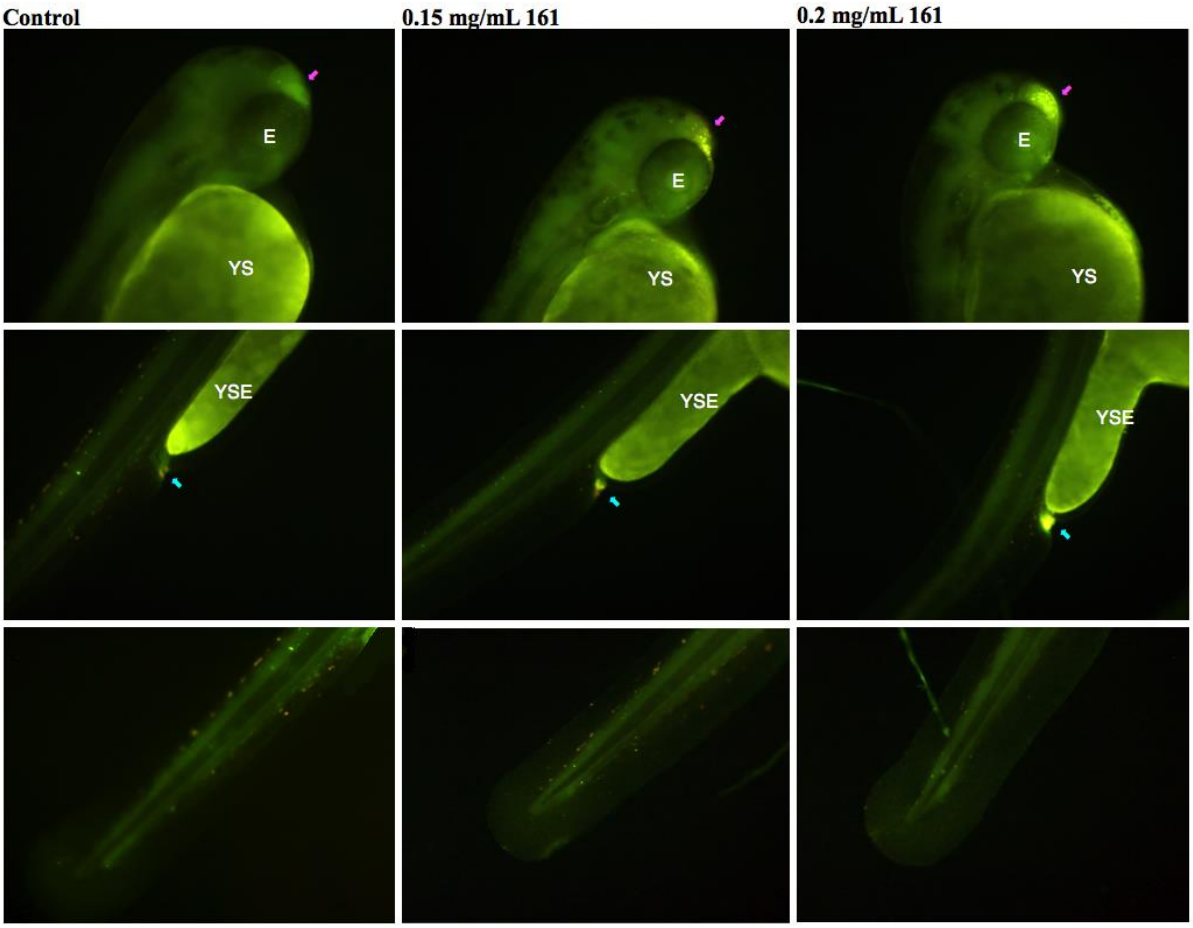


Figure 3-20: Treatment with derivative 161 from 24 to 48 hpf caused increased apoptosis in the olfactory placodes and the urogenital tract, similar to the effects seen at 24 hpf. Acridine orange staining showed a dose-dependent increase in apoptosis in both the olfactory placodes (pink arrows) and in the urogenital tract (blue arrows) following treatment with derivative 161 from 24 to 48 hpf. No differences in apoptotic activity could be identified in the tail region of treated embryos compared to controls (bottom panel).
E: eye, YS: yolk sac, YSE: yolk sac extension

3.1.2.4 Western blotting

As for dex treated embryos, we wanted to look at differences in proliferation (measured as the expression of PH3) and apoptosis (measured as the expression of active caspase-3) following treatment with derivative 161. Embryos incubated in drug concentrations around the LC_{50} values were collected from two independent experiments, and western blotting was performed.

Method A

Embryos incubated in derivative 161 from 3.3 to 24 hpf showed a marked decrease in expression of PH3 compared to controls, indicating a reduction in proliferating cells (Figure 3-21, A and C). A clear decrease in the expression of both pro-caspase-3 and active caspase-3 was also observed in treated embryos compared to controls, indicating a reduction in apoptotic activity following treatment with derivative 161 (Figure 3-21, B, D and E). Except for a marked drop in expression of pro-caspase-3 for embryos incubated in the highest drug concentration, differences in expression of the proteins in question seemed small between embryos incubated in the different concentrations of derivative 161.

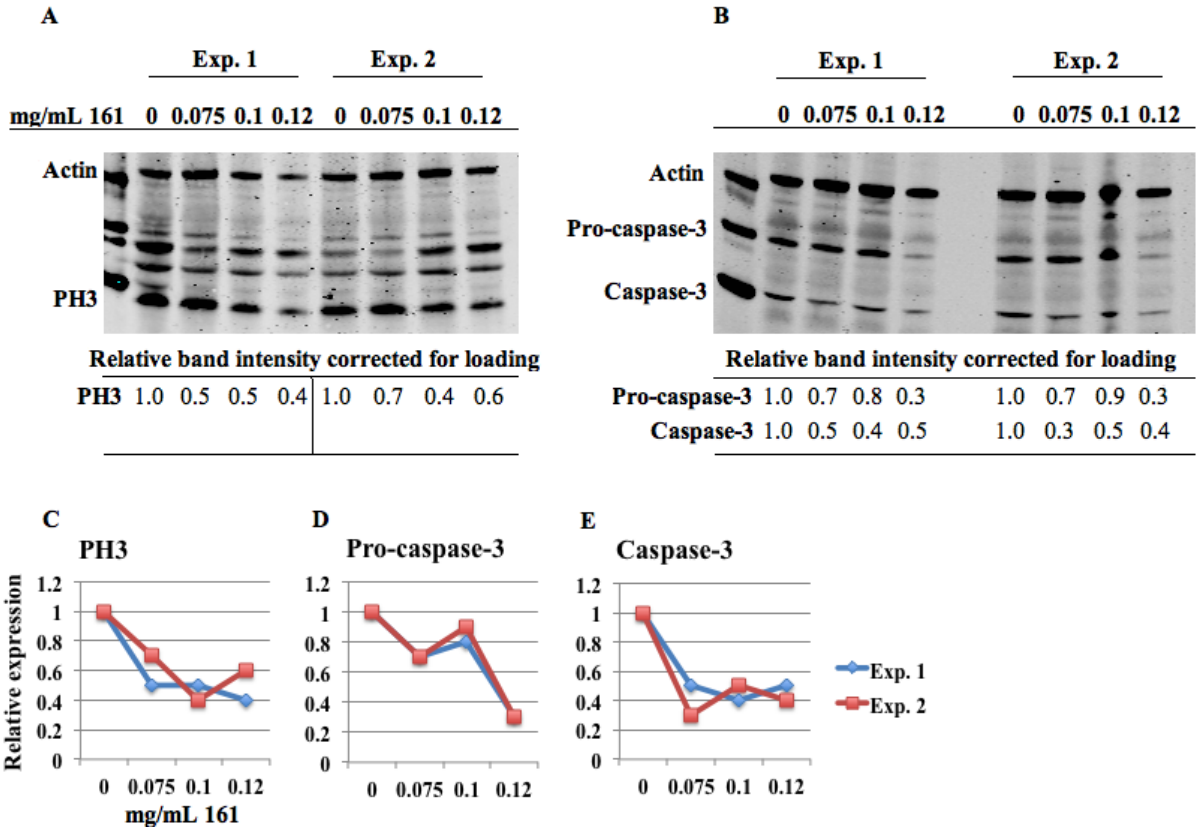


Figure 3-21: Western blotting of embryo extracts of embryos treated with derivative 161 from 3.3 to 24 hpf showed indications of decreased proliferation and apoptosis following treatment.
Possible differences in amount of sample put on the gel were adjusted for by using actin as a loading control. Band intensities were measured with Li-Cor Odyssey Sa 4.0 software (Section 2.2.6.5). Protein levels corrected for loading are here expressed relative to the control value in experiment 1 or 2.
Expected molecular weights of target proteins: PH3: 17 kDa, Pro-caspase-3: 32 kDa, Caspase-3: 17-20 kDa, Actin: 42 kDa

Method B

Embryos incubated in derivative 161 from 24 to 48 hpf showed a small increase in proliferation, as measured by the expression of PH3, compared to controls (Figure 3-22, A and C). Treatment with the highest concentration of derivative 161 caused increased expression of both pro-caspase-3 and active caspase-3 compared to controls, indicating a higher level of apoptotic activity in these embryos (Figure 3-22, B, D and E). Embryos obtained from experiment 1 also showed increased expression of these two proteins after treatment with 0.2 mg/mL derivative 161. Embryos from both experiments incubated in the lower drug concentrations showed a clear decline in the level of apoptotic markers compared to controls.

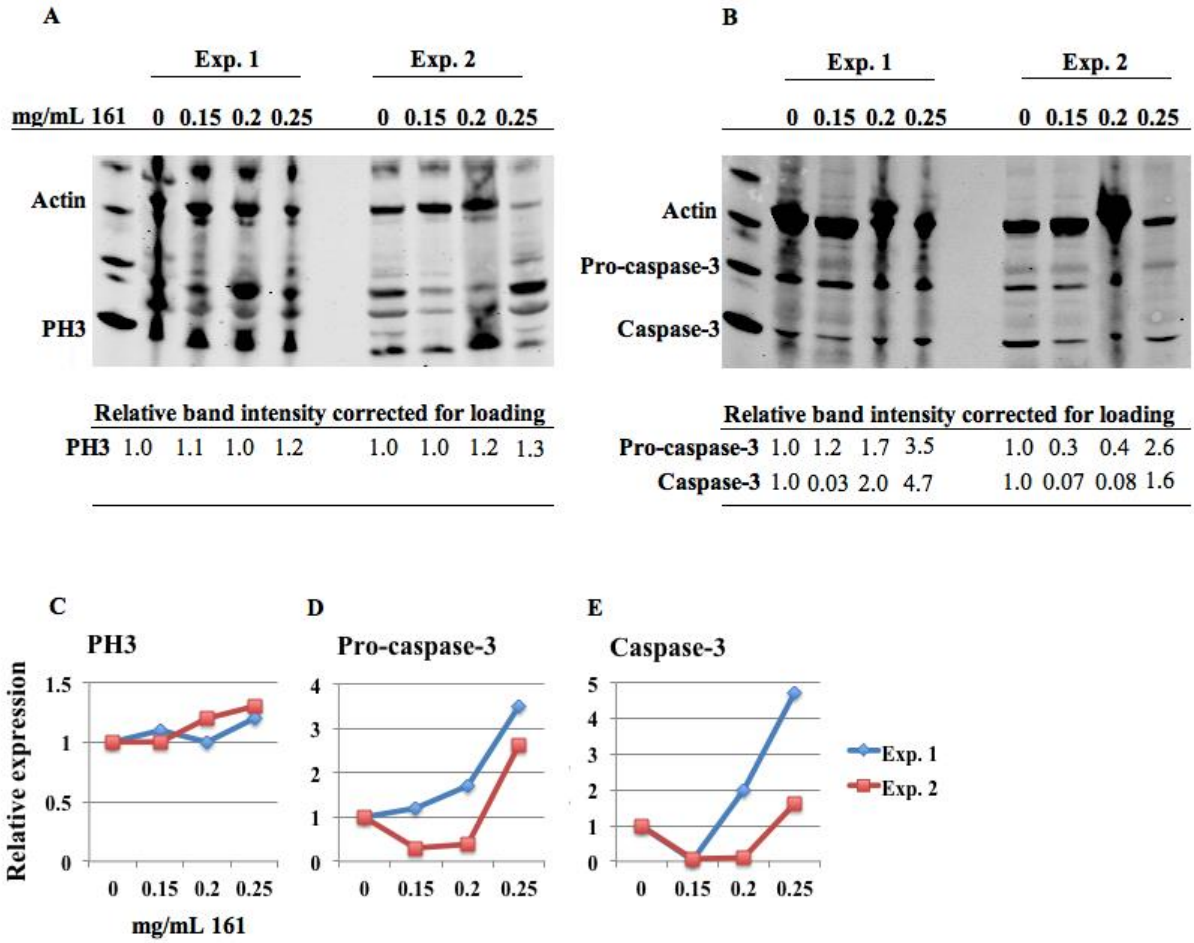


Figure 3-22: Western blotting of embryo extracts of embryos treated with derivative 161 from 24 to 48 hpf showed indications of increased proliferation and apoptosis following treatment with high concentrations of the drug, while decreased expression of apoptotic markers were seen after incubation in lower concentrations. Possible differences in amount of sample put on the gel were adjusted for by using actin as a loading control. Band intensities were measured with Li-Cor Odyssey Sa 4.0 software (Section 2.2.6.5). Protein levels corrected for loading are here expressed relative to the control value in experiment 1 or 2. Expected molecular weights of target proteins: PH3: 17 kDa, Pro-caspase-3: 32 kDa, Caspase-3: 17-20 kDa, Actin: 42 kDa

3.2 Cell culture experiments

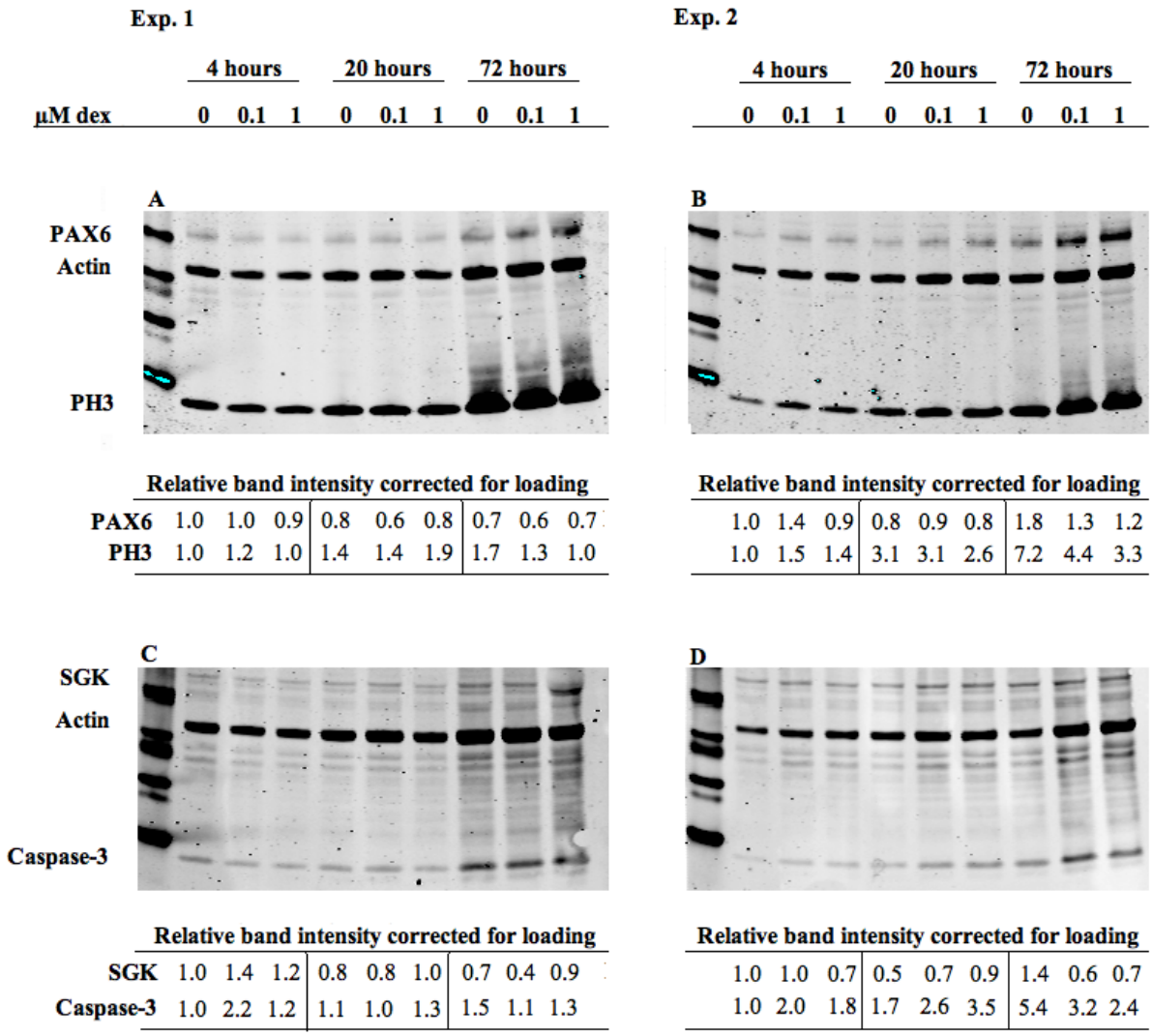
In parallel with zebrafish experiments, experiments with dexamethasone were carried out in mammalian cell lines as a side project. Human lens epithelial cells and human glioblastoma cells were used.

3.2.1 Western blotting - Human lens epithelial cells

Use of glucocorticoids increases the risk of developing cataract and glaucoma, but the pathophysiology behind this remains largely unknown. Anirida, a hereditary disorder that displays hyperplasia of the iris, leads to formation of cataract and development of glaucoma in about 50 % of cases (78). The disorder have been linked to decreased activity of the PAX6 gene (77, 78). As mentioned earlier, PAX6 is important for developmental processes of the eye and central nervous system, and abnormalities in many of these processes have been observed in mice with mutations of the Pax6 gene (104). We wanted to look into if human lens epithelial (B3) cells treated with dexamethasone would show decreased expression of PAX6. We also wanted to see if treatment with dexamethasone would influence cell proliferation (expression of PH3) and apoptosis (expression of caspase-3). Expression of serum- and glucocorticoid-inducible kinase-1 (SGK-1) was also evaluated. SGKs are a family of kinases that consists of three isoforms, which regulate the activity of many enzymes and transcription factors, and may play an active role in the pathophysiology of numerous diseases (105, 106). The name arises from the fact that both serum and glucocorticoids stimulate transcription of SGK-1 (105). In the eye, SGK-1 promotes activation of epithelial sodium channels that are essential for production of aqueous humor and preservation of intraocular pressure (IOP) (107). Increased IOP is a major risk factor for developing glaucoma, and is commonly seen after administration of glucocorticoids (73). SGK-1 was recently identified as a target gene downregulated by PAX6 in a pancreatic cell line (108). Because expression of SGK-1 is potentially regulated by both PAX6 and glucocorticoids, it was included here.

B3 cells were treated with 0.1 or 1.0 μM dexamethasone, and harvested for protein analysis 4, 20 or 72 hours following initiation of treatment. Controls were incubated in cell culture medium containing solvent (PBS). This was repeated so that western blotting could be carried out on cell extracts harvested from two independent experiments.

Results were very variable and difficult to reproduce (Figure 3-23 and Figure 3-24). Band intensities generally increased over time, but expression of the different proteins were for the most part not very different from time point to time point or between treatment groups when adjusted for loading. Although most results were not similar in the two experiments, exceptions were expression of PH3 and caspase-3 after 72 hours of drug treatment. Experiment 2 indicated a marked overall increase in proliferation over time, and a clear reduction in proliferation after dex treatment for 72 hours (Figure 3-23, B and Figure 3-24, D). Also in experiment 1, reduced expression of PH3 was seen for cells treated with dex for 72 hours compared to controls (Figure 3-23, A and Figure 3-24, C). The overall expression of active caspase-3 also increased over time in experiment 2, and apoptotic activity after 72 hours of treatment were clearly reduced in dex treated cells compared to controls (Figure 3-23, D and Figure 3-24, J). A small decrease in the marker for apoptosis was also seen in cells from experiment 1 treated with dex for 72 hours (Figure 3-23, C and Figure 3-24, I). Although the antibody used should recognize both pro-caspase-3 and active cspase-3, no bands were identified where pro-caspase-3 was expected to be seen (32 kDa) in either blot. Surprisingly, treatment with dexamethasone had very little influence on the expression of SGK-1, and in some cases even led to decreased expression in dex treated cells compared to controls (Figure 3-23, C-D and Figure 3-24, E-F). Incubation with dex also had little effect on expression of PAX6, but a reduction in expression was seen after 72 hours of treatment in the second experiment (Figure 3-23, B and Figure 3-24, B).



Relative band intensity corrected for loading

PAX6	1.0	1.0	0.9	0.8	0.6	0.8	0.7	0.6	0.7
PH3	1.0	1.2	1.0	1.4	1.4	1.9	1.7	1.3	1.0

Relative band intensity corrected for loading

	1.0	1.4	0.9	0.8	0.9	0.8	1.8	1.3	1.2
	1.0	1.5	1.4	3.1	3.1	2.6	7.2	4.4	3.3

Relative band intensity corrected for loading

SGK	1.0	1.4	1.2	0.8	0.8	1.0	0.7	0.4	0.9
Caspase-3	1.0	2.2	1.2	1.1	1.0	1.3	1.5	1.1	1.3

Relative band intensity corrected for loading

	1.0	1.0	0.7	0.5	0.7	0.9	1.4	0.6	0.7
	1.0	2.0	1.8	1.7	2.6	3.5	5.4	3.2	2.4

Figure 3-23: Western blots assessing expression of PAX6, PH3, SGK-1 and caspase-3 in human lens epithelial cells harvested after 4, 20 and 72 hours of treatment with dexamethasone. Possible differences in amount of sample put on the gel were adjusted for by using actin as a loading control. Band intensities were measured with Li-Cor Odyssey Sa 4.0 software (Section 2.2.6.5). Protein levels corrected for loading are here expressed relative to the control value after 4 hours of treatment. Expected molecular weight of target proteins: Pax6: 48 kDa, PH3: 17 kDa, SGK-1: 50-55 kDa, Caspase-3: 17-20 kDa, Actin: 42 kDa. No bands corresponding to pro-caspase-3 (32 kDa) showed up on the blots.

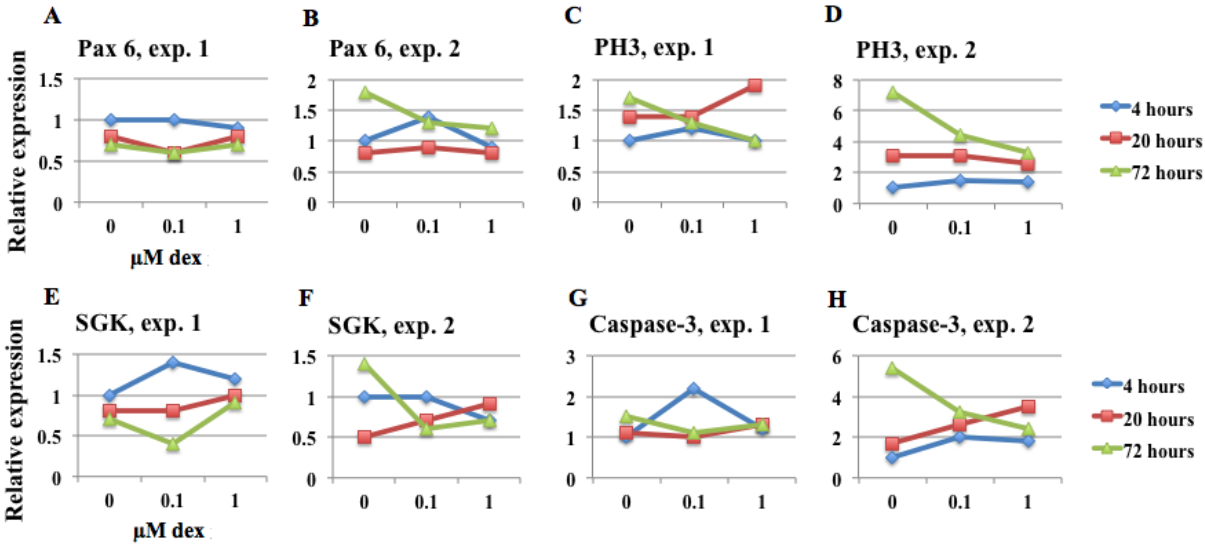


Figure 3-24: Illustration of relative expression of PAX6, PH3, SGK-1 and caspase-3 in human lens epithelial cells harvested after 4, 20 and 72 hours of treatment with dexamethasone. Visual presentation of relative band intensities calculated for western blots presented in **Figure 3-23**. Protein expression has been corrected for loading, and is here expressed relative to the control value after 4 hours of treatment. Band intensities were measured with Li-Cor Odyssey Sa 4.0 software (Section 2.2.6.5).

3.2.2 Western blotting - Human glioblastoma cells

The most common primary malignant brain tumor in adults, glioblastoma multiforme (GBM), is highly invasive and nearly always results in death shortly after diagnosis (104, 109). PAX6 has been shown to suppress GBM cells´ capability of causing tumors (104). Reduction of PAX6 expression is often seen in GBM cells, which may be why this type of cancer usually is so invasive (109). Many GBM patients receive dexamethasone to treat cerebral edema and relieve symptoms (110). However, it has been suggested that administration of dexamethasone may have a negative effect on survival (111). As increased activity of PAX6 seems to have a positive effect on GBM prognosis, while dexamethasone has been suspected to influence prognosis in a negative fashion, we wanted to investigate if treatment of human glioblastoma (U251) cells with dexamethasone would lead to reduced expression of PAX6. We also wanted to see if treatment with dexamethasone would influence proliferation (PH3), apoptosis (active caspase-3) and expression of SGK-1. As previously mentioned, SGKs are involved in many physiological and pathophysiological processes. They have been implied as important factors for cell proliferation, survival and migration, which are all key characteristics for cancer cells (106).

U251 cells were treated with 0.1 or 1.0 μM dexamethasone, and harvested for protein analysis 4, 20 or 72 hours following initiation of treatment. Controls were incubated in cell culture medium containing solvent (PBS). This was repeated so that western blotting could be carried out on cell extracts harvested from two independent experiments. Also here, results from the two experiments were inconsistent (Figure 3-25 and Figure 3-26). PAX6 was not detected after 4 or 20 hours of treatment in either experiment, but gave rise to a small band after 72 hours, possibly because a greater amount of sample was applied to the gel, as shown by stronger actin bands after 72 hours (Figure 3-25, A-B and Figure 3-26, A-B). However, no differences could be seen between dex treated cells and controls. Generally, there appeared to be small and non-systematic differences in proliferation and apoptosis, both between treatment groups and between the different time points, but indications of decreased apoptotic activity was seen after 20 and 72 hours of treatment in experiment 2 (Figure 3-25, D and Figure 3-26, H). No bands corresponding to pro-caspase-3 could be identified. Expression of SGK-1 was not induced by dexamethasone in this cell line either (Figure 3-25, C-D and Figure 3-26, E-F).

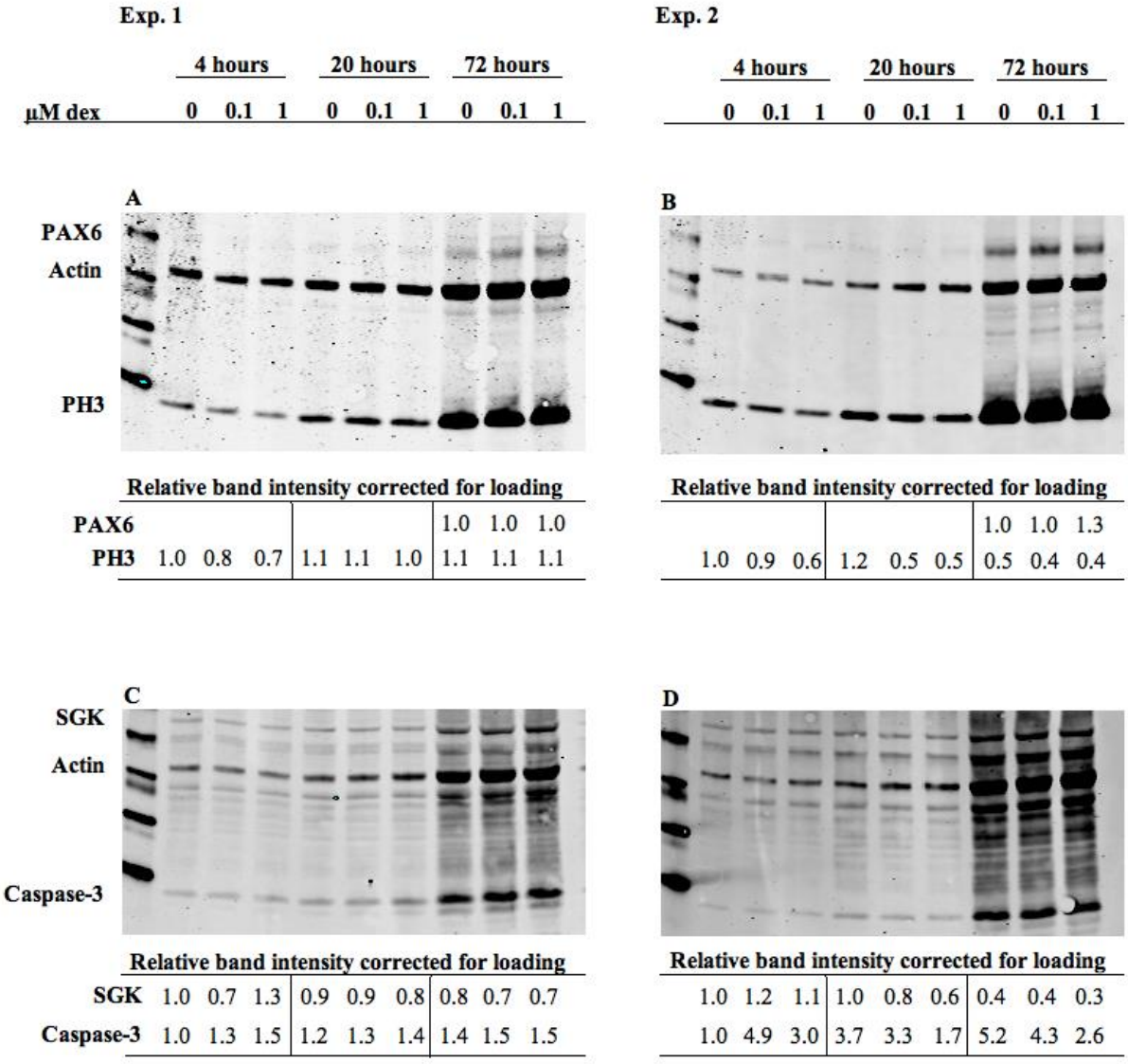


Figure 3-25: Western blots assessing expression of PAX6, PH3, SGK-1 and caspase-3 in human glioblastoma cells harvested after 4, 20 and 72 hours of treatment with dexamethasone. Possible differences in amount of sample put on the gel were adjusted for by using actin as a loading control. Band intensities were measured with Li-Cor Odyssey Sa 4.0 software (Section 2.2.6.5). Protein levels corrected for loading are here expressed relative to the control value after 4 hours of treatment. For PAX6, no bands were visible after 4 or 20 hours of treatment, and protein expression is therefore expressed relative to the control value at 72 hours (A-B). Expected molecular weight of target proteins: Pax6: 48 kDa, PH3: 17 kDa, SGK-1: 50-55 kDa, Caspase-3: 17-20 kDa, Actin: 42 kDa. No bands corresponding to pro-caspase-3 (32 kDa) showed up on the blots.

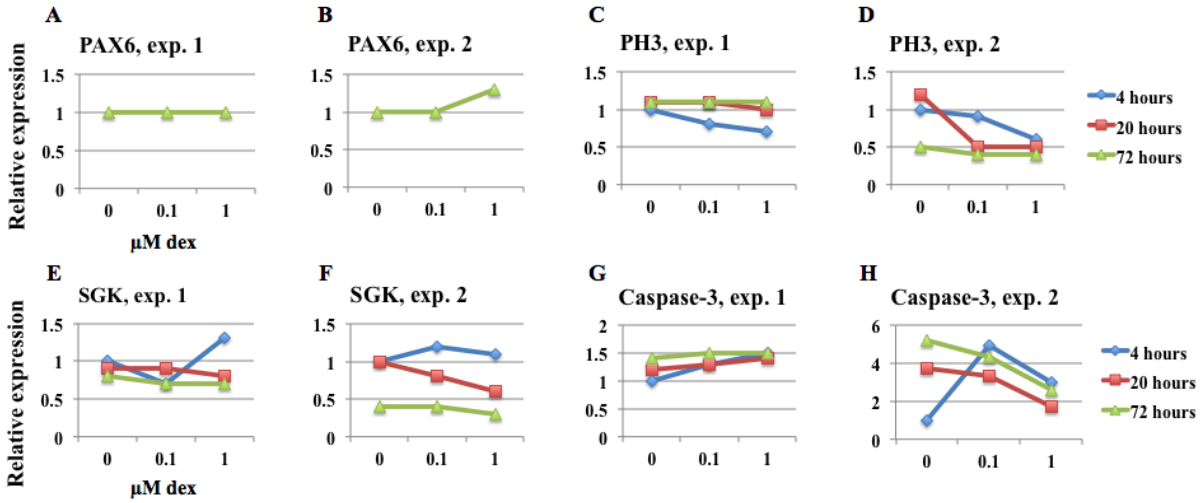


Figure 3-26: Illustration of relative expression of PAX6, PH3, SGK-1 and caspase-3 in human glioblastoma cells harvested after 4, 20 and 72 hours of treatment with dexamethasone. Visual presentation of relative band intensities calculated for western blots presented in Figure 3-25. Band intensities were measured with Li-Cor Odyssey Sa 4.0 software (Section 2.2.6.5). Protein expression has been corrected for loading, and is here expressed relative to the control value after 4 hours of treatment. For PAX6, no bands were visible after 4 or 20 hours of treatment, and protein expression is therefore expressed relative to the control value at 72 hours (A-B).

4. DISCUSSION

4.1 Summary of results

Using the zebrafish as a model system, the safety profile of two different drugs when administered to zebrafish embryos for up to three days were assessed. Zebrafish embryos treated with dexamethasone until 48 or 72 hpf showed a significant increase in the incidence of malformations compared to controls, and staining with acridine orange revealed altered apoptotic activity in brain tissues and the urogenital tract of dex treated embryos. Two different LC_{50}-values for $\beta^{2,2}$-amino acid derivative 161 were estimated for embryos treated with the drug from 3.3 to 24 hpf compared to treatment from 24 to 48 hpf, revealing that embryos were more sensitive to the drug at earlier stages of development. Truncation of the tail and damage to tail tissue were seen after treatment with derivative 161. Staining with acridine orange showed increased apoptosis in brain tissues and in the urogenital tract of embryos treated with derivative 161. Western blots of whole embryo extracts of embryos treated with dex or derivative 161 gave somewhat conflicting results, either with regard to repeatability or compared to results from acridine orange staining.

A side project was carried out in two different mammalian cell lines, human lens epithelial cells and human glioblastoma cells. Western blots were done to look at apoptosis, proliferation and expression of PAX6 and SGK-1 following dexamethasone treatment, but it was difficult to see any clear effects. Here, methods used in the project and results will be discussed in detail.

4.2 Zebrafish experiments

4.1.1 Zebrafish husbandry and egg quality

Due to construction work at the zebrafish facilities, some of the $\beta^{2,2}$-amino acid derivative 161 experiments were carried out on fish held at the ordinary lab. Presumably, these fish were kept under sub-optimal conditions. Since embryos obtained from the ordinary lab seemed to be more sensitive to the drug than embryos whose parents were kept at the zebrafish lab, results were analyzed separately. The LC_{50} values estimated for derivative 161 when embryos form the ordinary lab were used, were much lower than those estimated for the drug when

embryos from the zebrafish facility were used. Although few replicates for each test concentration was carried out using embryos from the ordinary lab, it indicates that zebrafish husbandry is of great importance for the outcome of toxicity tests. Mortality in the control group was higher for these embryos, indicating eggs of lesser quality. It is known that sub-optimal water temperature, pH and conductivity can affect the quality of eggs laid by the zebrafish females (17). Furthermore, it is possible that the stress of being moved to a different location also influenced the quality of the eggs laid by the females. Another possible explanation of increased sensitivity to the drug could be increased permeability of the chorion or of the embryos themselves. As such, it is plausible that the high sensitivity to 161 observed in embryos obtained from the ordinary lab can be ascribed to other factors than the drug itself. Consequently, derivative 161 appeared much more toxic than actually was the case. This highlights the importance of correct fish care if results from toxicity assays done at separate zebrafish facilities are to be compared. From here on out, only results obtained from experiments using zebrafish kept at optimal conditions will be discussed.

4.1.2 Drug administration

The desired concentration of the drug to be tested was prepared using E3 as solvent. Solutions of dexamethasone were stored protected from light at 4°C, and were given a shelf life of four weeks. This was done based on normal practice in the lab, but according to Sigma Aldrich, information on the durability of dex solutions kept at 4°C is lacking (112). Therefore, it is possible that the dex concentration was not constant during these four weeks. Stock solution of $\beta^{2,2}$-amino acid derivative 161 was prepared and divided into aliquot parts, before being stored at -20°C. Dexamethasone solutions and stock solutions of derivative 161 had to be remade several times during the course of the experiments. Vials of derivative 161 stock solution were thawed as needed, and diluted to the desired concentration. Exact drug concentrations were not measured in either stock or test solutions, and it is likely that they varied slightly. Furthermore, embryos were incubated in E3 until initiation of treatment. E3 was then removed from the wells by suction, and the desired drug solution was added. Obviously, some residue of E3 remained after removal, which could further have affected the drug concentration. Alas, the concentration of dex and derivative 161 were never exactly the same in each well or in each test.

Drug treatment was initiated at either 3.3 or 24 hpf. All embryos had intact chorions at time of treatment start. It is likely that results would have been different, especially for testing of 161 due to the high concentrations used, if embryos had been dechorionated before treatment began. It has been discussed that the chorion might be a barrier for embryo exposure to chemicals (113). The chorion has been reported to restrict uptake of large and lipophilic molecules such as polymers and surfactants (114, 115). However, it is not advisable to remove the chorion of zebrafish embryos younger than 24 hours (113). In this project, fairly small molecules (molecular weight < 500 Da) have been used, but it is still difficult to predict whether, and to what extent, the chorion has functioned as a barrier for drug penetration. It is also problematic to assess the amount of drug that has been absorbed by the embryo itself, and hence what dose of drug the embryo has received.

4.1.3 Mortality and visual morphological changes

Scoring for mortality was quite straightforward and easy to get right; either the embryo was dead, characterized by an opaque appearance, or it was not. Scoring for morphological changes on the other hand, required subjective evaluations. During the visual assessment, it was known at which drug concentration each well of embryos had been incubated. Thus, the possibility of biased evaluations cannot be excluded. To minimize the risk of this, the experiments could have been blinded so that the person doing the assessment had no knowledge of the drug concentrations used. In this project this was not feasible to arrange due to practical and temporal limitations. However, the malformations recorded were quite obvious traits, and all individuals, regardless of treatment group, were scrutinized. It is therefore considered probable that the recorded differences between treatment groups reflect real effects of the drug treatments.

4.2.1.1 Dexamethasone

Zebrafish embryos were incubated in 0.68 and 6.8 μg/mL dexamethasone, and assessed at 24, 48 and 72 hpf. Mortality rate did not differ from treatment groups and controls. At all time points, the rate of malformations or otherwise abnormal development increased with increasing concentrations of the drug. The increase was significant between treatment groups and controls at 48 and 72 hpf. The most common malformations observed were yolk sac

edema, pericardial edema, abnormal body axis curvature and kinked tail. This corresponds well with what others have described after treating zebrafish embryos with dexamethasone (116). In addition to the mentioned malformations, Hillegass and co-workers have also reported craniofacial changes and significant decreased embryo body length after exposure to dexamethasone. However, this was only seen for embryos treated with 100 μg/mL of the drug (116). In this project, no differences to the cranium were observed. Differences in body length were not seen either. Then again, only 10 embryos from each treatment group were measured, and much lower concentrations of the drug were used.

4.1.3.1 $\beta^{2,2}$-amino acid derivative 161

Zebrafish embryos were treated with derivative 161 from 3.3 to 24 hpf (method A) or from 24 to 48 hpf (method B). The two different methods gave rise to an LC_{50} for the peptide of 161 μM and 292 μM, respectively, indicating that embryos are more sensitive to the compound at earlier stages of development. This was as expected, as the zebrafish embryo at 3.3 hpf largely consists of dividing cells, making the embryo more vulnerable to chemical exposure (117). At 24 hpf, organogenesis of major organs has been established, and the embryo is not as sensitive to toxic effects anymore. Both LC_{50} values are well above the minimum inhibitory concentrations of derivative 161 previously measured against multi-resistant bacterial strains, as well as the half maximum inhibitory concentration measured against Ramos (Human Burkitt´s lymphoma) cells, see **Table 4-1** (91). Although a little lower, estimates of LC_{50} from the zebrafish experiments done in this project are also in accordance with early toxicity assessments of derivative 161, where toxicity was measured as hemolytic activity against red blood cells (RBCs) (91).

Table 4-1: Results from previous studies of derivative 161 (91)

Antimicrobial activity				Toxicity	Anticancer activity
MIC (μM)				EC_{50} (μM)	IC_{50} (μM)
MRSA	**MRSE**	***S. aureus***	***E. coli***	**RBC**	**Ramos**
7.4	15	15	52	425	7.1

MRSA: Methicillin resistant *S. aureus*, MRSE: Methicillin resistant *S. epidermis*, RBC: Red blood cells
MIC: Minimum inhibitory concentration of antimicrobial growth
EC_{50}: Half maximum effective concentration
IC_{50}: Half maximum inhibitory concentration

The mortality rates for the different concentrations of 161 varied quite a lot from experiment to experiment. It is known that dead embryos influences neighboring embryos in a negative fashion (98). Furthermore, different batches of embryos show variations in their sensitivity to chemicals (11). It is therefore possible that a combination of these two aspects is the reason for the somewhat inconsistent mortality rates. In experiments were some embryos by chance were more sensitive to chemical exposure, this could have set off a chain-reaction of embryo death, further inducing death in the surrounding environment. To eliminate the risk of dead or dying embryos promoting further embryo death within the well, embryos could have been kept in separate wells. In this project, such procedure was considered too time consuming, and hence 6-well plates were used, with about 50 embryos per well.

No visible morphological changes of treated embryos compared to controls could be seen through the dissecting microscope. However, measurements of embryo tail length showed a significant decrease compared to controls for embryos incubated in 0.25 mg/mL 161 from 24 to 48 hpf. There seemed to be a reduction in tail length at lower concentrations as well, but this was not significant compared to controls. However, measurements were done in retrospect, and only 10 embryos from each treatment group were measured. Thus, significance required very large differences between the groups, and it is therefore possible that significant effects on tail length would have been seen also at lower concentrations had more embryos been available for measurement.

4.1.4 Electron microscopy

Embryos treated with derivative 161 from 24 to 25 hpf showed severe changes to the tail tissue compared to controls when viewed by field emission scanning electron microscopy (FE-SEM). As described in section 3.1.2.1, embryos treated with high concentrations of derivative 161 regularly displayed tissue death in this area that could be identified through the dissecting microscope after about 2-3 hours of treatment. This correlates well with the observations done with FE-SEM, and indicates that absorption of the drug is higher in the tail area. A plausible explanation for this could be that the tissue is thinner in the tail, and easier to penetrate than tissue elsewhere on the embryo. It is however important to note that whereas embryos usually were exposed to derivative 161 with their chorion intact, embryos intended for observations with FE-SEM were dechorionated before treatment began. This will probably

have increased the uptake of the drug (113). Small blebs were identified on the tail tissue surface of treated embryos. These may be an indication of wound repair, showing damaged cells that have been extruded from the outer epithelial layer to protect underlying tissue. Morash and co-workers have observed similar protrusions, followed by neighboring cells migrating to the lesion sites to do damage control after administration of cancer selective antimicrobial peptides (103). They also investigated if tail tissue degeneration was due to apoptosis or ischemic cell death, and found that it was a combination of both (103).

4.1.5 Apoptosis assay

The risk of introducing bias is substantial with assessment of apoptotic activity through staining with acridine orange. If apoptotic activity is observed in the same areas in both controls and treated embryos, but the level of apoptosis appears different, it can be hard to quantify these differences. To do so, one would have to count the number of apoptotic cells in the area in question, but due to considerable individual variations in apoptotic activity between embryos, this would require a great number of observations for statistic significant differences to be found. Furthermore, such a time consuming process is not very suitable due to the fact that the level of fluorescence diminishes over time when exposed to light. In this project, only ten embryos per treatment group were assessed with regard to apoptosis, and results are based on subjective evaluation of fluorescence level rather than counted numbers of cells stained with acridine orange. For execution of future experiments, an automatic assessment of the relative fluorescence of larger pools of embryos using digital image analysis, as described by Tucker and Lardelli, could be considered (118).

4.2.1.2 Dexamethasone

At 24 hpf, a significantly larger number of dex treated embryos showed a small cluster of apoptotic cells in the epiphysis compared to the control group, where this was absent in most individuals. As apoptosis in this area at 24 hpf has been reported as part of normal development for zebrafish embryos, this was somewhat puzzling (119). Due to the small number of individuals evaluated, it is of course possible that the observed difference between treatment groups simply reflected individual variations around a normal process and could not be ascribed to effects of the drug.

At 48 hpf, there appeared to be an increase in the level of apoptosis around the eyes of dex treated embryos compared to controls. Apoptotic activity in the retina of normally developing zebrafish embryos has been reported to peak at 36 hpf, followed by a considerable decline by 48 hpf, and it has been suggested that the apoptosis may occur to create space for the growth of retinal ganglion cell axons (119). Glucocorticoids are known to induce retinal toxicity and cell death in other animals, but this appears to be through non-apoptotic mechanisms (120). More embryos would have to be assessed in order to conclude anything on the matter, but assuming that the observed difference in apoptotic activity between dex treated embryos and controls reflects reality; what would that mean? Either zebrafish embryos are sensitive to dexamethasone, responding with increased apoptosis around the eyes, or one could speculate if the increased apoptosis seen around the eyes of dex treated embryos could be due to delayed development. If normal apoptotic activity around the eyes of embryos follows the same pattern as retinal apoptosis, apoptotic activity in this area might already have peaked at 36 hpf and declined by 48 hpf for controls, whereas drug treated embryos lag behind in the developmental process and as such have just reached the height of apoptotic activity in this area. As mentioned, further experiments would be needed to test this notion, and it would also be necessary to evaluate apoptosis at 36 hpf to see if there actually is a peak in apoptotic activity around the eyes of normally developing embryos at this stage of development.

Both at 48 and 72 hpf, dex treated embryos showed decreased level of apoptosis in the olfactory placodes and in the urogenital tract compared to controls. Placodes are specialized tissue that develop into sensory neurons of the olfactory organ (121). From 22 hpf and onwards, immature sensory neurons are normally eliminated from the developing olfactory organ by apoptosis (119). Around the time of observation, normally developing embryos would also be expected to show apoptosis in the urogenital tract as this is important for opening up the urinary system to the outside environment (119). The observed decrease in apoptosis in these areas for dex treated embryos compared to controls can therefore possibly be an indication of reduced recognition and control of cells that should be eliminated.

4.1.5.1 $\beta^{2,2}$-amino acid derivative 161

As seen after dex treatment, apoptotic activity was observed in the epiphysis of a greater number of embryos treated with derivative 161 than in the control group at 24 hpf. Again, this was surprising as apoptotic activity in this region is associated with normal development, and was therefore expected to be seen in control embryos as well (119). At both 24 hpf (method A) and 48 hpf (method B), embryos treated with derivative 161 showed increased levels of apoptosis in the olfactory placodes and the urogenital tract compared to controls. This corresponds well with changes in apoptotic activity observed after administration of similar $\beta^{2,2}$-amino acid derivatives in a previous project (122). Very little apoptotic activity was detected in the tail, and no differences were seen between treatment groups. On one hand, this might indicate that the tissue damage detected with FE-SEM is not apoptotic. On the other, the apoptosis assay was done at 48 hpf after 24 hours of drug treatment, while assessment using FE-SEM was done after only one hour of treatment. Apoptotic activity can therefore not be excluded as responsible for damage to tail tissue observed in treated embryos; it could simply be a rapid response that is no longer seen at the time of apoptosis assay. It is also possible that damage to the tail tissue is a combination of both apoptotic and non-apoptotic events, which would correspond with what others have reported following administration of similar peptides to zebrafish embryos (section 4.1.4) (103).

4.1.6 Western blotting

The technique of western blotting is a fairly quick way to assess protein expression, and it allows easier quantification than *in situ* techniques. However, it does not provide any information as to where differences in expression are located. For instance, when assessing apoptotic activity through acridine orange staining, it has the advantage that localization of apoptotic cells becomes evident. This is completely lost when apoptosis is assessed through western blotting and staining with caspase-3 antibodies. In this project, extracts of 10 crushed whole embryos were used for western blotting. Having demonstrated through apoptosis assay that apoptotic cells amount to only a very small portion of the total cell count and that only small differences in apoptotic activity could be seen following treatment with either dex or derivative 161, local differences in expression of active caspase-3 between treated embryos and controls may be drowned in the overall body expression. This probably holds true for all proteins whose expression was assessed in this project; a substantial change in expression

must be present for differences to become evident. Furthermore, individual differences between embryos may also cloud the image. Therefore, tissue extracts used for western blotting should be based on many more embryos in future experiments. Also, it could be beneficial to use smaller tissue sections instead of whole embryos. For instance, if potential differences in protein expression in the embryo brain of treated embryos compared to controls are of the most interest, embryo heads can be separated from the rest of the body so that only cut-off heads make up the tissue extract used for analysis.

3.2.2.1 Dexamethasone

Two rounds of western blotting was done, using embryos obtained from two separate experiments, and proliferation (measured as expression of PH3), apoptosis (measured as expression of caspase-3) and expression of Pax 6 was assessed. The two rounds yielded quite different results, and few conclusions regarding protein expression can be drawn. The only reproducible outcomes were that the overall expression of all proteins assessed seemed to decrease over time, and that dexamethasone in the concentrations used had little influence on the level of expression in treated embryos compared to controls. This may be due to the low drug concentrations applied. Although our original intention was to treat embryos with 10 and 100 μg/mL dex, concentrations used were much lower, namely 0.68 and 6.8 μg/mL (Section 2.2.2.2). However, based on the assumption that the average human have a body volume of 60-70 L, the doses for humans which would roughly correspond to the incubation concentrations used in this project for zebrafish embryos are still considerably higher than what is normally administered to patients in the clinic, where doses usually lie in the range of 0.25-20 mg daily (Table 4-2) (123). Of course, this is a very simplified calculation that does not take in to consideration the actual plasma or tissue concentrations of dex in the embryos or ADME characteristics of the drug once administered to humans. Still, it indicates that the concentrations used are biological relevant even though previous studies have been based on higher treatment concentrations.

Table 4-2: Dexamethasone concentrations used in this project an their corresponding human dose, based on a very rough estimate

Incubation concentration of dex (μg/mL)	Roughly corresponding human dose (mg)
0.68	40.8 – 47.6
6.8	408 – 476

4.1.6.1 $\beta^{2,2}$-amino acid derivative 161

Protein analysis of embryos treated from both 3.3 to 24 hpf and from 24 to 48 hpf from two separate experiments showed much more reproducible effects than was seen after treatment with dexamethasone. Drug concentrations that to a much higher degree influenced embryos were used, the highest concentrations being greater than the LC_{50} values, underlining that the very variable effects seen after dex treatment might indeed be due to the fact that too low concentrations were used. Embryos treated with derivative 161 from 3.3 to 24 hpf showed a marked decrease in expression of PH3 compared to controls, indicating reduced cell proliferation. Embryos treated from 24 to 48 hpf however, displayed a small increase in PH3 expression compared to controls. Possibly, this could be a result of wound healing events of lesions to for instance the tail region, as observed by FE-SEM. As mentioned earlier, the zebrafish embryo at 3.3 hpf consists largely of dividing cells, and is therefore more sensitive to chemical exposure. It might be possible that coping mechanisms when exposed to stress differ at different time points in the embryogenesis, so that embryos exposed to derivative 161 during early development responds with reduced proliferation, while embryos that are older at time of exposure responds by trying to mend the damages inflicted by the drug. Levels of pro-caspase-3 and active caspase-3 were reduced in early treated embryos (method A) compared to controls, suggesting less apoptotic activity. Also later treated embryos (method B) showed reduced expression of active caspase-3 compared to controls for some of the drug concentrations used. This is contradictive of apoptosis assay results obtained *in vivo* in zebrafish embryos stained with acridine orange, were increased apoptosis in the olfactory placodes and urogenital tract was observed following treatment with the same drug concentrations. Again, this demonstrates the need for alterations to the methods used, such as using smaller tissue sections when doing western blots, and including more embryos both in western blotting and acridine orange staining.

4.2 Cell culture experiments

Glucocorticoid treatment is a risk factor for glaucoma and cataract (72, 73). Anirida, a hereditary disorder known to lead to these eye maladies have been linked to decreased activity of PAX6 (77, 78). We therefore wanted to investigate if human lens epithelial (B3) cells treated with dexamethasone would show decreased expression of PAX6. This transcription factor has also been linked to cancer outcome; increased activity of PAX6 seems to have a

positive effect on glioblastoma patients prognosis (109). In this regard, dexamethasone has been suspected to influence prognosis in a negative fashion (111). Again, we wanted to see how dexamethasone treatment of human glioblastoma (U251) cells would affect expression levels of PAX6. We also checked to see if treatment with dexamethasone would influence expression of SGK-1, caspase-3 and PH3 in either cell line.

Western blots of protein extracts from both B3 and U251 cells gave very variable results that were hard to reproduce for all proteins looked at. Disappointingly, dex treatment seemed to have very little or no effect on the level of expression of PAX6 in either cell line. Indications of reduced expression following dex treatment for 72 hours was seen for B3 cells in experiment 2, but not in experiment 1.

For serum- and glucocorticoid inducible kinase 1 (SGK-1), we had expected, as the name implies, to see increased levels of this protein after dex treatment. This was not seen in either B3 or U251 cells, and one can therefore question if dex stimulation of the cells were at all achieved. It is of course possible that the SGK-1 protein were not expressed in the cells we worked with. However, increased transcription of SGK-1 mRNA has been reported in B3 cells following exposure to dex, so this seems unlikely for at least one of the cell lines (124). Another explanation for the apparent absence of increased SGK-1 expression after dex treatment might be that the antibody, which the lab had little previous experience with, did not work, and that the bands we interpreted as SGK-1 were actually unspecific stained bands of a different protein.

For B3 cells, no reproducible differences were seen in apoptotic activity (caspase-3) and proliferation (PH3) after treatment with 0.1 or 1 μM dex for 4 or 20 hours. This corresponds with what Gupta and co-workers have reported after treatment for 12 hours with equal and higher concentrations of dex (74). After 72 hours of treatment however, there seemed to be a small decrease in both apoptosis and proliferations for treated cells compared to controls. Nevertheless, the experiments need to be repeated to confirm this. It would also be interesting to study cells treated for a longer time period, as effects to the lens are usually only seen after long-term use of glucocorticoids (72, 73).

For U251 cells, only small and non-systematic effects on apoptosis and proliferation could be seen following treatment with dex. Reduced cell proliferation have previously been reported for other glioblastoma cell lines following dex treatment (125). In this project, signs of reduced expression of PH3 for treated cells could be seen at some of the time points. However, the two experiments varied in this regard, and additional studies are needed to investigate if dex indeed does influence U251 proliferation. Glioblastoma cells harvested from the second incubation experiment in this project, showed decreased apoptotic activity with increasing concentrations of dex after 20 and 72 hours of treatment. This corresponds with other reports that treatment with dexamethasone inhibits apoptosis of glioma cells (126). However, further experiments would be needed to confirm this.

4.3 Future perspectives

Although the zebrafish has been established as an attractive model for drug safety studies, and much is already known about the species, additional knowledge is needed to further develop the model system. Although many known human effects of drug exposure can be reproduced in the zebrafish, predictivity for the human situation of effects first seen in zebrafish needs to be further explored (3). Early investigations suggest that ADME conditions of various compounds are different in zebrafish as opposed to humans, and the importance of this needs to be considered as well (127). As discussed, it is difficult to translate incubation concentration to the dose of drug administered to the zebrafish embryo, due to the fact that the concentration in the fish water would be expected to differ from the tissue or plasma concentration of the drug. For comparison of toxicity in zebrafish embryos and higher animals, knowledge of the amount of drug actually delivered to the embryos is therefore necessary (128). In accordance with our original plan, higher concentrations of dexamethasone should be used in future experiments to study effects of this drug to the zebrafish embryo. Zebrafish assays can never completely replace experiments in mammals, and $\beta^{2,2}$-amino acid derivative 161 will need to be further tested in for instance rodents to regain more insight into the drug´s potential toxic properties and for conclusions to be made. Regarding the cell lines included in the project, further studies are needed to assess regulation of PAX6 and the possible link between dex treatment and expression of PAX6.

5. CONCLUSION

In this project, zebrafish embryos were used as a model in safety pharmacology studies, and we observed that treatment with relative low concentrations of dexamethasone caused a significance increase in embryo malformation rate, while staining with acridine orange revealed altered apoptotic activity in brain tissues and in the urogenital tract of treated embryos. $\beta^{2,2}$-amino acid derivative 161, showed generally low toxicity to zebrafish embryos, as measured by the LC_{50} estimated in this project, compared to previously reported MIC and IC_{50} values of the drug against multi-resistant bacterial strains and cancer cells. However, truncation of the tail and damage to tail tissue were seen after treatment with the drug. Staining with acridine orange showed increased apoptotic activity in brain tissues and the urogenital tract of treated embryos. Western blots of whole embryo extracts of embryos treated with either drug gave conflicting result, and a refinement of the method is suggested for future experiments.

Parallel experiments with cell cultures to look into if treatment with dexamethasone would influence expression levels of PAX6 gave inconclusive results, and further studies are needed to answer this.

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APPENDIX 1: MORTALITY AND MALFORMATION RATES FROM THE INDIVIDUAL EXPERIMENTS

A1.1 Dexamethasone

Table A1-1: Mortality and malformation rate for embryos incubated in dexamethasone from 3.3 to 24 hpf

Exp.	n	Mort.	A.d	% Mort.	% A.d
Control					
1*	258	91	0	35.27	0.00
2	234	42	2	17.95	1.04
3*	284	104	1	36.62	0.56
4	236	24	0	10.17	0.00
5	49	7	0	14.29	0.00
6	42	6	0	14.29	0.00
7	25	3	0	12.00	0.00
8	139	19	0	13.67	0.00
9	164	17	0	10.37	0.00
			Mean	**13.25**	**0.15**
			STD	**2.70**	**0.39**
Dexamethasone 0.68 ug/mL					
1*	201	64	0	31.84	0.00
2	247	61	3	24.70	1.61
3*	293	114	0	38.91	0.00
4	259	63	2	24.32	1.02
5	40	14	0	35.00	0.00
6	46	9	1	19.57	2.70
7	25	1	0	4.00	0.00
8	162	18	0	11.11	0.00
9	161	20	0	12.42	0.00
			Mean	**18.73**	**0.76**
			STD	**10.39**	**1.07**
Dexamethasone 6.8 ug/mL					
1*	205	76	0	37.07	0.00
2	241	54	0	22.41	0.00
3	266	43	2	16.17	0.90
4	46	10	1	21.74	2.78
5	51	1	1	1.96	2.00
6	160	13	1	8.13	0.68
7	145	24	0	16.55	0.00
			Mean	**14.49**	**1.06**
			STD	**8.00**	**1.12**

Mort.: Mortality
A.d: Abnormal development
% A.d: percent abnormally developed embryos of survivors
STD: Estimated standard deviation
* Due to high mortality rate in the control group, marked entries were excluded from all calculations. Mean values and estimated standard deviations only takes into consideration the other entries.

Table A1-2: Mortality and malformation rates for embryos incubated in dexamethasone from 24 to 48 hpf

Exp.	n	Mort.	A.d	% Mort.	% A.d
Control					
1	113	0	2	0.00	1.77
2	126	1	4	0.80	3.20
3	117	0	4	0.00	3.42
4	124	0	2	0.00	1.61
5	19	0	1	0.00	5.26
6	14	0	0	0.00	0.00
7	81	0	2	0.00	2.47
8	96	0	2	0.00	2.08
			Mean	**0.10**	**2.48**
			STD	**0.28**	**1.55**
Dexamethasone 0.68 ug/mL					
1	92	0	4	0.00	4.35
2	111	2	3	1.80	2.75
3	125	1	3	0.80	2.42
4	126	0	15	0.00	11.90
5	9	0	2	0.00	22.22
6	15	0	4	0.00	26.67
7	99	0	9	0.00	9.09
8	99	0	8	0.00	8.08
			Mean	**0.33**	**10.94**
			STD	**0.66**	**9.03**
Dexamethasone 6.8 ug/mL					
1	86	0	9	0.00	10.47
2	130	2	7	1.54	5.47
3	142	0	19	0.00	13.38
4	17	0	3	0.00	17.65
5	29	0	7	0.00	24.14
6	96	0	11	0.00	11.46
7	82	0	5	0.00	6.10
			Mean	**0.22**	**12.67**
			STD	**0.58**	**6.56**

Mort.: Mortality
A.d: Abnormal development
% A.d: percent abnormally developed embryos of survivors
STD: Estimated standard deviation

Table A1-3: Mortality and malformation rates for embryos incubated in dexamethasone from 48 to 72 hpf

Exp.	n	Mort.	A.d	% Mort.	% A.d
Control					
1	39	0	2	0.00	1.77
2	58	2	4	3.45	3.20
3	58	0	4	0.00	3.42
4	77	0	2	0.00	1.61
5	19	0	1	0.00	5.26
6	14	0	0	0.00	0.00
7	42	0	2	0.00	2.47
8	50	0	2	0.00	2.08
			Mean	**0.43**	**5.81**
			STD	**1.22**	**4.29**
Dexamethasone 0.68 ug/mL					
1	38	0	4	0.00	4.35
2	54	1	3	1.85	2.75
3	62	0	3	0.00	2.42
4	86	1	15	1.16	11.90
5	8	0	2	0.00	22.22
6	13	0	4	0.00	26.67
7	49	0	9	0.00	9.09
8	49	0	8	0.00	8.08
			Mean	**0.38**	**15.04**
			STD	**0.72**	**8.11**
Dexamethasone 6.8 ug/mL					
1	39	2	9	5.13	10.47
2	58	3	7	5.17	5.47
3	96	1	19	1.04	13.38
4	17	0	3	0.00	17.65
5	29	0	7	0.00	24.14
6	53	0	11	0.00	11.46
7	40	0	5	0.00	6.10
			Mean	**1.62**	**23.25**
			STD	**2.44**	**6.06**

Mort.: Mortality
A.d: Abnormal development
% A.d: Percentage abnormally developed embryos of survivors.
STD: Estimated standard deviation

A1.2 $\beta^{2,2}$-amino acid derivative 161

Table A1-4: Mortality and malformation rates for embryos treated with various concentrations of derivative 161 from 3.3 to 24 hpf (method A)

Exp.	n	Mort.	A.d	%Mort.	%Mort, corr.	% A.d
Control						
1	50	5	0	10.00		0.00
2	50	3	0	6.00		0.00
3*	50	15	0	30.00		0.00
4	100	4	1	4.00		1.04
5	150	15	0	10.00		0.00
6	100	6	0	6.00		0.00
			Mean	**7.20**		**0.21**
			STD	**2.68**		**0.47**
0.01 mg/mL 161						
1	50	4	0	8.00	-2.22	0.00
2	50	3	0	6.00	0.00	0.00
			Mean	**7.00**	**-1.11**	**0.00**
			STD	**1.41**	**1.57**	**0.00**
0.02 mg/mL 161						
1	50	3	1	6.00	-4.44	2.13
2	50	6	0	12.00	6.38	0.00
			Mean	**9.00**	**0.97**	**1.06**
			STD	**4.24**	**7.66**	**1.50**
0.03 mg/mL 161						
1	50	5	0	10.00	0.00	0.00
2	50	9	1	18.00	12.77	2.44
			Mean	**14.00**	**6.38**	**1.22**
			STD	**5.66**	**9.03**	**1.72**
0.04 mg/mL 161						
1	50	7	0	14.00	4.44	0.00
2	50	8	0	16.00	10.64	0.00
			Mean	**15.00**	**7.54**	**0.00**
			STD	**1.41**	**4.38**	**0.00**
0.05 mg/mL 161						
1	50	5	0	10.00	0.00	0.00
2	50	9	0	18.00	12.77	0.00
3*	50	12	0	24.00	-8.57	0.00
4	100	8	0	8.00	4.17	0.00
5	150	22	1	14.67	5.22	0.78
6	100	7	0	7.00	1.06	0.00
			Mean	**11.54**	**4.64**	**0.16**
			STD	**4.67**	**5.02**	**0.35**
0.06 mg/mL 161						
1	50	7	0	14.00	4.44	0.00
2	50	11	0	22.00	17.02	0.00
3	100	4	2	4.00	0.00	2.08
			Mean	**13.33**	**7.16**	**0.69**
			STD	**9.02**	**8.83**	**1.20**

Table A1-4 continued

Exp.	n	Mort.	A.d	%Mort.	%Mort, corr.	% A.d
0.075 mg/mL 161						
1	50	17	0	34.00	26.67	0.00
2	50	10	0	20.00	14.89	0.00
3*	50	26	0	52.00	31.43	0.00
4	100	30	0	30.00	27.08	0.00
5	150	30	2	20.00	11.11	1.67
6	100	29	0	29.00	24.47	0.00
			Mean	**26.60**	**20.84**	**0.33**
			STD	**6.31**	**7.35**	**0.75**
0.1 mg/mL 161						
1*	50	24	0	48.00	25.71	0.00
2	100	14	0	14.00	10.42	0.00
3	150	55	1	36.67	29.67	1.05
4	100	58	0	58.00	55.32	0.00
			Mean	**36.23**	**31.80**	**0.35**
			STD	**22.00**	**22.53**	**0.61**
0.12 mg/mL 161						
1*	50	33	0	66.00	51.43	0.00
2	100	72	0	72.00	70.83	0.00
3	150	115	1	76.67	74.11	2.86
4	100	76	0	76.00	74.47	0.00
			Mean	**74.90**	**73.14**	**0.95**
			STD	**2.54**	**2.00**	**1.65**
0.14 mg/mL 161						
1	100	42	0	42.00	39.58	0.00
0.16 mg/mL 161						
1	100	66	0	66.00	64.58	0.00
2	150	129	0	86.00	84.44	0.00
3	100	84	0	84.00	82.98	0.00
			Mean	**78.67**	**77.34**	**0.00**
			STD	**11.02**	**11.07**	**0.00**
0.20 mg/mL 161						
1	100	75	1	75.00	73.96	4.00
2	150	131	0	87.33	85.89	0.00
3	100	94	0	94.00	93.62	0.00
			Mean	**85.43**	**84.49**	**1.33**
			STD	**9.64**	**9.90**	**2.31**
0.22 mg/mL 161						
1	150	150	-	100.00	100.00	-
2	100	100	-	100.00	100.00	-
			Mean	**100.00**	**100.00**	-
			STD	**0.00**	**0.00**	-
0.25 mg/mL 161						
1	150	149	0	99.33	99.22	0.00
2	100	100	-	100.00	100.00	-
			Mean	**99.65**	**99.61**	-
			STD	**0.49**	**0.55**	-

Table A1-4 Continued

Exp.	n	Mort.	A.d	%Mort.	%Mort, corr.	% A.d
0.25 mg/mL 161						
1	150	149	0	99.33	99.22	0.00
2	100	100	-	100.00	100.00	-
			Mean	**99.65**	**99.61**	-
			STD	**0.49**	**0.55**	-

Only results obtained from zebrafish kept at the zebrafish facility is shown.
Mort.: Mortality
Mort., corr: Mortality corrected for embryo death in control group
A.d: Abnormal development
% A.d: percent abnormally developed embryos of survivors
STD: Estimated standard deviation
* Due to high mortality rate in the control group, marked entries were excluded from all calculations. Mean values and estimated standard deviations only takes into consideration the other entries.

Table A1-5: Mortality and malformation rates for embryos treated with various concentrations of derivative 161 from 24 to 48 hpf (method B)

Exp.	n	Mort.	A.d	%Mort.	% A.d
Control					
1	50	0	0	0.00	0.00
2	50	0	0	0.00	0.00
3	50	0	0	0.00	0.00
4	100	0	0	0.00	0.00
5	150	0	0	0.00	0.00
6	150	0	0	0.00	0.00
			Mean	**0.00**	**0.00**
			STD	**0.00**	**0.00**
0.1 mg/mL 161					
1	50	0	0	0.00	0.00
2	50	0	0	0.00	0.00
			Mean	**0.00**	**0.00**
			STD	**0.00**	**0.00**
0.11 mg/mL 161					
1	50	2	0	4.00	0.00
2	50	2	0	4.00	0.00
			Mean	**4.00**	**0.00**
			STD	**0.00**	**0.00**
0.12 mg/mL 161					
1	50	1	0	2.00	0.00
2	50	0	0	0.00	0.00
			Mean	**1.00**	**0.00**
			STD	**1.41**	**0.00**
0.125 mg/mL 161					
1	50	5	0	10.00	0.00
2	50	0	0	0.00	0.00
			Mean	**5.00**	**0.00**
			STD	**7.07**	**0.00**

Table A1-5 continued

Exp.	n	Mort.	A.d	%Mort.	% A.d
0.13 mg/mL 161					
1	50	3	0	6.00	0.00
2	50	3	0	6.00	0.00
			Mean	**6.00**	**0.00**
			STD	**0.00**	**0.00**
0.135 mg/mL 161					
1	50	6	0	12.00	0.00
2	50	4	0	8.00	0.00
3	50	9	0	18.00	0.00
4	100	1	0	1.00	0.00
			Mean	**9.75**	**0.00**
			STD	**7.14**	**0.00**
0.14 mg/mL 161					
1	50	6	0	12.00	0.00
2	50	7	0	14.00	0.00
			Mean	**13.00**	**0.00**
			STD	**1.41**	**0.00**
0.15 mg/mL 161					
1	50	19	0	38.00	0.00
2	50	10	0	20.00	0.00
3	50	11	0	22.00	0.00
4	100	7	0	7.00	0.00
5	150	14	0	9.33	0.00
6	150	33	0	22.00	0.00
			Mean	**19.72**	**0.00**
			STD	**11.09**	**0.00**
0.175 mg/mL 161					
1	50	30	0	60.00	0.00
2	100	32	0	32.00	0.00
3	150	9	0	6.00	0.00
4	150	20	0	13.33	0.00
			Mean	**27.83**	**0.00**
			STD	**24.08**	**0.00**
0.19 mg/mL 161					
1	50	31	0	62.00	0.00
0.20 mg/mL 161					
1	50	24	0	48.00	0.00
2	100	51	0	51.00	0.00
3	150	58	0	38.67	0.00
4	150	88	0	58.67	0.00
			Mean	**49.10**	**0.00**
			STD	**8.27**	**0.00**
0.22 mg/mL 161					
1	150	84	0	56.00	0.00
2	100	55	0	55.00	0.00
			Mean	**55.50**	**0.00**
			STD	**0.71**	**0.00**

Table A1-5 continued

Exp.	n	Mort.	A.d	%Mort.	% A.d
0.25 mg/mL 161					
1	150	135	0	90.00	0.00
2	100	89	0	89.00	0.00
			Mean	**89.59**	**0.00**
			STD	**0.71**	**0.00**
0.3 mg/mL 161					
1	100	100	0	100.00	-
0.4 mg/mL 161					
1	100	100	0	100.00	-
0.5 mg/mL 161					
1	100	100	0	100.00	-

Only results obtained from zebrafish kept at the zebrafish facility is shown.
Mort.: Mortality
A.d: Abnormal development
% A.d: percent abnormally developed embryos of survivors
STD: Estimated standard deviation

APPENDIX 2: RESULTS FROM ACRIDINE ORANGE STAINING

A2.1 Dexamethasone

Table A2-1: The number of control embryos and embryos treated with dexamethasone showing apoptotic activity in the epiphysis at 24 hpf

µg/mL dex	Total n	Epiphysis	p-value
0	10	3	
0.68	10	7	n.s
6.8	10	10	< 0.01

Probability values given are for Fisher exact test
n.s: Difference between treated group and controls is not significant ($\alpha = 0.05$)

Table A2-2: The number of embryos treated with dex showing altered apoptotic activity in the specified tissues compared to controls at 48 hpf

µg/mL dex	Total n	Eyes	p-value	$\downarrow o.p_{control}$	p-value	u.t	p-value
0	10	5		-		10	
0.68	10	10	< 0.05	8	< 0.01	0	< 0.01
6.8	10	10	< 0.05	10	< 0.01	0	< 0.01

For apoptotic activity in the olfactory placodes, treated embryos have been compared to the control embryo showing the lowest level of apoptosis in the given area. The numbers listed are the number of treated embryos showing less apoptotic activity than this control embryo in the particular tissue.
Probability values given are for Fisher exact test
n.s: Difference between treated group and controls is not significant ($\alpha = 0.05$)
o.p: olfactory placodes, u.t: urogenital tract

Table A2-3: The number of embryos treated with dexamethasone showing altered apoptotic activity in the specified tissues compared to controls at 72 hpf

µg/mL dex	Total n	$\downarrow o.p_{controls}$	p-value	u.t	p-value
0	10	-		10	
0.68	10	7	< 0.01	4	< 0.01
6.8	10	10	< 0.01	0	< 0.01

For apoptotic activity in the olfactory placodes, treated embryos have been compared to the control embryo showing the lowest level of apoptosis in the given area. The numbers listed are the number of treated embryos showing less apoptotic activity than this control embryo in the particular tissue.
Probability values given are for Fisher exact test
n.s: Difference between treated group and controls is not significant ($\alpha = 0.05$)
o.p: olfactory placodes, u.t: urogenital tract

A2.2 $\beta^{2,2}$-amino acid derivative 161

Table A2-4: Number of embryos showing altered apoptotic activity in the specified tissues compared to controls, after treatment with derivative 161 from 3.3 to 24 hpf (method A)

mg/mL 161	Total n	Epiphysis	p-value	↑o.p$_{controls}$	p-value	↑u.t$_{controls}$	p-value
0	10	2		-		-	
0.075	10	5	n.s	7	< 0.01	7	< 0.01
0.1	10	8	< 0.05	10	< 0.01	7	< 0.01

For apoptotic activity in the olfactory placodes and in the urogenital tract, treated embryos have been compared to the control embryo showing the highest level of apoptosis in the given area. The numbers listed are the number of treated embryos showing greater apoptotic activity than this control embryo in the particular tissue. Probability values given are for Fisher exact test

n.s: Difference between treated group and controls is not significant ($\alpha = 0.05$)

o.p: olfactory placodes, u.t: urogenital tract

Table A2-5: Number of embryos showing increased apoptotic activity in the olfactory placodes and urogenital tract compared to controls, after treatment with derivative 161 from 24 to 48 hpf (method B)

mg/mL 161	Total n	↑o.p$_{controls}$	p-value	↑u.t$_{controls}$	p-value
0	10	-		-	
0.15	10	6	< 0.05	7	< 0.01
0.2	10	10	< 0.01	9	< 0.01

For apoptotic activity in the olfactory placodes and in the urogenital tract, treated embryos have been compared to the control embryo showing the highest level of apoptosis in the given area. The numbers listed are the number of treated embryos showing greater apoptotic activity than this control embryo in the particular tissue. Probability values given are for Fisher exact test

n.s: Difference between treated group and controls is not significant ($\alpha = 0.05$)

o.p: olfactory placodes, u.t: urogenital tract