Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
Department of Veterinary Sciences

Advancing the Zebrafish Embryo Developmental Toxicity Assay (ZEDTA) towards a sensitive screening assay

PhD thesis submitted for the degree of Doctor in Veterinary Sciences at the University of Antwerp to be defended by

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Table of Contents

List of Abbreviations

9ACA	9-acridine carboxaldehyde
aa	anguloarticular
AA	ascorbic acid
AB	alcian blue
ACN	acetonitrile
ADH	alcohol dehydrogenase
ADME	absorption, distribution, metabolism and excretion
AED	anti-epileptic drugs
AI	acridine
ALDH	aldehyde dehydrogenase
AO	acridone
AP	anteroposterior
AR	alizarin red
AR-AB	alizarin red-alcian blue
ASPA	Animals Scientific Procedures Act
AUC	area under curve
aud	auditory capsule
BA	blood accumulation
bb	basibranchial
BC	blood circulation
bh	basihyal
BOMR	benzyloxy-methyl-resorufin
BP	body parts
brs	branchiostegal rays
brs2	branchiostegal rays 2
c	cleithrum
C5	compound 5
C9	compound 9
CA	compound A
$Ca(NO_3)_2$	calcium nitrate
$CaCl_2$	calcium chloride
cb	ceratobranchial
cb5	ceratobranchial 5
CBZ	carbamazepine
ch	ceratohyal
chb	ceratohyal bone
C_{max}	maximal maternal plasma concentration
cot	circle saccular otolith

CYP	cytochrome P450
DAD	diode array detector
DART	developmental and reproductive toxicology
DEL	deep cell layer
den	dentary
DMSO	dimethyl sulfoxide
dpf	days post-fertilization
DV	dorsoventral
EB	embryoid bodies
EC50	50% effective concentration
ECA	etiracetam carboxylic acid
E-CBZ	carbamazepine-10,11-epoxide
ECVAM	European Centre for the Validation of Alternative Methods
EFD	embryofetal development
EGFP	enhanced green fluorescent protein
EM	embryo medium
en	entopterygoid
EROD	7-ethoxyresorufin
ES	embryonic stem cells
ESI	electrospray ionization
EST	Embryonic Stem Cell Test
eth	ethmoid plate
EVL	enveloping layer
FETAX	frog embryo teratogenesis assay: Xenopus
FMO	flavin-containing monooxygenase
GFP	green fluorescent protein
H2O2	hydrogen peroxide
hb	hypobranchial
HCl	Hydrochloric acid
HCT	hydrochlorothiazide
HDTA	hydra developmental toxicity assay
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hmb	hyomandibula
hpf	hours post-fertilization
HPLC	high-performance liquid chromatography
hsp	heat shock protein
HSS	High Strength Silica
hys	hyosymplectic
IC50	50% inhibitory concentration
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ID50	concentration that inhibited differentiation by 50%
ih	interhyal

IM	iminostilbene
KCl	potassium chloride
KOH	potassium hydroxide
L	left
LC	liquid chromatography
LC25	25% lethal concentration
LC50	50% lethal concentration
LC-MS	liquid chromatography–mass spectrometry
LC–MS/MS	liquid chromatography tandem mass spectrometry
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
LTC	levetiracetam
MAS	metabolic activation system
max	maxilla
MC	medium control
MCIG	minimum concentration to inhibit growth
mEST	mouse Embryonic Stem Cell Test
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
mk	Meckel's cartilage
MM	limb bud micromass
MOC	multi-organ-chip
MPO	myeloperoxidase
MPX	myeoloid-specific peroxidase
MS-222	methyl ethane sulfonate / tricaine methane sulfonate
mZEDTA	metabolic zebrafish embryo developmental toxicity assay
n	notochord
NA	not applicable
NaCl	natrium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	monosodium phosphate
$NaHCO_3$	sodium bicarbonate
NAM	new approach methodology
NaOH	sodium hydroxide
NHP	non-human primate
NOAEL	no observed adverse effect level
oa	occipital arch
OECD	Organization for Economic Cooperation and Development
OOC	organ-on-chip
op	opercle
OP	organophosphorous
pc	parachordal cartilage

pec fin	pectoral fin
PPAR	peroxisome proliferator–activated receptor
ps	parasphenoid
QTOF	quadrupole time of flight
qu	palatoquadrate
R	right
R1	replicate 1
R2	replicate 2
R^2	determination coefficients
RFP	red fluorescent protein
RO	reverse osmosis
RR	relative risk
Rt	retention time
rWEC	rat Whole Embryo Culture
SC	solvent control
SL	standard length
sZEDTA	skeletal zebrafish embryo developmental toxicity assay
t	pharyngeal teeth
tci	trabecula cranii
tco	trabecula communis
TI	teratogenic index
TP	transformation product
TQ	triple quadrupole
TQD	triple quadrupole detector
TRIS	tris(hydroxymethyl)aminomethane
UC'ed	ultracentrifuged
UGT	uridine 5'-diphosphoglucuronosyltransferase
uot	utricular otolith
UPLC	ultra high performance liquid chromatography
vb	vertebrae
WEC	Whole Embryo Culture
YSL	yolk syncytial layer
ZEDTA	zebrafish embryo developmental toxicity assay
ZeDTA	Zebrafish Developmental Toxicology Assay
zFET	(zebra)fish embryo acute toxicity test

CHAPTER 1:
General introduction

1.1 Developmental toxicity

1.1.1 Birth defects caused by chemicals and pharmaceuticals

In approximately 3% of all births major developmental defects are observed [1–3]. These are defined as defects that are life-threatening, require major surgery, or involve a serious disability. Examples include structural abnormalities (e.g. neural tube and heart defects), functional deficits (e.g. mental retardation), growth retardation (e.g. low birth weight) and pre- and postnatal death [1]. Birth defects can be caused by several factors, such as: genetic abnormalities, infectious diseases (e.g. Zika, rubella), maternal nutritional deficiencies (e.g. folate), radiation, pollutants and other environmental factors (e.g. drugs and chemicals). Although it is difficult to determine their exact cause [4], approximately 5 to 10% of congenital defects in newborns are known to be due to the teratogenic effects of chemicals and pharmaceuticals [2]. Teratogens are defined as '*agents that alter the growth or structure of a developing embryo or fetus, thereby causing birth defects*' [5]. During pregnancy, a fetus can be exposed to chemicals and pharmaceuticals taken by the mother via placental transport, or the fetus can experience an indirect adverse effect when the mother is exposed to teratogenic substances [2]. Even very short exposures to teratogens can already be long-lasting, profound and sometimes even be transgenerational [3]. To protect developing embryos and fetuses against teratogenicity (i.e., nowadays called 'developmental toxicity' [6]) caused by chemicals and pharmaceuticals, many efforts have been made to gain knowledge about these agents, and subsequently, to protect the developing embryos and fetuses against them [1].

1.1.2 History of developmental toxicity testing

Nowadays, pharmaceutical and chemical companies have to assess the potential developmental toxicity of their drug candidates in a rodent and non-rodent *in vivo* study before it can be taken by women of child bearing potential [7,8]. Before the 1960s, however, drug testing was very different from how it is done today [9]. The need for such specific guidelines for developmental toxicity testing of drugs was defined by the thalidomide tragedy in the late 1950s and early 1960s, as it greatly increased the concern of the occurrence of human malformations caused by chemicals and drugs [10,11].

Thalidomide, also known as Softenon, is a sedative and antinausea drug that was brought to the marked in West Germany in 1957 [10]. As it was found to be very effective in treating morning sickness and nausea, it was taken by thousands of pregnant woman [11,12]. Unfortunately, quickly after it was marketed, an increase in babies that were born with severe malformations such as phocomelia (i.e., malformations of the arms or/and legs) (see Figure 1), heart defects and craniofacial malformations was observed [12]. Due to the fact that 'teratogenicity' was not well known, it took until 1961 to discover that these birth defects were linked to the use of thalidomide [9,12]. It was estimated that by then more than 10,000 babies were affected. Moreover, also an increased number of miscarriages was reported [9]. Due to this tragedy, and in order to prevent

similar tragedies from happening in the future, the way that drugs were tested completely changed [9,11]. Up until then, only one test species, often a rodent, such as rats or mice, was used to assess the teratogenic potential of drugs. As the thalidomide tragedy revealed that rodents were less sensitive to the drug and testing in non-rodents (such as the rabbit and the nonhuman primate) did reveal its teratogenicity, it became mandatory to test a drug candidate *in vivo* in a rodent and a non-rodent species [9,12,13].

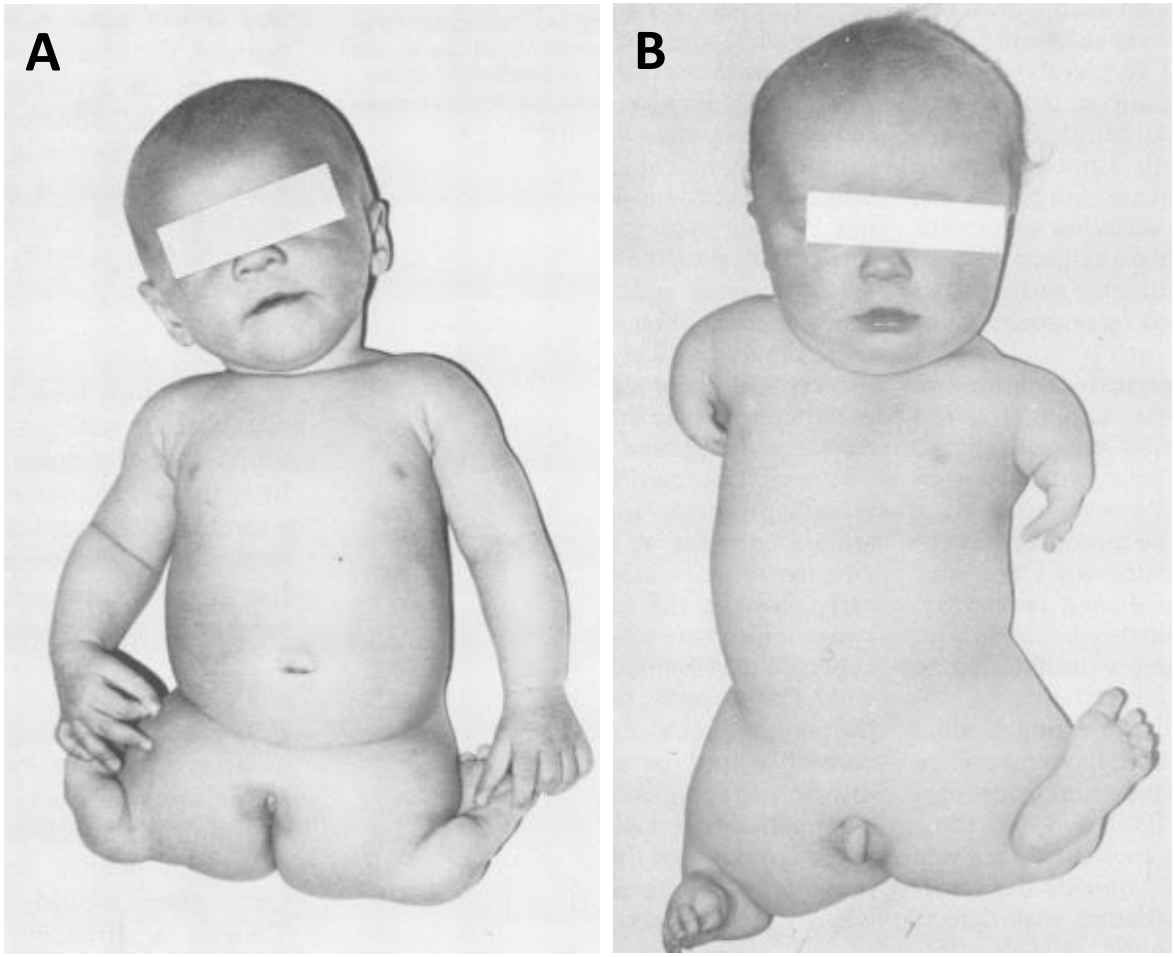

Figure 1. Babies with phocomelia. A) shows a baby with reduction deformities of both legs, and B) shows a baby with reduction deformities of all four limbs [14].

1.1.3 *In vivo* developmental toxicity testing in rodents and non-rodents

An evaluation of developmental toxicity of drugs and chemicals in laboratory animals allows researchers to gain more insights into the potential negative impact of these agents on developing human embryos and fetuses [10,15]. In traditional embryofetal developmental (EFD) toxicity studies, mostly rats are used as rodent species and rabbits as non-rodent species [16]. In these studies, the test compound of interest is administered daily to the pregnant mammals during the period of organogenesis, which is gestation day 6/7-17 for rats and 6/7-19 for rabbits [17]. In this way, effects on the entire period of organogenesis (i.e., from implantation to closure of the hard

palate [10]), can be assessed [16,18]. Usually, the administration is done orally via gavage at approximately the same time each day, however, other routes can be used too if justified [16]. One day before the expected parturition, the dam is euthanized and submitted to a cesarean section [17] and the fetuses are assessed for external, skeletal and visceral (i.e., soft tissues) abnormalities [10,16,18,19]. External examination will be performed on all fetuses. At least half of the total number of fetuses needs to be assessed for skeletal malformations, while the other half needs to be assessed for visceral malformations [10,11,16].

For many years, the traditional *in vivo* developmental toxicity studies were considered as the gold standard [18]. After all, using a rodent and non-rodent species proved to be very effective to predict human developmental toxicity. Indeed, after these guidelines were applied, no pharmaceutical was brought to the market without previous signs of developmental toxicity in at least one of the two species [8]. Despite the undeniable success and value of *in vivo* studies, they also have some limitations and disadvantages that should be taken into account. In contrast to what is needed for rapid testing of drugs, *in vivo* studies are costly, time-consuming, laborious and require a lot of test compound. Moreover, they raise ethical concerns as many animal lives are sacrificed during these studies [18,20]. It was estimated that approximately 1500 testing animals are required for assessing developmental toxicity of only one chemical. Therefore, during the last decades, many efforts have been made to reduce the amount of laboratory animals, to create more awareness and to improve animal welfare. A well-known example that tried to improve the quality of life of laboratory animals and reduce the number of animals used in scientific research, is the principle of the 'Three Rs' (i.e., Replacement, Reduction and Refinement) that was introduced in 1959 by Russel and Burch [21,22]. In 2010, an updated *Directive 2010/63/EU on the protection of animals used for scientific purposes* was implemented. The directive aimed to provide help on implementing the principle of the 3Rs into animal experiments, as well as providing updates to further improve the welfare and protection of laboratory animals by encouraging the use of alternative methods, but also updating the minimum housing standards, ethical review and harm-benefit analysis [23,24]. Also at this moment, the use and development of alternatives to animal testing for developmental toxicity is still a hot topic and receiving a lot of attention. Proof of this is the very recently approved third revision of the ICH S5 guideline on detection of toxicity to reproduction for human pharmaceuticals, as it opens opportunities for the use of alternative methods to defer or replace conventional *in vivo* studies in certain circumstances [17].

1.1.4 Alternative methods for developmental toxicity testing

Over the years, alternative methods for toxicity assessment of drugs and chemicals became very important, as they can replace or at least reduce some of the traditional *in vivo* studies in animals. In addition to that, they have many more advantages such as: being simple, cost-effective, require a small amount of test compound, and being more high-throughput than *in vivo* studies [18]. In the first place, alternative methods can be used in drug discovery or early drug development as screening assays. By using screening assays, the compounds that already appear to be teratogenic in alternatives can be filtered out early on and need no further testing in animals, unless a false positive result is suspected. In this way, not only several animal lives, but also a significant amount

of money and time will be saved during drug development, as only the drug candidates that were considered to be non-teratogenic will be subjected to *in vivo* mammalian testing. In the long term, alternative assays may even have the potential to replace the regulatory *in vivo* mammalian developmental toxicity studies, most probably when used together with other alternatives in a tiered or testing battery approach [17]. In the next sections, several alternative methods to developmental toxicity testing in mammals will be discussed.

1.1.4.1 *In vitro* models

In vitro (Latin for 'in glass') models rely on the use of components that were isolated out of an organism, instead of the use of the living organism itself. For developmental toxicity testing, there are three types of *in vitro* models available: cell cultures, organ cultures, and whole embryo cultures [22,25]. Cell cultures that use continuous immortalized cell lines have the advantage that they are the most easy to perform and that no (or very few) animal material is used. Unfortunately, only effects on single mechanisms, such as cell adhesion, can be studied. Cell cultures that use primary cells, on the other hand, retain *in vivo* characteristics and are therefore better in mimicking the in *vivo* state of cells than immortalized cell lines. However, they require more animal material and have a limited lifespan. Organ cultures or organoids are more complex than cell cultures, and can more closely represent the complexity of pattern formation. They are, however, more laborious and require more animal material. Of these three types, the whole embryo culture is considered to be the most complex and most complete alternative, since it incorporates several developmental endpoints and mechanisms. Unfortunately, it also requires the most animals, is the most laborious and still covers only a part of the organogenesis period as embryos can only be cultured for a limited time (e.g. 48 hours, see further) [22,26].

Up until now, only three *in vitro* alternative models for developmental toxicity testing have been validated by the European Centre for the Validation of Alternative Methods (ECVAM): the embryonic stem cell test (EST), the limb bud micromass (MM), and the whole embryo culture (WEC) [27].

The EST uses mouse embryonic stem cells (ES cells) from the D3 cell line [28]. ES cells are pluripotent and have the ability of self-renewal [29,30]. They are isolated from the inner cell mass of blastocysts from mice and are cultured *in vitro*. In this cultivated system, the ES cells form multicellular aggregates, also called embryoid bodies (EB). These EB are able to differentiate into cells from each of the germ layers (i.e., ectoderm, endoderm and mesoderm) [28]. In this assay, three endpoints are assessed after 10 days of exposure: 1) the inhibition of differentiation into beating cardiomyocytes, 2) the cytotoxic effects on differentiating ES cells, and 3) the cytotoxic effects on differentiated 3T3 fibroblasts [29,31]. A such, it can be used to investigate potential cytotoxic, mutagenic, embryotoxic, and teratogenic effects of test compounds [18]. An accuracy of 78% for the EST was obtained in the ECVAM validation study [27]. In later studies, however, much lower accuracies were obtained [29]. The advantage of the EST is that no live animals are needed, since only commercially available cell lines are used. However, the assay lacks the complexity and pattern formation of a whole organism, and is therefore too simple to mimic the *in vivo* situation [29,30].

In the MM test, limb bud cells from a rat, mouse or chicken embryo are dissected and seeded in culture medium as high-density spots, also called 'micromass'. In this culture medium, different concentrations of test compounds are present. The differentiation and growth of the limb bud cells is evaluated after 5 days of cultivation. In normal circumstances, these high density spots of limb bud cells will differentiate into chondrocytes. Exposure to teratogenic compounds, however, can lead to inhibitory effects on differentiation into chondrocytes and to cytotoxic effects [27,32]. Therefore, two endpoints are assessed in the MM test: the concentration that inhibited differentiation by 50% (ID50), and the concentration that reduced growth by 50% (IC50) [32]. An advantage of the MM test is that only a small amount of animal material is needed to prepare for a large numbers of test cultures. Unfortunately, also this assay lacks the complexity of a whole organism [18,27,32]. In addition, the accuracy of the MM is estimated to be around 70%, and is therefore the least accurate of all three validated alternatives [27].

The last validated test, the WEC, is rather *ex vivo* than *in vitro*, because of the use of whole embryos. In this test, explanted post-implantation embryos of rats or mice are cultured for 24 to 48 hours in medium with serum, and exposed to a test compound. Cultivation is carried out in a roller bottle system, which allows a constant movement of the bottles, which facilitates oxygenation of the culture medium. In the WEC, three endpoints are assessed: mortality, malformation and growth inhibition [18,29,33]. Mortality is determined by the presence of a heartbeat and yolk sac circulation, malformations are determined through a morphological scoring that evaluates 17 different parameters, and growth is measured by the crown-rump length or by protein content [18,29]. Of the three validated alternatives, the WEC is considered to mimic the *in vivo* situation the most, as it allows the examination of a whole embryo [22]. Its disadvantages, however, are the need for animal material (i.e., pregnant dams), the complexity of isolating and explanting the embryos, and the fact that only 48 hours of the entire organogenesis period is represented [22,29,33]. The accuracy of the WEC for pharmaceutical agents is estimated to be around 80% [26,27,34,35].

1.1.4.2 *In vivo* nonmammalian models

Besides *in vitro* models, also *in vivo* nonmammalian models can be used in developmental toxicity studies [25]. Invertebrate species, such as the fruit fly (*Drosophila melanogaster*) [36], nematode (*Caenorhabditis elegans*) [37] and fresh-water polyp (*Hydra attenuate*) [38,39], as well as vertebrate species, such as frogs (*Xenopus laevis*) [40,41] and zebrafish (*Danio Rerio*) [42,43] can be used. In contrast to toxicity assays using *in vitro* models, the use of *in vivo* nonmammalian models provides data from whole organisms [37].

Alternative tests using invertebrates were considered to be the first alternatives to the traditional *in vivo* tests in mammals. *Drosophila* embryo cell cultures, for example, have been used as a teratogen screening method by determining the teratogenic effect of chemicals on neuron and muscle differentiation, stress proteins, and neurotransmitter levels [36]. Another invertebrate species, *C. elegans,* has been used in ranking studies to assess viability, larval growth, and reproductive output for developmental toxicants [37]. A third example of a vertebrate species used as an alternative, is the *Hydra attenuate*. This is a fresh-water polyp that has been used in the hydra developmental toxicity assay (HDTA) to detect teratogenic substances. The HDTA is based upon the

difference in toxicity between the adult hydra and a regenerating stage (i.e., adult/developmental ratio) [38,39]. Although being an animal as well, the use of invertebrates raises less ethical concerns than the use of higher animals, such as (lower) vertebrates or mammals. Moreover, they have a rapid reproduction rate, are inexpensive and are easy to maintain [44]. However, due to differences in metabolism, physiology and anatomy, as well as problems with extrapolation of data from invertebrates to vertebrates, they cannot be used to replace studies in vertebrates [36].

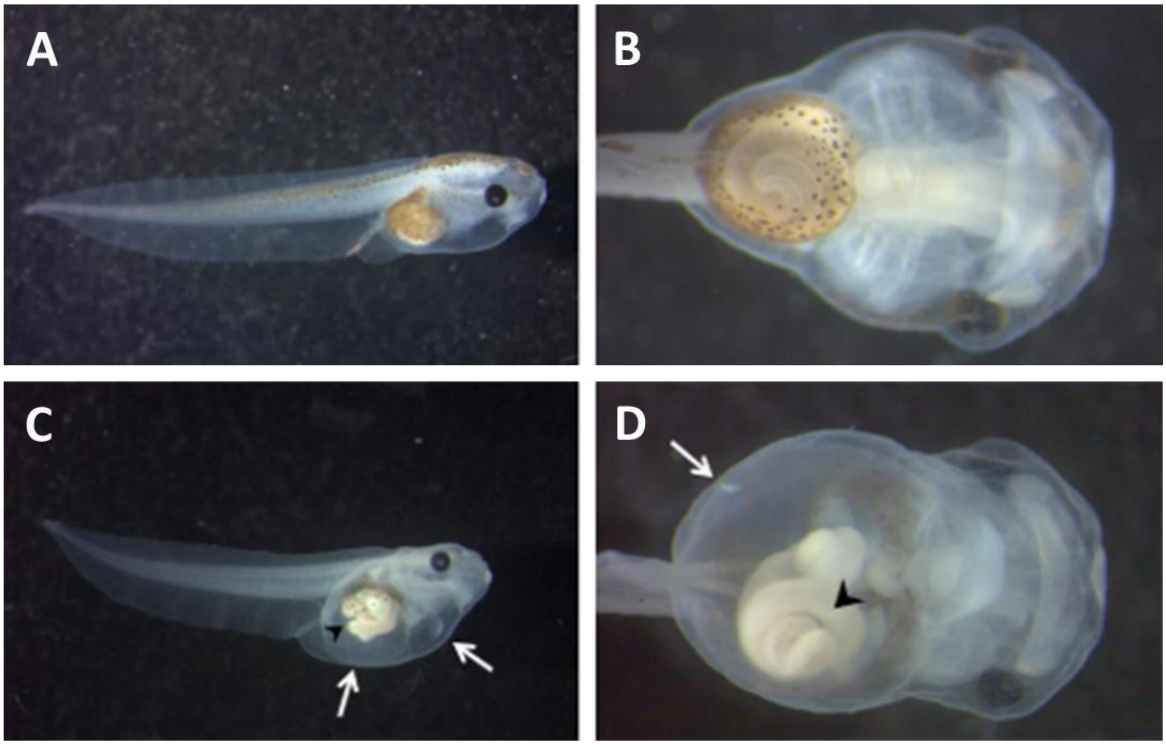

Figure 2. *Xenopus laevis* larvae at the end of the FETAX. A control larva in A) lateral and B) ventral position, and a to 50 mg/L bZnO exposed larva in C) lateral and D) ventral position. The exposed larva (C, D) shows the following malformations: abnormal gut coiling (arrow head), abdominal and cardiac edemas (arrow) and a slight dorsal tail flexure (adapted from [47]).

The Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) is an alternative test that uses embryos of *Xenopus laevis*, a vertebrate species, to evaluate the teratogenicity of test compounds (see Figure 2) [41,45]. In this assay, mid-blastula-stage embryos from the South African clawed frog are exposed to different concentrations of potential teratogenic compounds for 96 hours [46]. Subsequently, three endpoints are assessed: mortality, malformation and growth inhibition. The 50% lethal concentration (LC50) and the effective concentration for malformations (EC50) are calculated. Also the teratogenic index (TI) is calculated (i.e., LC50/EC50). The minimum concentration to inhibit growth (MCIG) is calculated by comparing the head to tail length of treated and control embryos [45]. In contrast to the three validated *in vitro* alternatives, this assay allows the assessment of a whole vertebrate animal during the main organogenesis period. Other advantages include the high fecundity, the short organogenesis period and the need for only small amounts of test compound [46]. Moreover, due to the fact that frogs are nonmammalian vertebrates, the FETAX is not considered as an animal model until the stage of independent feeding is reached [24]. One of the

disadvantages is the lack of maternal metabolism due to the external development of the embryos [41]. Moreover, an expert panel that evaluated the use of the FETAX for developmental toxicity testing concluded that the assay is not sufficiently optimized and validated, and needs further standardization to reduce variability, as well as an increase in the number of endpoints for evaluation [45]. Moreover, a relatively low accuracy of ~60% was obtained when comparing the FETAX to *in vivo* studies in mammals [45].

The Zebrafish Embryo Developmental Toxicity Assay (ZEDTA) is another alternative that uses a vertebrate species as a model. In this assay, zebrafish embryos are exposed to xenobiotics during the main organogenesis period (i.e., from 5.25 until 96-120 hours) to evaluate their potential teratogenicity (see Figure 3) [42,43]. This assay is based on the, in ecotoxicology used, validated (zebra)Fish Embryo Acute Toxicity test (zFET), in which the acute toxicity of chemicals is tested in zebrafish embryos [21,48]. In contrast to the zFET where only four indicators of lethality are assessed [21,48], both lethality and morphology are assessed in the ZEDTA [42,43]. An overview of the endpoints that are evaluated in the ZEDTA is shown in chapter 3.

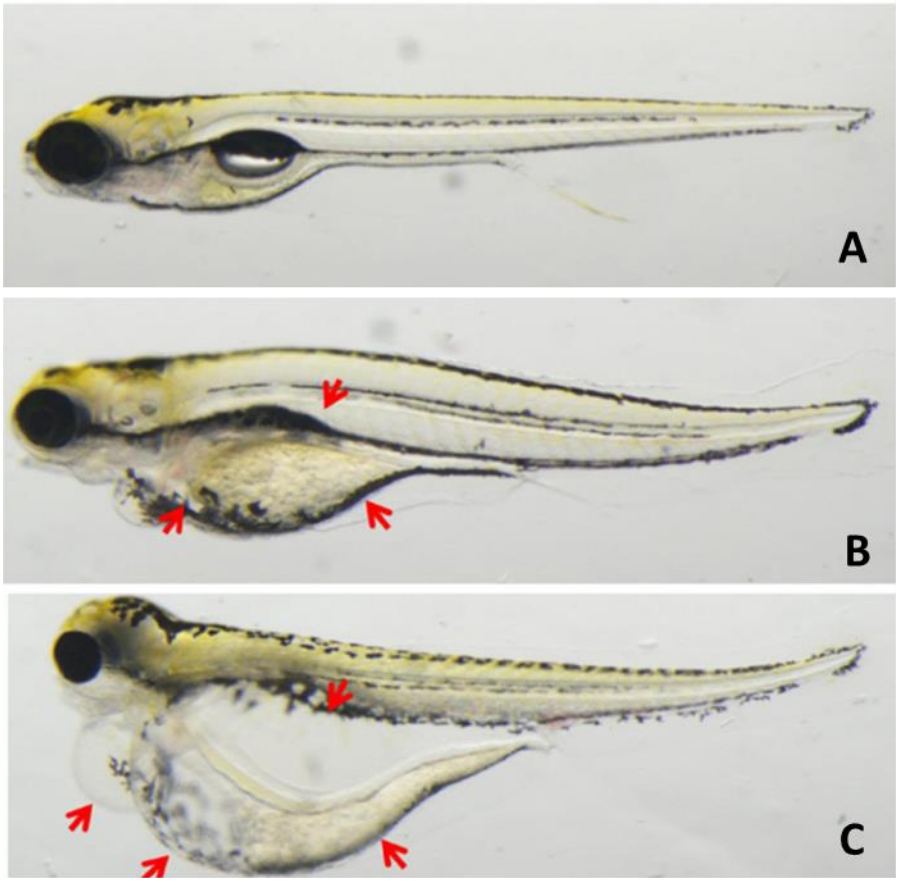

Figure 3. 5 days post-fertilization (dpf) zebrafish larvae at the end of the ZEDTA. A normal control larva is depicted in A. B and C show larvae with several edemas due to exposure to 0.1 µM of triptolide (B) and 100 µM of aconitine (C) (adapted from [54]).

The ZEDTA is considered to be a very promising alternative to evaluate the teratogenicity of xenobiotics, because of its many advantages when compared to other alternatives and to *in vivo* studies in mammals. The short generation time of 2-3 months, high fecundity and short organogenesis period of zebrafish makes their use less time-consuming than the traditional studies in mammals [49,50]. Moreover, zebrafish are small, easy to maintain and do not require high demands for infrastructure or food, which makes the maintenance very cheap in comparison to other laboratory animals [50]. The combination of the *ex utero* development and the optically transparency of embryos and chorions facilitates a detailed examination of early developmental processes and morphological changes during their development [49–51]. Other advantages over *in vivo* mammals studies are the need for only a small amount of the test compound [49] and the ability of high-throughput screening [49,51]. Moreover, just as frog embryos, zebrafish embryos are not considered as an animal model until the free feeding stage, which is 120 hpf (hours post-fertilization) at 28.5°C (EU Directive 2010/63) [24]. Compared with nonmammalian invertebrate models such as the fruit fly and nematode, there is a strong conservation between zebrafish and humans, which allows the study of complex biological processes [50], including processes related to embryonic development [52]. The biggest advantage of the ZEDTA when compared to *in vitro* assays such as the EST, WEC and EST, is that the morphological evaluation can be done in a whole embryo during the main period of organogenesis (see Table 1) [42]. In contrast to the FETAX, comparative studies have shown a high concordance (80-85%) between the ZEDTA and *in vivo* studies in mammals [26,53].

Despite the extensive list of advantages, the ZEDTA still suffers from some flaws and limitations. First of all, differences in study design can cause discordances in classification of identical compounds in different laboratories. For example, the teratogenic index (TI) can be calculated as LC50/EC50 or as LC25/NOAEL [2,34]. As a consequence, this can lead to a different sensitivity of the assay and, consequently in discordances in compound classification. Also the use of different or limited exposure windows may result in discordant classifications, as the exposure duration may be too short or the critical window for exerting teratogenic effects may be missed [55]. Moreover, also differences in incubation medium composition, which impacts compound uptake and distribution, and the use of different experimental parameters were observed between several laboratories [54]. So, there is a clear need for standardization and harmonization of the ZEDTA protocols.

Second, false negative and false positive results for, respectively, mammalian teratogens and non-teratogens have been reported [2,34,43,55–60]. Especially false negatives are of concern in view of human safety, as teratogenic compounds will be missed. A first cause of false negative results are issues with (or the lack of) compound uptake by zebrafish embryos [56]. This may result in insufficient exposure to the compound of interest, and consequently, to misclassification as false negative. However, by performing an uptake analysis, the uptake and internal concentration of a compound can be determined. In case no or only a small amount of compound is detected internally, a false negative result due to compound uptake is likely [56]. Also inter-species differences in mode of action are a potential cause of false negative results in zebrafish assays. Ribavirin, for example, did not cause malformations in zebrafish as they have nucleated erythrocytes and, therefore, no accumulation of ribavirin triphosphate can occur. This is in contrast

to the situation in mammals, where accumulation can occur, as they have erythrocytes without a nucleus [60]. Another cause for false negative results in the ZEDTA is the immature intrinsic cytochrome P450 (CYP)-mediated biotransformation capacity of zebrafish embryos during a large part of organogenesis. As zebrafish embryos develop externally, they cannot rely on the maternal metabolic capacity [42,61,62]. Consequently, compounds that require bioactivation to exert their teratogenic potential can be missed [7]. Also the limited number of morphological endpoints in zebrafish assays is a potential cause for false negative results. In rat and rabbit EFD studies an exhaustive list of external, visceral and skeletal malformations are assessed (see Table 1) [19]. As no skeletal staining is performed in zebrafish embryo assays and for some compounds mainly skeletal malformations were observed in EFD studies, the lack of a skeletal assessment may cause false negative results [43,56,57].

Table 1. A comparison of the ZEDTA, the three validated alternative assays, and the EFD studies. Abbreviations: embryofetal development (EFD), embryonic stem cell test (EST), gestation day (GD), metabolic activation system (MAS), limb bud micromass test (MM), non-human primate (NHP), whole embryo culture test (WEC), zebrafish embryo developmental toxicity assay (ZEDTA).

Test	Organism/cells	Endpoints	Metabolism	Exposure period
ZEDTA	Whole zebrafish embryos	Several external and visceral endpoints (see chapter 3 – 3.2 Introduction)	Exogenous MAS can be added (see chapter 3 – mZEDTA)	5 days (5.25 - 120 hpf)
EST	Pluripotent mouse embryonic stem cells from the D3 cell line (isolated from the inner cell mass of blastocysts) [28,29,31]	1) The inhibition of differentiation into beating cardiomyocytes 2) The cytotoxic effects on differentiating ES cells 3) The cytotoxic effects on differentiated 3T3 fibroblasts	Exogenous MAS can be added	10 days (from GD4 onwards)
WEC	Post-implantation whole rat or mouse embryos [18,29,33]	1) Embryonic death 2) Structural and functional abnormalities 3) Growth retardation	Exogenous MAS can be added	24-48 hours (from GD6/7 onwards)
MM	Limb bud cells from a rat, mouse of chicken [27,32]	1) Differentiation and growth of the limb bud cells into chondrocytes (ID50) 2) Growth of the limb bud cells (IC50)	Exogenous MAS can be added	5 days (for rat from GD14 onwards)
EFD	Rodent and non-rodent fetuses (and the dam to a limited extent) [17,19]	- Antemortem endpoints in dam (clinical observations/mortality, body weight, food consumption) [17] - Postmortem endpoints in dam (macroscopic evaluation, corpora lutea, implant sites, live and dead conceptuses, resorptions, gross evaluation of placenta) [17] - Postmortem endpoints in fetuses (body weight, sex, and an extensive list of external, visceral and skeletal evaluations [19]) [17]	Maternal metabolism will generate metabolites	Depends on animal species [17]: - Rat GD6/7-17* - Mouse GD6/7-15* - Rabbit GD6/7-19* - NHP ~GD20-50** *implantation to closure of hard palate **confirmation of pregnancy to end of major organogenesis

Despite its shortcomings, it is clear that the ZEDTA has many advantages when compared to other alternatives and has great potential as an alternative method for developmental toxicity testing.

Hence, in this thesis efforts are made to further standardize and optimize the ZEDTA. For the optimization part, we will focus on tackling the limited number of skeletal endpoints and the immature biotransformation capacity of zebrafish. Therefore, knowledge on the embryonic development and metabolism of the zebrafish is pivotal and will be discussed in the next sections.

1.2 Zebrafish reproduction and embryonic development

1.2.1 Zebrafish reproduction and breeding in laboratory settings

Zebrafish are oviparous organisms that are sexually mature at approximately 3 months. As the average sperm cell count of males peak around the age of 10 months, and from the age of 1.5 years onwards gametes will start to deteriorate, optimal breeding age is from 6 months to 1 year [63].

Ovulation in female zebrafish, which typically takes place overnight, is stimulated by male gonad pheromones. After ovulation, the females release female gonad pheromones to attract males and stimulate them to initiate courtship. Courtship consists of three stages: the initiatory stage, the receptive/appetitive stage and the spawning stage. In the first stage, the males start to swim close to the females and make abrupt movements. In the next stage, they touch the side or tail of the females with their heads, while circling around the female. In the final stage, males and females swim next to each other to align their genital pores. By performing rapid tail movements against the side of the female, the male triggers oviposition in the female. The female is able to release more than hundred eggs in a single spawning. At the same time, the male releases its sperm to externally fertilize the eggs [63].

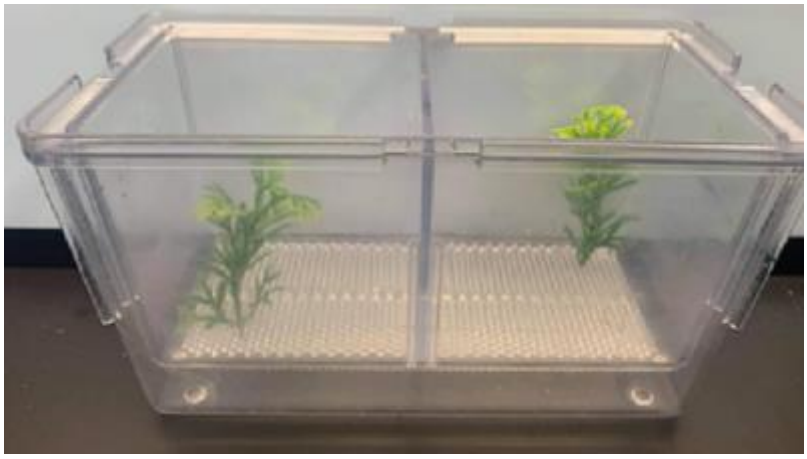

Figure 4. A zebrafish breeding tank with a double bottom. The top bottom is perforated, allowing the eggs to fall through [64].

Spawning can be affected by many factors, including the light cycle, stress levels and health status of zebrafish [63]. In contrast to the situation in nature where spawning mainly occurs during the monsoon season, spawning in laboratory settings can take place all year long [64]. However, overspawning will result in decreases in egg quantity and quality. Therefore, the optimal breeding

frequency is estimated to be around every 10 days. As zebrafish mating is photoperiodic, mating will take place within the first hours of daylight. However, if needed, zebrafish breeding can also take place later in the day by using isolation cabinets with adapted light:dark cycles [63]. In laboratory settings, the breeding will typically take place in special breeding tanks with a double bottom to prevent predation of the eggs, as adult zebrafish will try to eat their eggs in captivity. By using the special tanks, the released eggs are protected as they fall through the perforated top bottom (see Figure 4). Subsequently, the eggs can be collected from the bottom and used for experimentation [64–66].

1.2.2 Embryonic development

Once a zebrafish egg is fertilized, it will go through different periods of embryonic development. These periods are: the zygote period, the cleavage period, the blastula period, the gastrula period, the segmentation period, the pharyngula period, the hatching period, the larval period, the juvenile period, and the adult period. The timing and most important events of each period will be discussed in the next section (see 1.2.2.1), as knowledge on the development of organs is crucial for scoring the different morphological endpoints used in the ZEDTA (see chapter 3). Moreover, as discussed before (see 1.1.4.2), the lack of a skeletal assessment is a potential cause of false negative results in the ZEDTA. To include skeletal endpoints in the ZEDTA, also a thorough understanding of the embryonic development of the zebrafish skeleton is pivotal and will therefore be discussed as well (see 1.2.2.2). It is, however, important to know that the timing of each stage is dependent on the temperature to which the zebrafish embryos are exposed to [67]. In the next sections, the embryonic development at 28.5°C will be discussed.

1.2.2.1 Development of gross structures and soft tissues

The first period is the **zygote** period, which starts at 0 hpf and ends at 0.75 hpf. During this period, the cytoplasm will move towards the animal pole, resulting in segregation of cytoplasm from the blastodisc (see Figure 5) [67].

The **cleavage** period (0.75 hpf – 2.25 hpf) is characterized by 6 synchronous and rapid cleavages of the cytoplasm by means of mitosis. These divisions are meroblastic[1], and will form blastomeres. At the end of the cleavage period a 64-cell stage is reached (see Figure 5) [67].

During the **blastula** period (2.25 - 5.25 hpf) more divisions occur. When a 512-cell stage is reached, the marginal cells collapse, resulting in the release of their cytoplasm and nuclei in the cytoplasm of the yolk. This gives rise to the formation of the yolk syncytial layer (YSL), which is located between the cells of the blastodisc and the yolk. The YSL is important for staging the embryo. At 1,000-cell stage, the blastodisc will consist of two layers: the enveloping layer (EVL), which is an outer single

[1] Meroblastic cleavage: an incomplete cleavage in which sister cells are only partially separated and remain connected by cytoplasmic bridges.

cell layer that becomes epithelial, and the deep cell layer (DEL), which consists of the remaining cells. The cells of DEL will become important during the gastrulation period, as they will form the germ layers of the embryo. Next, the shape of the blastodisc changes. First, the cells of the blastodisc raise until far above the yolk, then the blastodisc gets more spherical, and at the end of the blastula period, the blastodisc is shaped as a dome. The change of the blastodisc to a dome shape indicates the start of epiboly. This is a process in which the cells of the DEL migrate outwards, resulting in blastodisc thinning and spreading. Subsequently, the blastodisc and YSL migrate over the yolk, until this last one is completely covered. However, a complete coverage will only be obtained at the end of the gastrula period. At the end of the blastula period only a 30%-epiboly[2] stage is reached (see Figure 5) [67].

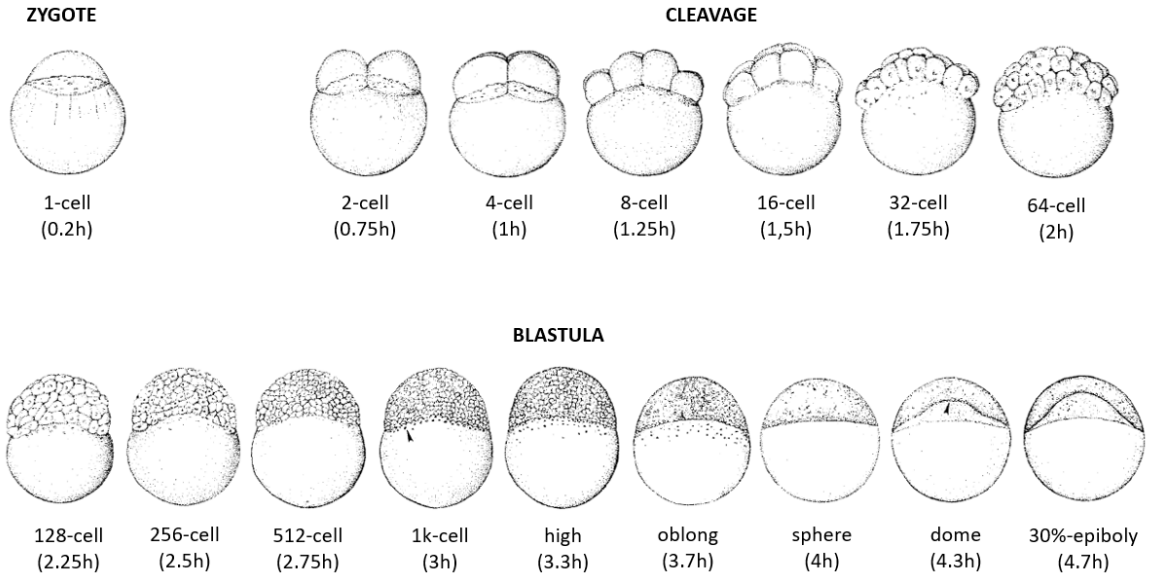

Figure 5. Stages of embryonic development of the zebrafish during the zygote, cleavage and blastula period. The chorion is not depicted here, but is still present (adapted from [67]).

Epiboly continues during the **gastrula** period (5.25-10 hpf). Moreover, the three germ layers and the embryonic axes are produced by specific cell movements: involution, convergence and extension. At 50%-epiboly, involution movements produce a germ ring, which is a thick marginal region that appears around the blastoderm. The germ ring consists of EVL and DEL (i.e., epiblast and hypoblast). Then, convergence movements produce the embryonic shield, which is a local accumulation of DEL cells at one position along the germ ring. As this embryonic shield positions at the dorsal part of the embryo, it determines the dorsoventral (DV) and anteroposterior (AP) axes. Due to the formation of the germ ring, embryonic shield, and the embryonic axes, the fate map is established and the position of the three germ layers is determined. After finishing these processes, epiboly continues. At 75%-epiboly stage, the embryonic shield becomes less pronounced as the DEL cells are spread over the AP-axis. Moreover, at this stage the hypoblast is divided into axial

[2] 30%-epiboly: 30% of the yolk is covered by blastoderm.

hypoblast and paraxial hypoblast. Axial hypoblast is located at the dorsal side and will give rise to the notochord and endoderm. Anterior paraxial hypoblast will give rise to the muscles that are responsible for the movements of the eyes, jaws, and gills, and posterior paraxial hypoblast will give rise to the somites. At 90%-epiboly stage, only a small part of the yolk is not covered with blastoderm. This part is located at the vegetal pole and is called 'yolk plug'. During this stage, also rudiments of the brain and the notochord starts to develop as the epiblast thickens at the anterior side to form the neural plate. The gastrula period ends when the 100%-epiboly stage is reached. At this moment, the tail bud develops dorsally from the yolk plug, and the neural plate is thickened along the embryonic axis. The thickening is the most prominent at the vegetal pole, which will be the future head region (see Figure 6) [67].

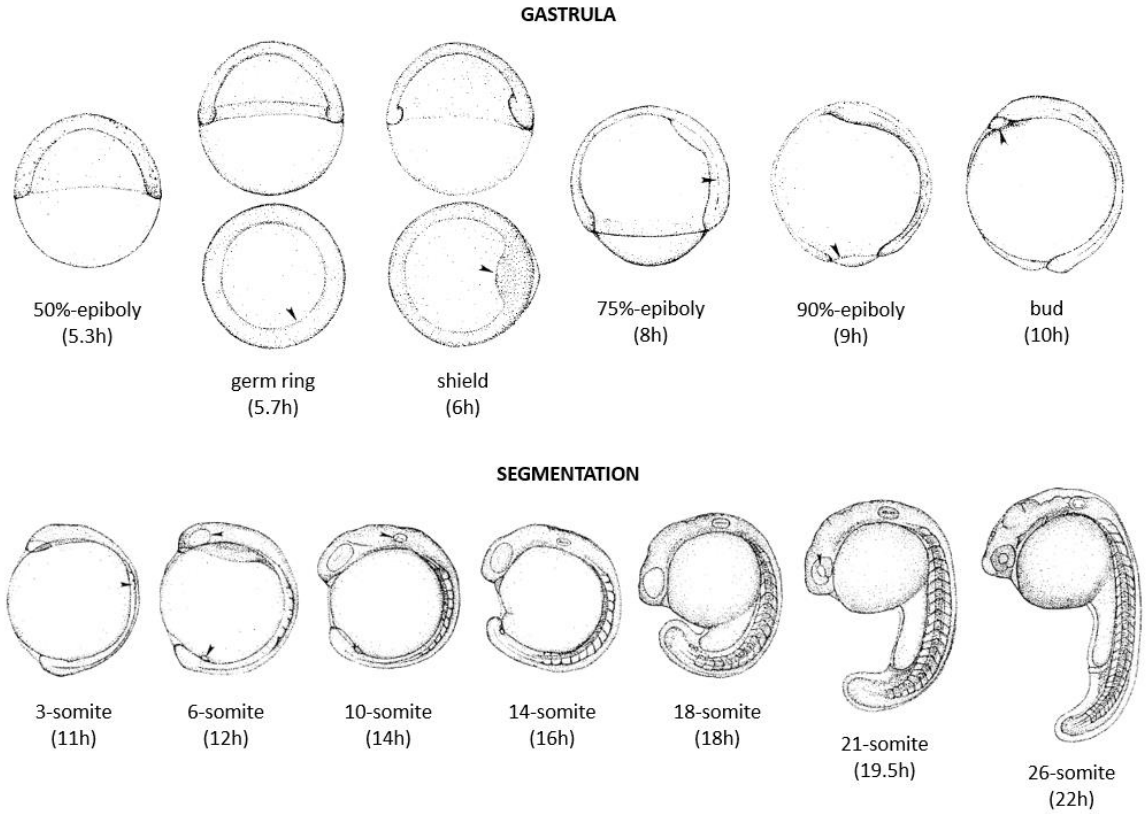

Figure 6. Stages of embryonic development of the zebrafish during the gastrula and segmentation period. The chorion is not depicted here, but is still present (adapted from [67]).

The next period is the **segmentation** period (10-24 hpf). During this period the embryo grows in the length. First, the tail grows in the direction of the head, and at the end of the segmentation period, the tail straightens and grows further in the length. Moreover, also the somites start to form. A total of 30 somites are formed, from anterior to posterior. The somites develop as myotomes or muscle segments, and sclerotomes, which gives rise to vertebral cartilage. Also an important process, the neurulation, takes place during the segmentation period. Zebrafish have a secondary neurulation, in which first a neural keel and neural rod are formed out of the neural plate. The neural tube is formed later on, at the end of the segmentation period. This in contrast to the primary neurulation that occurs in mammals, in which the neural tube is directly formed from the neural

plate by neural folds that come together. Moreover, also several structures start to develop during this period, being: the early rudiment of the pronefros, the otic placode, which becomes an otic vesicle with two otoliths (i.e., circle saccular otolith and utricular otolith), the lens placode, and the parts of the brain. The yolk changes its shape and becomes more elongated in the direction of the tail, forming the yolk extension (see Figure 6). Also the first spontaneous myotomal contractions can be observed. These movements, however, are still uncoordinated. The first coordinated movements will occur during the next period [67].

The embryo has a well-developed notochord and five brain lobes at the beginning of the **pharyngula** period (24-48 hpf). During this period, the 7 pharyngeal arches develop. The first two will form the jaws and the operculum, and the arches that are located more posterior form the gills. Also the liver, swim bladder, intestine, pectoral fins, and heart begin to form. As zebrafish have no lung circulation, the heart consist of only one atrium and one ventricle, and the gills take over the function of the lungs. From this moment onwards, a heartbeat and blood circulation in the tail can be noticed. Moreover, the distance between the lens placode and the otic vesicle will decrease, and the pigment cells (i.e., epithelium of the retina and melanophores in the skin) will start to differentiate. These melanophores form the longitudinal stripes that are typical for zebrafish. The formation occurs from anterior to posterior. Another important event, which is used to characterize the pharyngula period, is the straightening of the head of the larva (see Figure 7) [67].

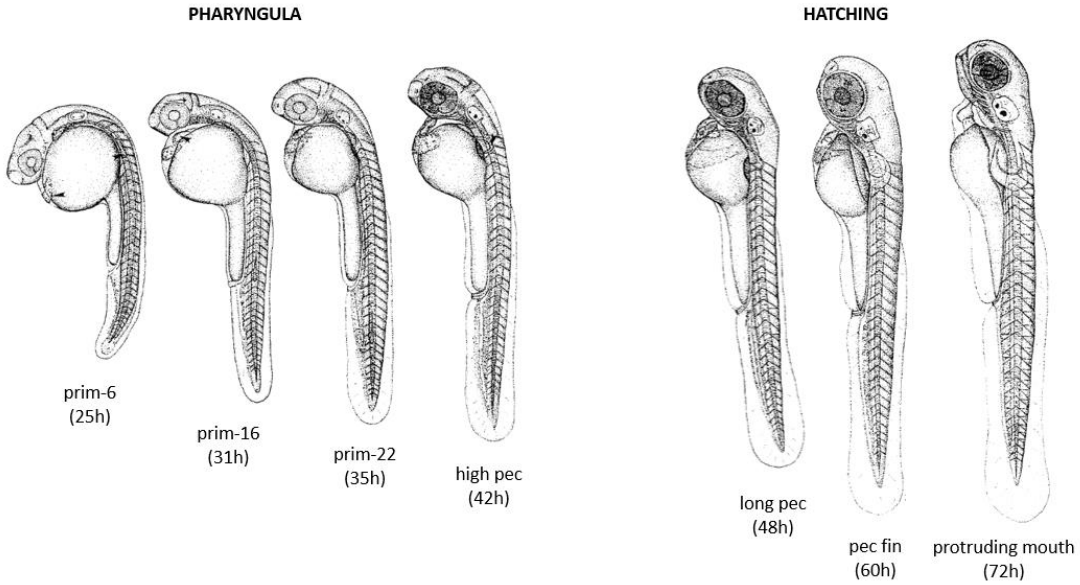

Figure 7. Stages of embryonic development of the zebrafish during the pharyngula and hatching period (adapted from [67]).

The embryo hatches out of its chorion during the **hatching** period (48-72 hpf). Hatching is facilitated by the combination of controlled tail movements and enzymes of the hatching gland that digest the chorion. The timing of hatching differs between individuals, and is very dependent on the temperature. When embryos are incubated at 26°C, instead of at 28.5°C, the hatching is delayed and takes place between 72 and 96 hpf. During the hatching period, morphogenesis of many organ

rudiments (except for the digestive system) is (almost) complete and slows down. The pectoral fins, which already started to develop during the pharyngula period, become longer (see Figure 7). From 72 hpf onwards, the embryo is called 'larva'. This is independent on whether the larva is hatched or not [67].

The **larval** period starts at 72 hpf and ends at 30 dpf [68]. From 120 hpf onwards, the zebrafish is considered as a laboratory animal [24]. At that time, the intestines are functional, the swim bladder is inflated and yolk is partly resorbed by the embryo, meaning the larval zebrafish can start to become self-feeding by preying small organisms [67,69]. During the larval period, the digestive tract develops further, opens, and becomes functional [67,70]. Also the liver and pancreas develop further, and pronephros development is completed [70–72]. At approximately 11 dpf, the mesonephros starts to develop. Its development will be completed at approximately 35 dpf (i.e., the juvenile period) [73].

The **juvenile** period (30-90 dpf) is characterized by the development of the gonads. During early life, zebrafish have undifferentiated ovary-like gonads. During the juvenile period, the ovary-like gonads differentiate into ovaries (for females) or testes (for males) [74,75]. The timing of the gonad differentiation is dependent on the strain of the zebrafish, and on environmental factors, such as stocking density, feeding conditions, and water temperature [74]. The juvenile period is the last period before the **adult** period is reached [68].

1.2.2.2 Development of the skeleton

Skeletal development is highly conserved among vertebrates [76,77]. The same skeletal cell types, such as chondroblasts, chondrocytes, osteoblasts, osteocytes, and osteoclasts are present in both human and zebrafish adults [76,78]. Nevertheless, there are some differences, such as endochondral ossification being rare in zebrafish, that should be taken into account when using the zebrafish as a model [78]. This section will mainly focus on the development of the skeleton, and especially the craniofacial skeleton, in zebrafish embryos and young larvae, as we are interested in including skeletal endpoints to the ZEDTA.

Cartilage development

The craniofacial skeleton of zebrafish consists of the neurocranium, which supports the brain (see Figure 8), and the viscerocranium, which is the so-called pharyngeal arch skeleton (jaws and gills) (see Figure 9). The cartilage of the craniofacial skeleton develops between 45 and 72 hpf [76]. The pectoral fin cartilage develops between 42 and 48 hpf [68].

Between 45 and 48 hpf, the first neurocranial cartilage, the parachordal cartilage, is formed near the anterior part of notochord. Between the eyes, the trabecula cranii cartilages chondrify. At approximately 52 hpf, the trabecular cranii fuse in the middle of the eyes to form the trabeculae communis, and more laterally, they form the ethmoid plate. Subsequently, the parachordal cartilage and trabeculae communis fuse and form the basal plate [76,79]. Anteriorly, the edges connect to the auditory capsules [79]. The occipital arches, located at the end of the parachordal cartilage, and the auditory capsule cartilages surround the ear [80].

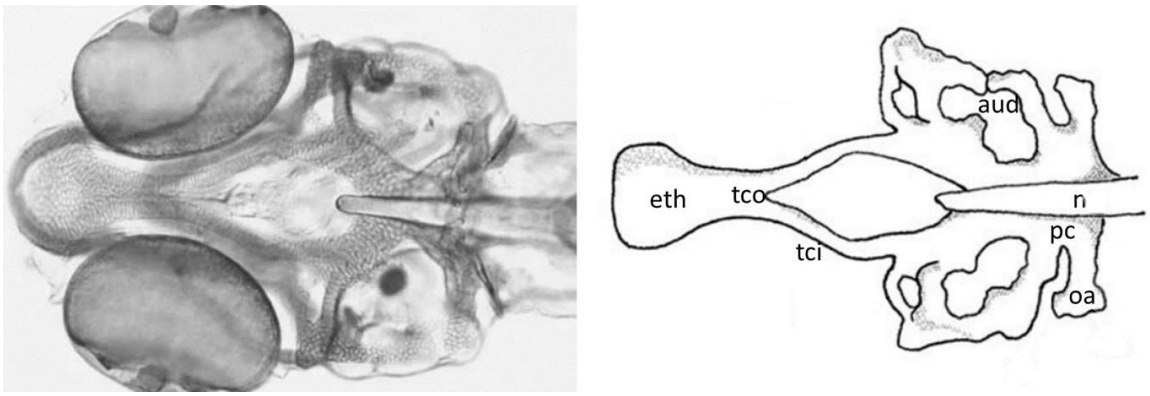

Figure 8. Early cartilage pattern of the zebrafish neurocranium at 5 dpf. The zebrafish is positioned in dorsal view. Abbreviations: auditory capsule (aud), ethmoid plate (eth), notochord (n), occipital arch (oa), parachordal cartilage (pc), trabecula cranii (tci), and trabecula communis (tco) (both figures are adapted from [76]).

Soon after the first neurocranial cartilages start to chondrify, also the pharyngeal cartilages develop. Zebrafish have seven pharyngeal arches: the mandibular arch (#1), the hyoid arch (#2), and 5 branchial arches (#3-7). Cartilages of the pharyngeal skeleton; the Meckel's cartilages, the palatoquadrate cartilages, the hyosymplectic cartilages, the ceratohyal cartilages, and the basihyal cartilage, start to chondrify from approximately 48 hpf onwards [68,76,81]. Between 60 and 72 hpf, the five ceratobranchial cartilages and the interhyal cartilages form [68,76]. Subsequently, at the early larval stage (72-96 hpf), also the hypobranchial cartilages and the pterygoid process of the quadrate chondrify [68]. The exact timing of chondrification of the basibranchials is not specified in literature, however, it is known to be present in 5 dpf old zebrafish larvae [82].

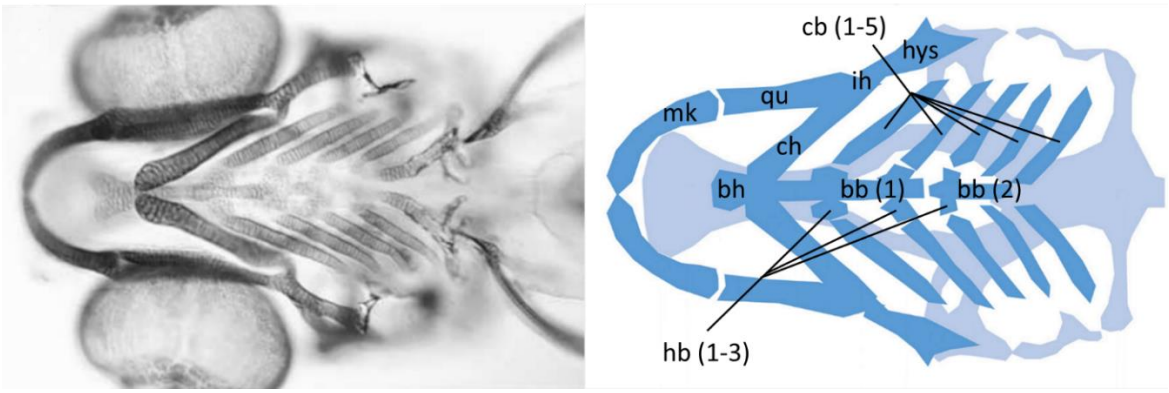

Figure 9. Early cartilage pattern of the zebrafish viscerocranium at 5 dpf. The zebrafish is positioned in ventral view. Abbreviations: basibranchial (bb), basihyal (bh), ceratobranchial (cb), ceratohyal (ch), hypobranchial (hb), hyosymplectic (hys), interhyal (ih), Meckel's cartilage (mk), and palatoquadrate (qu) (photo adapted from [76], schematic image adapted from [82]).

Bone development

There are three types of ossification: intramembranous, perichondral and endochondral ossification [78]. The first type, the dermal bones (i.e., intramembranous bones) have no cartilage intermediate and form directly from differentiation of mesenchymal cells into osteoblasts. The second type, the endochondral bones, develop by replacing cartilage intermediates [76] as mesenchymal cells first differentiate into chondroblasts and chondrocytes, and are then replaced

by bone matrix [78]. This type of ossification is uncommon in teleosts, but very common in mammals. Type I endochondral ossification occurs in the ceratohyal and pectoral fin radials, and type II endochondral ossification occurs in the hypomandibula, branchial arches, ethmoid, and hypuralia [78]. The third type, the perichondral bones, develop in the perichondrium, which is the connective tissue that surrounds cartilage [68,76,78].

Standard length (SL), rather than age, is used as an indicator for bone formation (see Figure 10). In the ZEDTA, however, the embryos and larvae are evaluated at certain timepoints, thus use age as an indicator. Therefore, the age vs. standard length is shown in Table 2, which allows an estimation of the age at which the different structures ossify [79,83].

Table 2. Zebrafish age vs. standard length. Based on the graphs of [79] and [83].

Age	Standard length (mm)
3 dpf	~ 3.3
4 dpf	~ 3.6
5 dpf	~ 3.8
6 dpf	~ 4.1
7 dpf	~ 4.4
8 dpf	~ 4.6
9 dpf	~ 4.9
10 dpf	~ 5.2
12 dpf	~ 5.6
14 dpf	~ 6.1

Before the bones of a zebrafish start to develop, a special type of bones, the otoliths, appear. In total, an adult zebrafish has three otoliths at each side of its head: the circle saccular otolith (also called sagitta), utricular otolith (also called lapillus), and asteriscus (also called lagonar otolith). Otoliths are calcium carbonate structures that are responsible for the detection of movement and sound. From 18.5 hpf onwards, the circle saccular and the utricular otoliths start to develop [68]. The third otolith, the asteriscus, forms around 11-12 dpf [84].

The paired cleithra and the tips of the teeth that are located on the paired ceratobranchials 5, the so-called pharyngeal or ceratobranchial teeth, are the first bone structures that ossify [79]. The cleithrum is a dermal bone that connects the dorsal occipital region with the ventral pectoral gridle (see Figure 11) [68]. Ventrally, the cleithrum curves in anteromedial direction, whereby an ~120° angle is formed. Right after, when the larvae are 3 mm SL, the ceratobranchials 5 can start to ossify (see Figure 10) [79]. At this length, zebrafish are around 3 dpf [79,83]. Approximately a day later (3.6 mm SL), the ceratobranchials 5 are already present in all larvae. When the pharyngeal teeth grow further ventrally, they join with the ceratobranchials 5 and become ankylosed (i.e., separately formed bones that join in such a way that no motion can take place between them) (see Figure 11). Additional pharyngeal teeth will form later on, so that in total two or three rows of pharyngeal teeth are present in adult zebrafish. Pharyngeal teeth are the only kind of teeth that form in zebrafish. Besides ceratobranchial 5, there are four more ceratobranchials, the ceratobranchials 1-4. However, they start to ossify much later (5.8 mm SL) [79]. Also the opercle starts to ossify at 3 mm SL (~3 dpf [79,83]). Its ossification starts in the membrane close to where it articulates with the

hyomandibular cartilage (see Figure 11). In adult zebrafish the paired opercles have a concave, trapezoidal shape so they fit with the shape of the cranium [79]. The timing of ossification of the notochord varies greatly between individuals. Although ossification can already start at 3 mm SL (~3 dpf [79,83]), it only appears to be present in all zebrafish individuals much later on at 4.8 mm SL (~9 dpf [79,83]) [79]. The notochord is a rod-shaped perichondral bone (see Figure 11). Ossification starts with the formation of two rings of bone that surround the notochord, one at the tip of the notochord, and one in the cranial region. Subsequently, these two ossification regions fuse [79]. Around 3-4 dpf [79,83] (3.4 mm SL), the parasphenoid and the branchiostegal rays start to ossify. In adults, the parasphenoid is a rod-shaped unpaired dermal bone that is pointy at the anterior side (see Figure 11). Its ossification starts in the connective tissue of the interorbital septum [79]. The branchiostegal rays are dermal bones that form ventrally from the ceratohyal cartilages (see Figure 11). An adult zebrafish has 3 paired branchiostegal rays. The ray that is located most posteriorly ossifies first (3.4 mm SL) and the most anterior one ossifies last (5.5 mm SL). In rare cases, inbreeding may result in the formation of a fourth branchiostegal ray, which is then located anteriorly from the three other rays [79]. The entopterygoid (i.e., dermal bone; paired) starts to ossify in the membrane that is located dorsomedially from the palatoquadrate cartilage (see Figure 9 and Figure 11). Although the ossification can already start at 3.8 mm SL (~5 dpf [79,83]), only at 5.5 mm SL (~11-12 dpf) the bone is present in all individuals [79].

The paired exoccipitals are the first bones that start to develop after 5 dpf, so when the zebrafish is already considered to be a laboratory animal. The exoccipitals are endochondral bones which form the posterior margin of the cranium [68]. The development starts at 4.0 mm SL and the structure is present in all individuals at 4.8 mm SL (6-7 dpf and 8-9 dpf, respectively [79,83]) [79]. The timing of ossification of the basioccipital and the ceratohyal bones varies greatly between individuals. Although these structures can form from 4.2 mm SL (~6-7 dpf [79,83]) and 4.6 mm SL (~8 dpf [79,83]), respectively, they are present in all zebrafish individuals at, respectively, 5.6 mm SL (~12 dpf [79,83]) and 6.0 mm SL (~13-14 dpf [79,83]) [79]. The basioccipital is an unpaired endochondral bone that forms the posteroventral margin of the cranium [68]. The ossification of the ceratohyal bone, which is a paired endochondral bone, starts in the anterior part of the ceratohyal cartilage. Subsequently, it ossifies further in anterior and posterior direction. At 8.8 mm SL (>14 dpf [79,83]), both the anterior and posterior end broaden, and the anterior end terminates in two heads that join together by cartilage with the ventral and dorsal hypohyal bones [68,79]. Around 8 dpf (4.6 mm SL) [79,83], the hyomandibula, which is a paired endochondral bone of the hyoid arch, starts to develop. Ossification starts at the dorsal end of the hyosymplectic cartilage and, from there, spreads through the rest of the cartilage [79]. Around 13-14 dpf (6 mm SL), the bone structure is present in all individuals [79,83]. The maxilla is a dermal bone that starts to develop at 4.8 mm SL (~8-9 dpf). The ossification begins in the lateral upper jaw where it can be observed as a small rod of bone. Further ossification at the anterior part makes that the structure becomes S-shaped. Another dermal bone, the dentary, starts to ossify at the anterior end of Meckel's cartilage at 5.1 mm SL (~9-10 dpf). It forms the anterolateral part of the lower jaw and is connected to the maxilla via a ligament. Zebrafish have no maxillary nor dentary teeth [79].

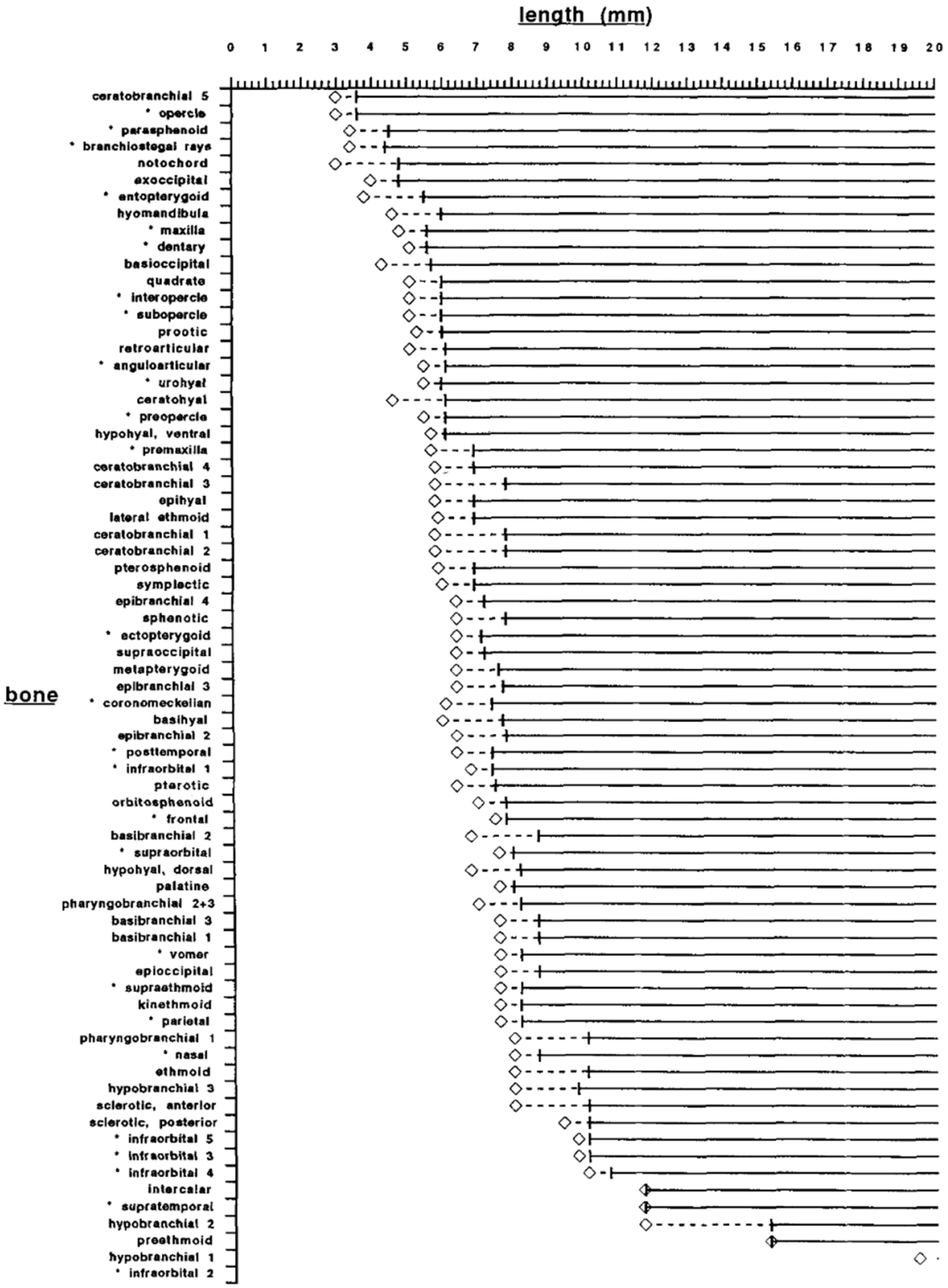

Figure 10. Overview of the ossification sequence in the zebrafish skull. The white diamond represents the smallest length at which a structure of interest was ossified. The dotted line represents the length range in which a structure may or may not be ossified. The solid line represents the length range in which a structure is always ossified in control zebrafish. The length is measured as standard length (mm) (i.e., distance from snout to caudal peduncle, or to posterior tip of the notochord in pre-flexion larvae [83]) [79].

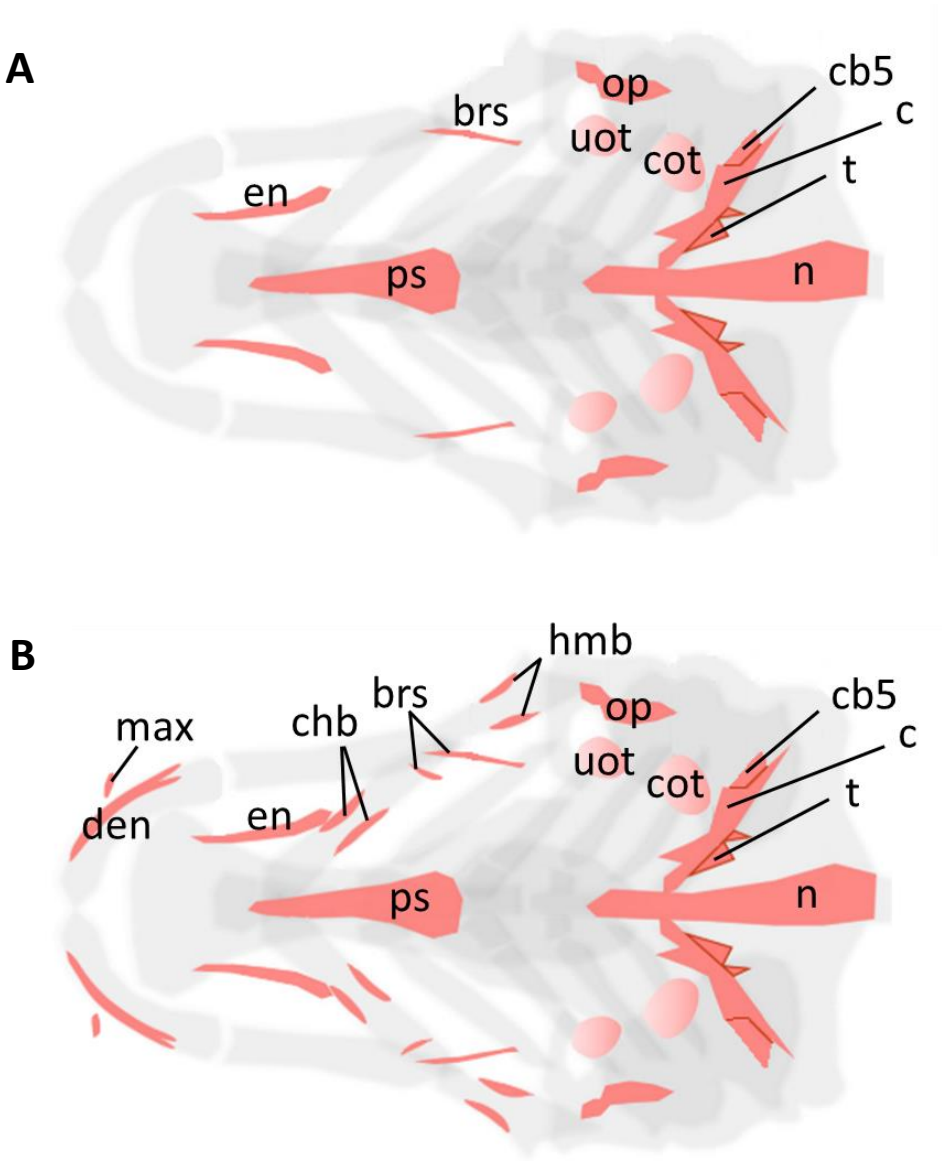

Figure 11. Early bone pattern of the zebrafish cranium at A) 5 dpf and B) 9 dpf. The zebrafish cranium is positioned in dorsal view. Abbreviations: branchiostegal rays (brs), cleithrum (c), ceratobranchial 5 (cb5), ceratohyal bone (chb), circle saccular otolith (cot), dentary (den), entopterygoid (en), hyomandibula (hmb), maxilla (max), notochord (n), opercle (op), parasphenoid (ps), pharyngeal teeth (t), utricular otolith (uot). The exoccipital and basioccipital are not indicated (adapted from [82]).

1.3 Biotransformation of xenobiotics by zebrafish embryos and young larvae

Zebrafish embryos develop externally. Therefore, they cannot rely on the maternal biotransformation capacity as an *in utero* developing organism would do. Hence, they depend on their own biotransformation capacity for biotransforming xenobiotic compounds such as drugs and chemicals. Multiple studies have indicated that, between the different stages of development, the biotransformation capacities of zebrafish can be quantitatively (i.e., metabolite formation rate) and/or qualitatively (i.e., used biotransformation pathway) different [85]. Therefore, in this section, the disposition of xenobiotics in zebrafish, with in particular the metabolization (biotransformation) in zebrafish embryos and young larvae will be discussed.

1.3.1 ADME

The disposition of xenobiotics within an organism is described by ADME, which is an acronym for Absorption, Distribution, Metabolism and Excretion [86].

Absorption is the movement from a xenobiotic compound from the administration site into the blood [87]. In the ZEDTA, zebrafish will be exposed to xenobiotics via the incubation medium. Until 3 dpf, the main route for absorption is dermal (i.e., via the skin). From 3 to 14 dpf, absorption occurs via the skin, mouth and gills, as from approximately 3 dpf onwards the zebrafish mouth opens and from 14 dpf the gills become functional [88].

Distribution is defined as the transfer of a xenobiotic compound from one compartment (i.e., tissue or organ) to another [87]. Once absorbed in the blood, the xenobiotic is carried further into the body to reach its site of action. Xenobiotics can be distributed to several organs and tissues, where they can be stored for a varying amount of time and/or elicit biological responses [87]. The distribution can be affected by many factors, such as plasma protein binding and membrane permeability, depending on the physicochemical properties of the compound [89]. Not much is known yet about the distribution of xenobiotics in zebrafish. However, it is known that from 3 dpf onwards [90], zebrafish start to develop a blood brain barrier that is found to be very similar to that of mammals [88].

Metabolism is a transformation process in which xenobiotics such as drugs (i.e., parent compounds) are converted into more polar products (i.e., metabolites) with the help of enzymes in order to facilitate their elimination from the body [91]. We will further elaborate on this in section 1.3.2.

Excretion is defined as the removal of metabolic waste from the body [92]. In humans, excretion of xenobiotics mainly occurs via the kidneys, bile/feces and the lungs. The bile and gills of zebrafish, however, are not completely functional until 4 and 14 dpf, respectively. As the pronephros of a zebrafish is already formed and functional at 2 dpf [88], renal excretion is considered to be the main elimination route for xenobiotics in zebrafish larvae [87,93].

1.3.2 Xenobiotic metabolism: an overview

The liver is the most important drug-metabolizing organ. However, also many other organs and tissues contain enzymes important for biotransformation reactions. The enzymes that are responsible for these reactions are classified in two groups; the phase I and phase II enzymes [94]. The key phase I reactions are oxidation, reduction and hydrolysis reactions, and they act by introducing polar functional groups to the molecules or by demasking already existing polar groups [85,87,94,95]. The formed metabolites can be inactive or less active than the parent compound (i.e., detoxification), or can be more active than the parent compound, or even have toxic effects (i.e., toxification) [91,95]. The most important phase I enzymes for biotransformation of xenobiotics are the cytochrome P450 enzymes (see 1.3.3.1) [91]. Other enzymes that are often involved in phase I reactions include, but are not limited to: flavin-containing monooxygenases, alcohol dehydrogenases, aldehyde oxidases, aldehyde dehydrogenases, carboxylesterases, xanthine oxidases, xanthine dehydrogenases, monoamine oxidases, etc. [96]. Some of the polar and water-soluble phase I metabolites are the final products and ready for excretion, but most of them are substrates for phase II metabolism [85]. Phase II reactions primarily involve conjugation, and act by adding endogenous hydrophilic groups (e.g. glucuronate, sulfate,…) to the molecules to form water-soluble, inactive, metabolites that can be easily excreted [87,94,95]. In this thesis, we will focus on phase I metabolism and enzymes.

1.3.3 Phase I enzymes

1.3.3.1 Cytochrome P450

Cytochrome P450 enzymes, a superfamily of cysteine thiolate-ligated heme-containing monooxygenase enzymes, are considered to be the most important enzymes in the phase I biotransformation of many xenobiotics [85,97]. Around 75% of the drugs that is used for clinical purposes is metabolized via these enzymes [98]. They are membrane-bound and mainly present in the endoplasmic reticulum of liver cells, followed by the small intestine [85,99]. However, they also can be found in other tissues and organs such as the brain, skin, kidney, and gonads [85]. The name “cytochrome P450” is derived from “Pigment-450 nm”, which is their maximum absorbance when bound to carbon monoxide [97]. The CYP-superfamily is classified into families and subfamilies based on similarities in amino sequence. CYPs within the same family share ≥40% amino-acid identity, whereas CYPs within the same subfamily share ≥55% amino-acid identity. Similar to humans, also in adult zebrafish the CYP1, CYP2 and CYP3 families are mainly involved in the biotransformation of xenobiotics [49,100]. The cytochrome P450 enzymes function by catalyzing the incorporation of one oxygen atom, originating from molecular oxygen, into a substrate, while the other oxygen atom is reduced by two electrons and used to produce a water molecule (see Equation (1)) [97,101].

$$RH + O_2 + 2e^- + 2H^+ \xrightarrow{\text{Cytochrome P450}} ROH + H_2O \quad (1)$$

In humans, intrinsic CYP-mediated biotransformation showed to be immature during embryofetal development [49]. Interestingly, also recent studies in zebrafish embryos show an immature intrinsic CYP-mediated biotransformation capacity [42,49,62,102]. A study using both a non-specific CYP substrate, benzyloxy-methyl-resorufin (BOMR), and a CYP1-specific substrate, 7-ethoxyresorufin (EROD), showed that the transcripts and/or the activity of CYPs is low or undetectable before 72 hpf [62]. Moreover, a study using targeted liquid chromatography–mass spectrometry (LC-MS) confirmed this late onset of biotransformation capacity, as via CYP enzymes formed metabolites were only detected from 96 hpf onwards in whole-embryo extracts and culture medium [42]. Thus, CYP enzymes become active at the end of the organogenesis period, i.e., around 72-96 hpf when the liver becomes functional [103]. In contrast to the *in utero* development of human embryos, zebrafish embryos develop externally. As a consequence, zebrafish embryos will not be exposed to the formed maternal biotransformation products (see Figure 12). The absence of maternal biotransformation products combined with the lack of an intrinsic biotransformation capacity in early zebrafish stages is a problem as it is known that CYPs can change the toxicological properties of xenobiotics by forming metabolites that are more reactive, teratogenic, toxic or carcinogenic than the parent compound [85]. As zebrafish embryos and young larvae are only exposed to the parent compound, false negative results will be obtained for proteratogens that require CYP-mediated bioactivation [62]. Therefore, our research group, as well as other research groups, have been exploring the use of an exogenous metabolic activation system (MAS) to circumvent the limited biotransformation capacity of young zebrafish. In this MAS, liver microsomes are used to metabolically activate the drugs and chemicals that cannot be activated by the embryo itself. In this way, the embryos can be exposed to the metabolites of a compound of interest [7,104,105] (see chapter 3).

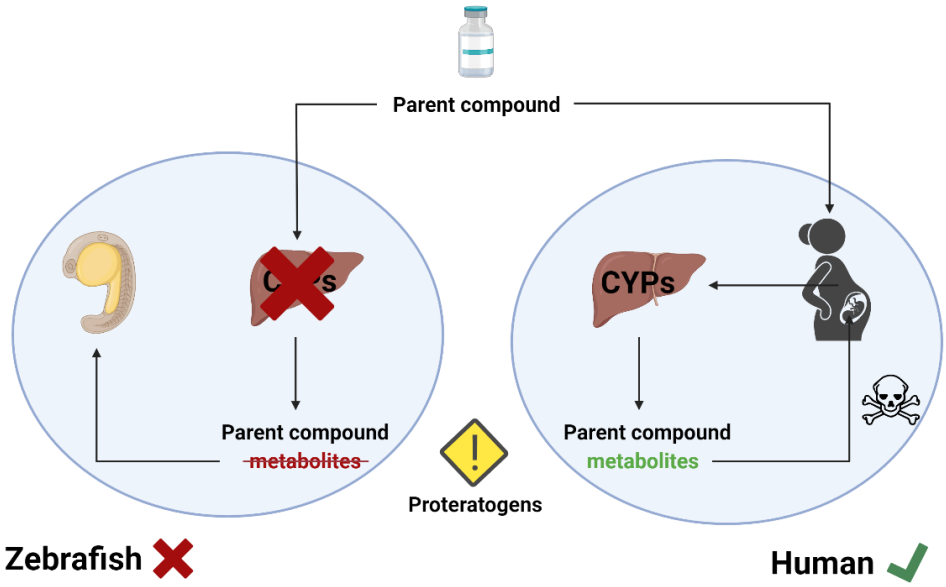

Figure 12. The cytochrome P450 (CYP)-mediated biotransformation in zebrafish (left panel) and humans (right panel). Both zebrafish embryos and human embryos have an immature intrinsic CYP-mediated biotransformation. As zebrafish develop externally, zebrafish embryos will only be exposed to the parent compound, and not to the metabolites formed via maternal metabolization. Human embryos, however, are exposed to both the parent compound and the maternal metabolization products (created with BioRender.com).

1.3.3.2 Non-CYP phase I enzymes

Although less intensively studied than CYPs, knowledge on the activity and/or expression of some non-CYP phase I enzymes in zebrafish embryos and young larvae is available in the public domain.

Esterases

Esterases are enzymes that can hydrolyze compounds containing an ester, thioester and/or amide group. The esters will subsequently break down into an alcohol and an acid, along with the production of a water molecule. 10% of the drugs on the market are considered to be metabolized via these enzymes [106]. Esterases are present in several tissues and organs, including the liver, plasma, small intestine, and kidney [98]. Based upon the interaction with organophosphorous (OP) compounds, they are classified into 3 categories: A-esterases, which hydrolyse OP compounds, B-esterases, which are inhibited by OP compounds, and C-esterases, which do not interact with OP compounds. A-esterases include arylesterases, and B-esterases include cholinesterases and carboxylesterases, which both belong to the serine esterase superfamily [98,107]. Up until now, no drug-metabolizing C-esterases have been identified [98]. From all esterases, the carboxylesterases appear to be most involved in drug hydrolysis [98,106]. Besides detoxifying drugs, esterases are also known to be able to activate prodrugs [106].

Multiple studies showed the presence of esterase activity in zebrafish at young age. Otte et al. (2017) reported intrinsic esterase activity already from 2.5 hpf onwards by investigating the fluorescein diacetate-dependent esterase activity in the subcellular S9 fraction[3] of whole organism homogenates. Interestingly, at 96 and 120 hpf, the esterase activity appeared to be two times higher than at 2.5 and 72 hpf. Up until 72 hpf, the esterase activity was not significantly different from 2.5 hpf [108]. So, although already present at younger stages, the esterase activity increased with increasing age. Also Küster (2005) investigated the esterase activity in zebrafish embryos. In this study, the activity of cholinesterases (including the acetylcholinesterases and the butyrylcholinestererases) and carboxylesterases was explored up until 48 hpf by using specific substrates and inhibitors. The results of the cholinesterase inhibitor studies showed that mainly the acetylcholinesterases, and not the butyrylcholinestererases, are responsible for the cholinesterase activity in zebrafish embryos [109]. This is in agreement with an earlier study by Bertrand (2001), where no butyrylcholinesterase gene or activity was found in zebrafish embryos [109,110]. Küster reported the presence of acetylcholinesterase and carboxylesterase activity in zebrafish embryos from 12 hpf onwards. At this age, the activity of the carboxylesterases was 40 times higher than that of the cholinesterases. Up until 24 hpf, the carboxylesterase activity remained higher, and at 30 hpf, the activity of both esterases became equally high. From 36 to 48 hpf, however, the carboxylesterase activity showed to be around 30% lower than the cholinesterase activity [109].

[3] S9 fraction: The supernatant fraction that contains cytosol and microsomes, which is obtained from an organ homogenate (usually the liver) by centrifuging for 20 minutes at 9000 x g in a suitable medium [123].

Myeloperoxidases

Myeloperoxidases (MPO), which are heme peroxidases, are lysosomal phase I enzymes that are present in granulocytic cells [111,112]. They are known to be involved in the biotransformation of several xenobiotics [113]. The MPO catalytic cycle involves the reaction of hydrogen peroxide and chloride ions to form hypochlorous acid, which, on its turn, oxidizes substrates [111].

Already at 18-19 hpf, expression of a homologue of the human myeloperoxidase enzyme, the myeloid-specific peroxidase (MPX) was found in the axial intermediate cell mass of zebrafish by in situ hybridization. From 24 hpf onwards, MPX was found to be expressed throughout the embryo, especially at the surface of the yolk and in the head and pharyngeal regions. However, at this age, no enzymatic activity was found by using an histochemical staining. Only several hours later, at 33 hpf, the first peroxidase activity was reported. The activity was also mainly found in cells at the surface of the yolk, and in the region where the ventral vein is located. By 48 hpf, peroxidase activity was found in cells throughout the entire embryo, with in particular in the ventral venous plexus [114].

Alcohol dehydrogenases

Alcohol dehydrogenases (ADH) are cytosolic enzymes that catalyze the oxidation of primary and secondary alcohols to aldehydes or ketones, respectively, by transferring an anion to NAD+, while a proton is released [115]. They are known to be involved in xenobiotic metabolism of several compounds that contain alcohol groups (e.g. hydroxyzine and abacavir) [116]. In humans, ADH enzymes are mainly present in the liver, but are also found in other tissues and organs, including the gastrointestinal tract and adipose. However, between species there are differences in tissue distribution, as well as in genes, proteins and enzymatic activity [116].

The activity and/or expression of alcohol dehydrogenase enzymes in zebrafish embryos has been investigated in several studies. Otte et al. (2017) reported that no intrinsic ADH activity above the limit of detection (LOD) was found by looking at the activity in both the subcellular refined S9 and the cytosolic fraction of whole organism homogenates of 2.5, 24, 48, 72, 96, and 120 hpf old zebrafish [108]. The lack of expression of a specific alcohol dehydrogenase isoenzyme, ADH8a, in zebrafish embryos was reported by Klüver et al. (2014). In their study, qPCR experiments showed that adult zebrafish had a thousand times more ADH8 mRNA than 24 and 120 hpf zebrafish embryos. Moreover, in zebrafish embryos no metabolic conversion of allyl alcohol by ADH8, and thus no toxicity, was observed. This was in contrast to the situation in adult zebrafish [117]. Some other ADH isoenzymes, however, showed to be expressed in zebrafish embryos. mRNA from ADH5 (formerly called ADH3 [68]), for example, could be detected in whole zebrafish embryos from 4 hpf onwards. Interestingly, the expression showed to vary during development, as a decrease in ADH5 mRNA was found between 8 and 24 hpf, and from 30 hpf onwards it increased again. Interestingly, the expression in 8, 16, 20 and 24 hpf zebrafish embryos was significantly lower than at 96 and 120 hpf [118]. In another study, ADH5 showed to have a significantly lower expression as well. Here, the expression was lower at 24 hpf than at 48-120 hpf [117]. Also ADH8b showed to have a higher expression at 120 hpf, than at 24 hpf [117]. For ADHFE1, the expression was similar at 24, 48, 72, 96 and 120 hpf [117]. Based on these results, it appears that zebrafish embryos and young larvae

may have a limited alcohol dehydrogenase activity and/or expression for at least some of the isoenzymes.

Aldehyde dehydrogenases

Aldehyde dehydrogenases (ALDH) catalyze the oxidation of both exogenous (i.e., drugs and ethanol) and endogenous (i.e., amino acids, lipids,…) aldehydes into carboxylic acids [119,120]. ALDH enzymes are present in the cytosol, mitochondria and microsomes of different tissues and organs, such as the liver, kidneys and the mucosa of the gastrointestinal tract [121,122].

Otte et al. (2017) investigated the enzymatic ALDH activity of zebrafish embryos in the cytosolic and the refined S9 fraction of whole organism homogenates at, respectively, 2.5, 24, 48, 72, 96, and 120 hpf and 2.5, 48, and 96 hpf. In the cytosolic fraction, an intrinsic aldehyde dehydrogenase activity above the limit of quantification (LOQ) was found at 96 and 120 hpf. In the refined S9 fraction, this was at 2.5 and 96 hpf [108].

As shown in this paragraph, data on the activity of other phase I enzymes (i.e., different from CYPs) involved in the biotransformation of xenobiotics in zebrafish embryos is rather limited or not available at all. Potentially, there are more enzymes that, just like the CYPs, are only active at the end of the organogenesis period. This may result in false negative results for proteratogens. Therefore, in chapter 6 of this thesis, the biotransformation capacity of zebrafish embryos and young larvae and the teratogenic potential of the formed metabolites will be further investigated.

1.4 References

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CHAPTER 2:
Aims of the doctoral project

Within Europe, alternative methods for toxicity assessment of xenobiotics become very important. Several pharmaceutical, (agro)chemical and cosmetic companies are currently using the zebrafish embryo as an alternative for animal testing to screen new compounds for developmental toxicity. However, false negative and false positive results are reported in the Zebrafish Embryo Developmental Toxicity Assay for known mammalian teratogens and non-teratogens, respectively. For safety reasons, false negative results are more critical, as teratogens may be missed. **Therefore, the main goal of this project was to further standardize and optimize the ZEDTA in order to increase its sensitivity.** This has led to the following research objectives (see also Figure 1).

The first two objectives contribute to the standardization of the ZEDTA.

Objective 1: Develop a standardized ZEDTA protocol that can be extended with a metabolic activation system and a skeletal staining protocol.

The current ZEDTA protocol varies between labs in design, exposure window, endpoints, etc., resulting in discordant data for identical compounds. Moreover, the assay has some known limitations, such as low biotransformation capacity and fewer morphological endpoints in comparison with the *in vivo* mammalian developmental toxicity studies. Consequently, there is a need to standardize and further optimize the assay for developmental toxicity testing. Our goal was to develop a standardized ZEDTA protocol that can be extended with a metabolic activation system (i.e., metabolic ZEDTA or mZEDTA) and/or skeletal staining (i.e., skeletal ZEDTA or sZEDTA). In this way, the ZEDTA can be used as a modular system depending on the compound of interest.

Objective 2: Determine the maximal concentration of DMSO that can be safely used as a solvent.

For further standardization of the ZEDTA, it was investigated which concentrations of DMSO can be safely used as a solvent. A possible combined effect of DMSO and two non-teratogenic chemicals, hydrochlorothiazide and ascorbic acid, on zebrafish larvae was also investigated.

The next objectives contribute to the further optimization of the ZEDTA.

Objective 3: Evaluate stained skeletal endpoints with compounds that show skeletal malformations in the *in vivo* mammalian developmental toxicity studies.

As the number of skeletal endpoints in zebrafish embryo developmental toxicity assays is very limited compared to the *in vivo* mammalian studies, it was investigated whether the sensitivity of the ZEDTA could be increased by including a skeletal staining method. Three staining methods (i.e., alizarin red-alcian blue, alizarin red live and calcein) were tested on zebrafish larvae that were exposed to four mammalian skeletal teratogens, levetiracetam and proprietary compounds 5, 9 and A.

Objective 4: Investigate whether anti-epileptic drugs that require bioactivation to exert their teratogenic potential are biotransformed by non-CYP enzymes in zebrafish embryos and young larvae, and whether these metabolites cause developmental toxicity.

Recent studies in zebrafish embryos showed an immature intrinsic CYP-mediated biotransformation capacity, as most CYPs were only active at the end of the organogenesis period. This may result in false negative results in case of proteratogens, as they need bioactivation to exert their teratogenic potential. Data on other phase I enzymes involved in the biotransformation of xenobiotics in zebrafish embryos is limited. Therefore, it was investigated whether carbamazepine and levetiracetam, two anti-epileptic drugs that presumably require non-CYP mediated bioactivation to exert their teratogenic potential, are biotransformed into metabolites in the zebrafish embryo and whether one or more of these metabolites cause developmental toxicity in this species. In this way, more information will be obtained about the biotransformation capacity of zebrafish embryos and young larvae and the teratogenic potential of the formed metabolites.

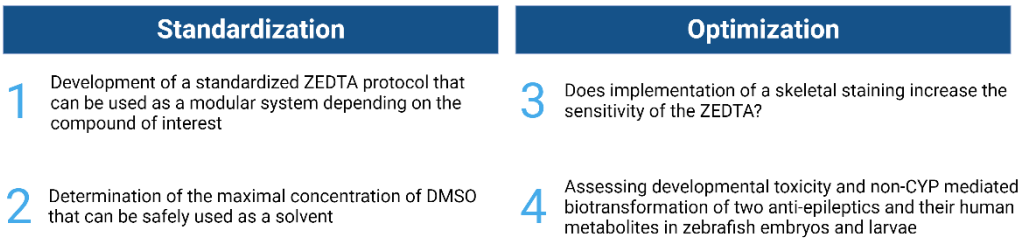


Figure 1. Overview of the different chapters/objectives (created with BioRender.com).

CHAPTER 3:
Development of a standardized ZEDTA protocol that can be used as a modular system depending on the compound of interest

Adapted from: J. Hoyberghs, C. Bars, C. Pype, K. Foubert, M. Ayuso Hernando, C. Van Ginneken, J. Ball, S. Van Cruchten. Refinement of the zebrafish embryo developmental toxicity assay. MethodsX. 7 (2020) 101087, doi: 10.1016/j.mex.2020.101087.

Abstract

Several pharmaceutical and chemical companies are using the zebrafish embryo as an alternative to animal testing for early detection of developmental toxicants. Unfortunately, the protocol of this zebrafish embryo assay varies between labs, resulting in discordant data for identical compounds. The assay also has some limitations, such as low biotransformation capacity and fewer morphological endpoints in comparison with the *in vivo* mammalian developmental toxicity studies. Consequently, there is a need to standardize and further optimize the assay for developmental toxicity testing. We developed a Zebrafish Embryo Developmental Toxicity Assay (ZEDTA) that can be extended with a metabolic activation system and/or skeletal staining to increase its sensitivity. As such, the ZEDTA can be used as a modular system depending on the compound of interest.

- Our protocol is customized with a metabolic activation system for test compounds, using human liver microsomes. This system ensures exposure of zebrafish embryos to metabolites that are relevant for human risk and safety assessment. As human liver microsomes are toxic for the zebrafish embryo, we developed a pre-incubation system with an ultracentrifugation and subsequent dilution step.
- Additionally, we developed a skeletal staining protocol that can be added to the ZEDTA modular system. Our live alizarin red staining method detects several bone structures in 5-day old zebrafish larvae in a consistent manner.

3.1 Introduction

Developmental toxicity testing mainly relies on *in vivo* studies in rodent and non-rodent species. As these studies are ethically under discussion, time-consuming and costly, require a lot of test compound, and have a low throughput, several pharmaceutical and chemical companies are using *in vitro* and/or *in vivo* screening assays for early detection of developmental toxicity. One of the alternatives to animal testing that has gained a lot of interest in the last decade is the zebrafish embryo assay. This alternative model has already been validated for assessing acute toxicity of chemicals in view of environmental risk assessment in the so-called (zebra)Fish Embryo acute Toxicity (zFET) test [1]. Several industrial and academic groups also noted the potential of the zebrafish embryo for developmental toxicity testing. Still, the number of morphological endpoints and other factors (such as medium, exposure window, etc.) in this developmental toxicity assay vary between labs, despite harmonization efforts [2,3]. This has led to discordant results for identical compounds. So, there is a clear need for standardization and optimization of this assay. To increase the sensitivity of the assay, we adapted the protocol used by Ball et al. [3] and developed a Zebrafish Embryo Developmental Toxicity Assay (ZEDTA) that can be extended with a metabolic activation and/or skeletal staining protocol (see Table 1, Figure 1 and procedure section). These additional steps will be further referred to as the metabolic (m)ZEDTA and skeletal (s)ZEDTA, respectively (see Figure 1). The metabolic activation protocol was developed and added to our ZEDTA, because we showed previously that zebrafish embryos have a low biotransformation capacity during a major part of organogenesis and, in contrast to mammals, they cannot rely on

the maternal metabolic capacity due to their external development [4,5]. Consequently, test compounds that require bioactivation to exert their toxicity could be missed (and cause false negative results) in a standard ZEDTA. Busquet et al. [6] were the pioneers in developing a metabolic activation system (MAS) by using rat liver microsomes, but the zebrafish embryos could only be exposed for 1 h (2–3 hpf) to test compounds in this MAS, due to its inherent embryotoxicity. Others tried to expose zebrafish embryos to MAS during the entire period of organogenesis, but they reached only a maximum of 4 h co-incubation with MAS and this only for the older developmental stages [7]. As these groups showed that co-incubation of zebrafish embryos with MAS during the entire exposure period is not feasible, we developed a pre-incubation system with human liver microsomes as MAS (see mZEDTA procedure below). We used human liver microsomes, as our mZEDTA is aimed for human safety/risk assessment. In addition to the mZEDTA, we developed the sZEDTA in order to extend the number of morphological endpoints in the ZEDTA (see Table 2 and Figure 2). These endpoints are much more limited than in the *in vivo* mammalian developmental toxicity studies [8] and especially the skeletal endpoints are scarce, as no skeletal staining is performed in a standard ZEDTA. For the skeletal endpoints, we evaluated several skeletal staining methods described in literature (including an alizarin red staining protocol for fixed and live larvae and a calcein staining) in collaboration with the University of Exeter. The alizarin red staining protocol of live larvae (based on Bensimon-Brito et al. [9] and personal communications from Dr. C. Hammond (Bristol University, UK)) showed the most consistent results (see sZEDTA procedure below). With this staining protocol, we are currently evaluating several proprietary and non-proprietary compounds showing skeletal malformations in rat and/or rabbit as part of a consortium exercise within the European Teratology Society. This evaluation falls out of scope of this methodology paper, which focuses on the staining method and the bones that can be detected in 5-day old zebrafish larvae.

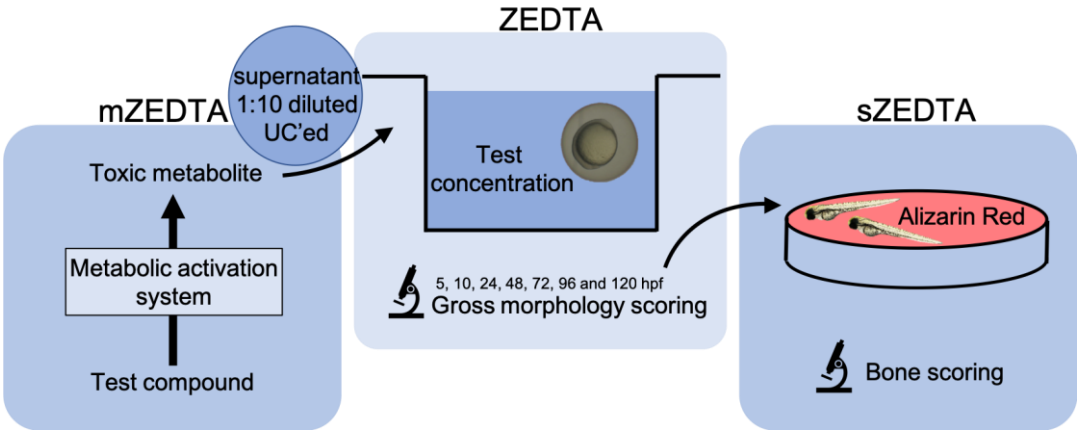


Figure 1. Schematic representation of our ZEDTA, the metabolic (m)ZEDTA and the skeletal (s)ZEDTA. The mZEDTA panel shows the pre-incubation of the test compound with the metabolic activation system for 1 h. After 1 h, this pre-incubation mix is ultracentrifuged (UC'ed) at 100,000 x g for 1 h at 4 °C and the supernatant is pipetted and diluted 1:10 to obtain the test solution, as represented in the upper right circle. Zebrafish embryos are then exposed to the test concentration until 120 h post fertilization (hpf) in our ZEDTA protocol (mid panel) and the embryos are scored for gross morphology. For the sZEDTA (depicted in the right panel), the zebrafish embryos are transferred after gross morphology scoring in the ZEDTA to a small petridish with 0.005% alizarin red solution. After 1 h the solution is removed, the zebrafish larvae are washed in embryo medium, anesthetized and embedded in agar to be imaged. Finally, the larvae are euthanized and the images are processed and evaluated for bone scoring.

Table 1. Comparison between the protocol of the Zebrafish Developmental Toxicology Assay (ZeDTA) by Ball et al. [3] and our Zebrafish Embryo Developmental Toxicity Assay (ZEDTA).

Name of the assay	ZeDTA	ZEDTA
Strain	Several wild type strains	**Wild type AB**
Selection and number of eggs	Select fertilized eggs undergoing cleavage and no signs of irregularities	Select fertilized eggs undergoing cleavage and no signs of irregularities
	12 embryos per concentration, 2 replicates	**20** embryos per concentration, 2 replicates
Temperature	28°C ± 1°C	28.5°C ± 0.2°C
Chorion	Intact	Intact
Test chamber	24 well-plate (one embryo per well)	**48 well-plate** (one embryo per well)
	1,000 µL per well	**500-1,000 µL per well**
Exposure length	Start at gastrulation (4-6 hpf)	Start at gastrulation (from 5.25 hpf)
	Ends at 120 hpf	Ends at 120 hpf
Choice of concentrations	Highest concentration 1,000 µM or 100 µM – lowest concentration 0.1 µM	Based on a range finding test with different concentrations
Number of concentrations	4-5 concentrations	By default 3 concentrations, can be reduced or extended
Exposure method	Static	Static, unless nominal chemical concentration <20% at end of test
Use of solvent	Survival of solvent control should be ≥ 90% at 120 hpf	Survival of solvent control should be ≥ 90% at 120 hpf
Morphology scoring system	Morphological scoring based upon Panzica-Kelly et al. [10]	Extended morphological scoring (see Table 2)
Medium	0.3x Danieau 's solution	**TRIS-buffered medium**
pH	7.1-7.3 ± 0.2	7.4 ± 0.2
Conductivity	Not defined	**490-510 µS/cm**
Internal concentrations	Yes	Yes

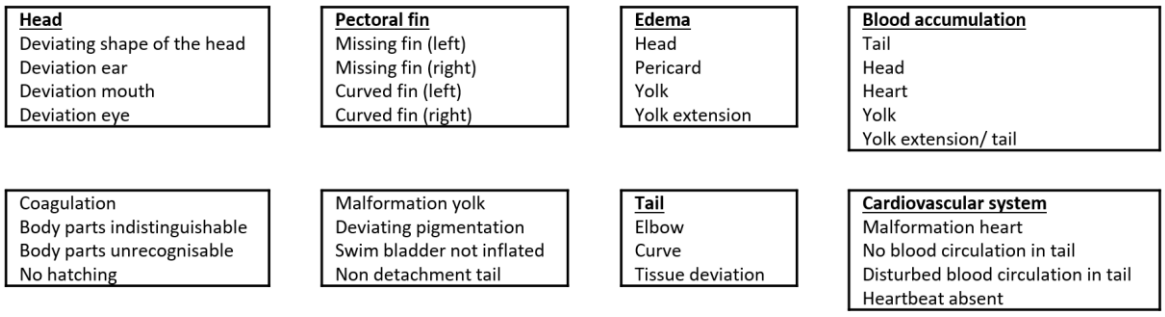


Figure 2. Detailed overview of all morphological endpoints in our ZEDTA.

Table 2. General overview of morphological scoring of zebrafish embryos at different developmental stages in our ZEDTA.

	Stage (hpf)						
	5.25	10	24	48	72	96	120
Coagulation	+	+	+	+	+	+	+
Hatching				+	+	+	+
Tail deviation			+	+	+	+	+
Edema			+	+	+	+	+
Blood accumulation			+	+	+	+	+
Malformation of the cardiovascular system			+	+	+	+	+
Malformation of the head			+	+	+	+	+
Malformation of the pectoral fins					+	+	+

hpf = hours post-fertilization

3.2 Procedure

3.2.1 ZEDTA

Assess stability and uptake of the test compound (by the zebrafish embryos). Outsource this analysis or perform internally depending on the availability of bioanalytical tools and/or expertise.

Perform the following steps first:

1. For stability assessment, prepare test concentrations in embryo medium (see step 14 in the ZEDTA protocol) and incubate at 28.5 °C ± 0.2 °C.
2. Take samples for analysis at 0 h, 24 h and 120 h and send to the bioanalytical lab. The required volume and dilution steps for sample analysis depend on the bioanalytical method established by the lab.
3. When the test solution is already degraded at 24 h with more than 20% of the nominal concentration (i.e., concentration at 0 h), the test compound is too unstable in the embryo medium. Do not proceed further.
4. When the test solution is degraded at 120 h with more than 20% of the nominal concentration, use a semi-static exposure approach in step 15 of the ZEDTA protocol, i.e., renew your test solutions every 24 h.
5. For assessment of uptake, i.e., the concentration of the test compound in zebrafish embryos, sample zebrafish embryos (see steps 1–11 of the ZEDTA protocol for embryo collection) that have been exposed to the test concentrations (from 5.25 hpf onwards) at 24 hpf and at 120 hpf. The required number of embryos depends on the established bioanalytical method.
6. Use the washing protocol and further preparatory steps provided by the bioanalytical lab.
7. Analysis and reporting of the internal concentrations by the bioanalytical lab.
8. When the analytical data show no uptake over the exposure period (at 24 hpf and 120 hpf), do not proceed further.
9. When lower or higher internal concentrations of the test compound are noted at 120 hpf than at 24 hpf and this cannot be explained by the stability data, the test compound was

metabolized by or accumulated in the zebrafish embryos, respectively, and this has to be considered when interpreting the data in step 16 of our ZEDTA protocol.

10. Proceed to the ZEDTA protocol below.

Follow these steps of our ZEDTA protocol:

1. Use an adult zebrafish breeding stock (Danio rerio, AB zebrafish line, GIGA, University of Liège) kept in tanks of approximately 50 L.
2. Set water temperature to 28.5°C ± 0.2°C; conductivity 500 ± 25 µS/cm (adjusted with Instant Ocean sea salts), pH 7.5 ± 0.3 (adjusted with $NaHCO_3$).
3. Renew water when ammonia, nitrite and nitrate reach detectable levels.
4. Apply a light cycle with 14/10 h light/dark.
5. Feed fish daily with thawed food (*Artemia nauplii*, *Daphnia*, *Chironomidae* larvae or *Chaoborus larvae*) twice a day, as well as granulated food at a rate of 2% of their mean wet weight per feeding, twice a day.
6. Put the fish in a net in the tank the day before mating to avoid eating of the eggs.
7. The following morning, allow fish to spawn eggs and fertilize them for about 45 min.
8. Collect fertilized eggs from the bottom of the tank by siphoning them out with a tube.
9. Remove feces and coagulated eggs and rinse remaining embryos in TRIS buffered medium, i.e., 0.294 g $CaCl_2.H_2O$, 0.123 g $MgSO_4.7H_2O$, 0.059 g $NaHCO_3$, 0.005 g KCl, and 0.1M TRIS-HCl (pH 7.5) dissolved in 1 L reverse osmosis water (further referred to as embryo medium).
10. Check embryos under a stereomicroscope for normal cell division within 2 hpf.
11. Discard unfertilized eggs or asymmetric embryos and replace them with healthy eggs until sufficient embryos are obtained (see step 14).
12. Transfer all selected embryos randomly into a 48-well plate (one embryo per well), filled with min. 500 µL embryo medium per well.
13. The experiment is valid when the fertilization rate is >90% and mortality of the controls is ≤ 10% at the end of the experiment.
14. Use 20 embryos per replicate (2 replicates, performed in different weeks) of each control group (medium and/or solvent) and each test group. Classically 3 concentrations (Low, Mid, High) of the test compound are used in order to determine a NOAEL (no observed adverse effect level), but the number of test groups can be reduced (e.g. for back-up compounds) or extended with more concentrations of the test compound (and its human metabolites after pre-incubation in a metabolic activation system, when applicable (see mZEDTA)). To establish concentration-response curves, 5 or more concentrations of the test compounds may be needed.
15. Rear medium control embryos in embryo medium until 120 hpf. Transfer solvent controls and test groups at 5.25 hpf from embryo medium into the solvent concentration or test solution, respectively, and they remain in the solvent/test solution until 120 hpf. When using solvents other than 0.01% DMSO, perform a solvent control experiment first, as the solvent (concentration) may be toxic for zebrafish embryos.

16. Evaluate the zebrafish at 5.25, 10, 24, 48, 72, 96, and 120 hpf for the morphological parameters depicted in Table 2. Use the earliest timepoint (5.25 hpf) to monitor embryo quality at the start of exposure. Larvae at 96 and 120 hpf must be evaluated under anesthesia with MS-222 (0.2 g/L in embryo medium) due to their ability to swim, which hampers the morphological evaluation. Use Supplementary Figures 1–8 for the binary classification of the gross morphology endpoints. Use score 0 for normal morphology and score 1 for abnormal morphology. Figure 3 depicts some of the most common malformations in zebrafish at 96 hpf. The percentage of malformed embryos is calculated by dividing the number of alive larvae having one or more malformations with the total number of living larvae, multiplied by 100.
17. Euthanize the larvae by means of an overdose of MS-222 (1 g/L in embryo medium) after the last gross morphology scoring. Alternatively, use hypothermic shock (in e.g. ice water) for at least 12 h.

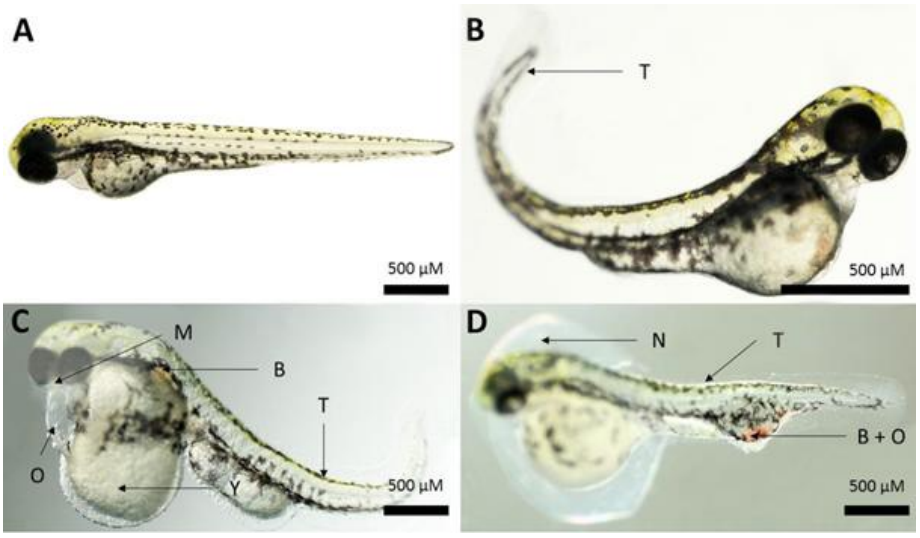


Figure 3. Overview of several malformations that can be observed in 96 hpf zebrafish larvae. A) Normal zebrafish larva. B) Tail malformation (curve). C-D) Several malformations are present. Abbreviations: blood accumulation (B); malformation mouth (underdeveloped) (M); non-hatching (N); Edema (O); tail malformation (curve) (T); malformation yolk (Y). Adapted from [11].

3.2.2 Metabolic (m)ZEDTA

Follow steps 1 to 13 of our ZEDTA protocol and then continue with the following steps:

14. Pre-incubate the test compound (one or more concentrations) with human liver microsomes (pooled, 50 donors, Thermo Fischer scientific, USA) at 200 µg/mL and NADPH tetrasodium salt (1.25 mM) in embryo medium for 1 h at 28.5 °C. In addition to the test suspension(s), include a medium control and a blank control, i.e., the pre-incubation mix without test compound.

14'. After 1 h pre-incubation, the test suspension and blank control are ultracentrifuged at 100,000 x g for 1 h at 4 °C.

14''. Pipet the supernatant of the blank control and the test suspension, containing the test compound and its metabolites, and dilute the supernatant 1:10 in embryo medium.

15-17. Follow the remaining steps in our ZEDTA. In case of negative results, proceed to step 18.

18. Increase the concentration of the test compound (up to substrate inhibition) in step 14 and follow the remaining steps in the mZEDTA. In case of negative results, proceed to step 19.

19. Determine the metabolite concentrations in the test solution of step 18. In case of low metabolite concentrations (in most cases < µM range), concentrate the test solution. Different procedures can be applied. Discuss with a (bio)analytical expert for your compound(s) of interest.

3.2.3 Skeletal (s)ZEDTA

Follow steps 1 to 16 of our ZEDTA protocol and then continue with the following steps:

16'. Transfer the larvae at 120 hpf into a small petridish and remove the solution.

16''. Add 10 mL 0.005% alizarin red solution to the petridish.

16'''. Remove the solution after 1 h and wash the larvae in embryo medium.

17. Transfer the larvae in MS-222 (0.2 g/L in embryo medium) until they lose their dorsoventral balance.

18. Embed the larvae in 1% low gelling temperature agar containing 0.2 g/L MS-222 and image them with an Olympus SZX16 (6.3x mag) scope (Olympus, UK) in lateral and dorsoventral position with Prior 200 Lumen illumination (100%) with RFP at 620 nm for 100 ms capture in µManager (v1.4) with 8 images per stack (1 stack per position and thus 16 images in total) at 3 second intervals using a Zyla 4.2 sCMOS camera (Andor, UK).

19. Euthanize the larvae by means of an overdose of MS-222 (1 g/L in embryo medium). Alternatively, use hypothermic shock (in e.g. ice water) for at least 12 h.

20. Process the images in ImageJ and evaluate the intensity and shape of the bone structures depicted in Table 3 and Figure 4. For intensity, use score 0 when the structure is not stained, score 1 when the structure is weakly stained, score 2 when the structure is moderately stained and score 3 when the structure is heavily stained (see Figure 5). Select for each bone structure of each larva in each group the image with the highest intensity score to determine the final score of the bone. For shape, use score 0 when the structure is normal and use score 1 when malformed.

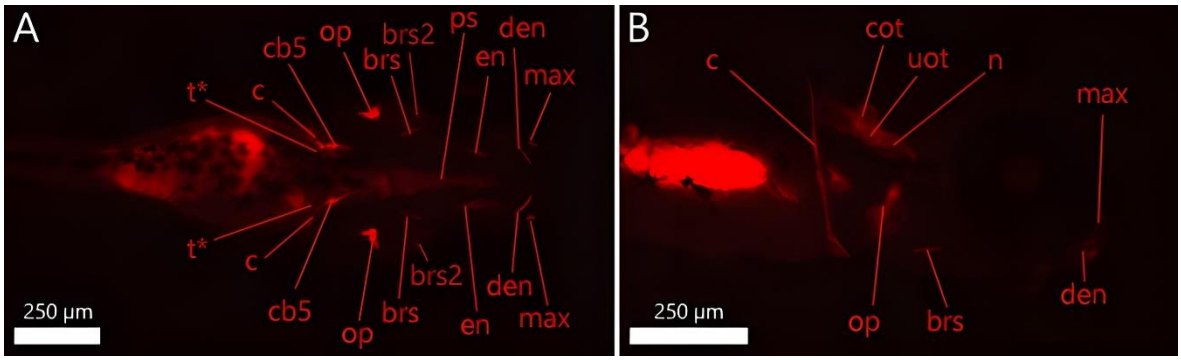


Figure 4. Zebrafish larvae at 120 hpf with bone structures that are stained with 0.005% alizarin red. Left panel (A) shows a dorsal view. Right panel (B) shows a lateral view. The abbreviations are depicted in Table 3. * t should be present at 120 hpf, but cannot be distinguished from cb5.

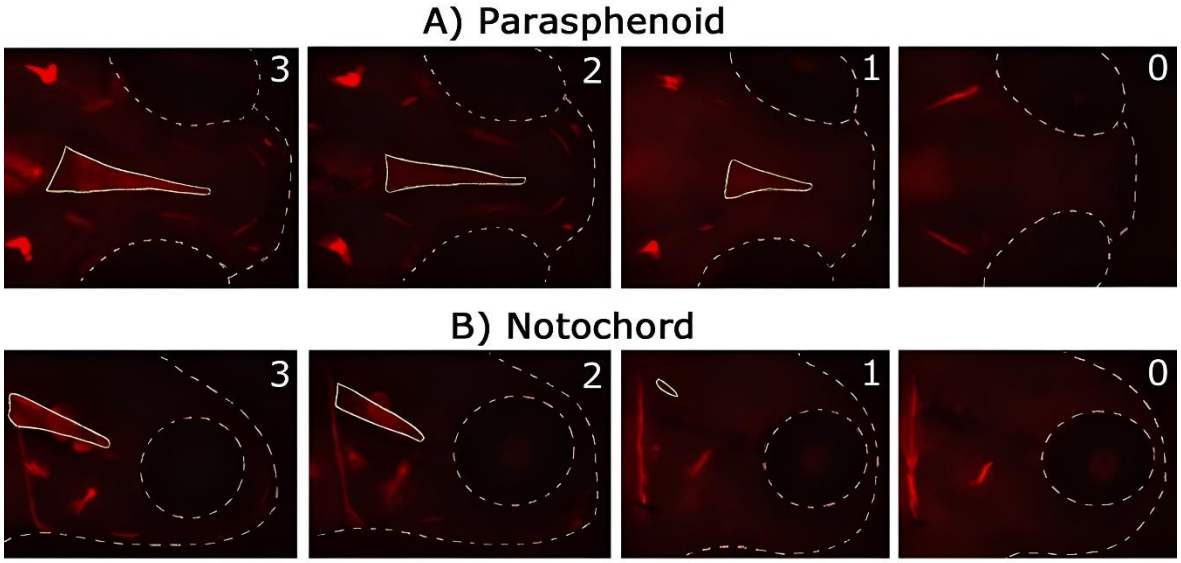


Figure 5. Illustration of the intensity scoring system for 2 bones at 120 hpf (0 = not stained; 1 = weakly stained; 2 = moderately stained; 3 = heavily stained). The upper panel (A) is a dorsal view, the lower panel (B) is a lateral view. The stained bone of interest is delineated with a full line. The head and eye are delineated with a dotted line.

Table 3. Bone structures in zebrafish at 120 hpf.

Structure (+ abbreviation)	120 hpf
Entopterygoid (en)	+
Operculum (op)	+
Parasphenoid (ps)	+
Cleithrum (c)	+
Notochord (n)	+
Ceratobranchial v (cb5)	+
Pharyngeal teeth (t)	+
Utricular otoliths (uot)	+
Circle saccular otoliths (cot)	+
Branchiostegal rays (brs and brs2)	+
Dentary (den)	/
Maxilla (max)	/

+ stained at 120 hpf, / can be stained as the structure starts to develop around 120 hpf.

3.3 Method validation

3.3.1 ZEDTA

In order to validate our ZEDTA protocol, we used 30 mM trimethadione (Tebu-bio, Boechout, Belgium) in embryo medium as test solution. Trimethadione is an anticonvulsant and human teratogen. We used one concentration in our ZEDTA, as Weigt et al. [12] already showed that exposure of zebrafish embryos to 20 mM and 40 mM trimethadione from 2 hpf until 72 hpf caused 33% and 88% malformed embryos, respectively. Our experiment was valid, as the medium controls had a coagulation of less than 10% (see Table 4) and the fertilization rate was above 90% (data not shown). The statistical analysis was carried out with GraphPad Prism 7 (San Diego, CA, USA). Morphological data were analyzed by means of the Fisher's exact test. Results were considered significant at $p \leq 0.05$. After comparing the morphological effects in the replicates, no replicate showed more or less malformations than the other for any of the parameters ($p > 0.05$). Therefore, the data of the replicates were pooled. The results are depicted in Table 4. 30 mM trimethadione clearly showed malformations at the end of the exposure period with several organs affected. The higher than expected percentage of affected embryos (93%) with 30 mM trimethadione in our experiment compared to the study by Weigt et al. [12] can be explained by the longer exposure period in our ZEDTA, as the last morphological scoring in [12] was done at 72 hpf. At 72 hpf, we obtained 60% of malformed embryos in our ZEDTA and several morphological endpoints were not affected yet (see Table 5). These findings emphasize the importance of the exposure duration (i.e., the main period of organogenesis) and not only the dose/concentration of the developmental toxicant.

Table 4. Overview of coagulation and malformations in the group exposed to 30 mM trimethadione at the end of the experiment.

Affected parameter	Medium control	30 mM trimethadione
Coagulation	8%	0%***
Malformed embryos	2%	93%***
Hatching	0%	52%***
Curved tail	2%	60%***
Edema pericard	2%	75%***
Blood accumulation yolk	0%	12%*
Malformation yolk	2%	33%***
No blood circulation	2%	50%***
Absence of a heartbeat	2%	13%*
Deviating shape of mouth	2%	22%**

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$

Table 5. Overview of coagulation and malformations in the group exposed to 30 mM trimethadione at 72 hpf.

Affected parameter	Medium control	30 mM trimethadione
Coagulation	8%	0%
Malformed embryos	2%	60%***
Hatching	38%	80%*
Curved tail	4%	42%**
Edema pericard	2%	55%***
Blood accumulation yolk	0%	5%
Malformation yolk	2%	10%
No blood circulation	2%	18%**
Absence of a heartbeat	0%	3%
Deviating shape of mouth	2%	0%

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$

3.3.2 mZEDTA

For the mZEDTA, we performed morphological experiments with undiluted and 1:10, 1:20 and 1:30 diluted supernatant of an ultracentrifuged blank control (i.e., pre-incubation mix of human liver microsomes at 200 µg/mL and NADPH tetrasodium salt (1.25 mM) in embryo medium). Our experiment was valid, as the medium controls had a coagulation of less than 10% (see Table 6) and the fertilization rate was above 90% (data not shown). The morphological data analysis was carried out as described above in the ZEDTA experiment with 30 mM trimethadione. The results are depicted in Table 6. When using the undiluted supernatant of the blank control, a slight increase in yolk malformations was noted ($p < 0.01$), although the total number of affected embryos did not reach statistical significance ($p = 0.0528$). Exposing zebrafish embryos to the 1:10 or higher diluted supernatant of the ultracentrifuged pre-incubation system showed no effect on any of the morphological parameters. These data confirm that our pre-incubation protocol is not embryotoxic and consequently zebrafish embryos can be exposed during the main organogenesis period to 1:10 diluted supernatant of our ultracentrifuged metabolic activation system. However, as the 1:10 dilution may also dilute the effect of the toxic metabolite in the supernatant, one must be cautious

and the activity of metabolic activation system, i.e., the obtained metabolite concentrations, must be assessed, especially in case of negative results.

Table 6. Overview of coagulation and malformations in the group exposed to the blank control after ultracentrifugation.

Affected parameter	Medium control	Ultracentrifuged blank control
Coagulation	5%	10%
Malformed embryos	3%	17%
Edema pericard	3%	3%
Edema yolk	0%	3%
Blood accumulation yolk	0%	3%
Malformation yolk	0%	17%**
Malformation heart	0%	3%
No blood circulation	0%	3%
Deviating shape of head	0%	3%
Deviating shape of mouth	0%	3%

** $p < 0.01$

To test this part of our protocol, we used carbamazepine as tool compound. Carbamazepine is an anti-epileptic drug and a human teratogen. It is believed that its metabolite carbamazepine-10,11-epoxide is causing the malformations. As depicted in Table 7, we used 4 conditions, i.e., 250 µM carbamazepine (Sigma-Aldrich, Diegem, Belgium) and 125 µM carbamazepine-10,11-epoxide (Sigma- Aldrich, Diegem, Belgium) as internal controls for our analytical measurement, undiluted supernatant after step 14' to assess consumption of carbamazepine, and our test solution, 1:10 diluted supernatant after ultracentrifugation of the pre-incubation mix, to determine the actual concentration of the metabolite carbamazepine-10,11-epoxide in our mZEDTA. Prior to the analysis of the test conditions, we determined the stability of 500 µM carbamazepine in embryo medium (according to the stability protocol in our ZEDTA). Uptake by the zebrafish embryos was not assessed, as a dose-response in morphological defects had already been established by others for this compound [12].

Table 7. Identification of the samples that were analyzed with LC-MS for presence of carbamazepine and carbamazepine-10,11-epoxide.

Sample content	Estimated molar concentration (µM)	Estimated mass concentration (µg/mL)
Parent (carbamazepine)	250	59.0
Ultracentrifuged (UC'ed) pre-incubation mixture (carbamazepine)	250	59.0
1/10 ultracentrifuged (UC'ed) pre-incubation mixture (carbamazepine)	25	5.90
Human metabolite (carbamazepine-10,11-epoxide)	125	31.5

The following analytical protocol was used:
First, the carbamazepine samples (parent and ultracentrifuged pre-incubation mixture in Table 7) were diluted in water (HPLC grade) by a factor of 10. These diluted samples were once again diluted by a factor of 10 in 70:30 (v/v) water:acetonitrile (HPLC grade) containing 0.39 µM lamotrigine (Sigma-Aldrich, Diegem, Belgium) as an internal standard. In order to quantify the concentrations

of carbamazepine and carbamazepine-10,11-epoxide in these samples, standard curves (in replicate) for carbamazepine (17.5 - 50,000 ng/mL) and carbamazepine-10,11-epoxide (1.7 – 50,000 ng/mL) were prepared. These samples were subsequently diluted by a factor of 10 in water:acetonitrile containing 0.39 µM lamotrigine. The samples were then stored at −80 °C upon use.

The analytical investigation was realized on an Acquity Ultra Performance LC with sample manager, binary solvent manager, diode array detector (DAD) and a triple quadrupole (TQ) detector (ACQUITY UPLC-TQ detector, Waters, Milford USA), equipped with MassLynx software (version 4.1). Chromatographic separation was performed on an Acquity UPLC HSS T3 (2.1 × 100 mm; 1.8 µm) column (Waters, Milford USA) and elution was conducted with a mobile phase consisting of water with 0.1% formic acid (A) and acetonitrile containing 0.1% of formic acid (B). Chromatographic separation of the three analytes was accomplished in 6 min, using a flow rate of 0.5 mL/min and the solvent gradient program was set as follows: 85% A / 15% B (0–0.5 min); 85–0% A / 15–100% B (0.5– 3.3 min); 0% A / 100% B (3.3–4.4 min); 0–85% A / 100–15% B (4.4–4.5 min); 85% A / 15% B (4.5–6 min). The column was set at 40 °C and the injection volume was 10 µL. As mass spectrometric conditions, the following parameters were used for data acquisition in positive ionization mode: capillarity voltage 3.5 kV, extractor voltage 3 V, cone voltage 35 V, Rf lens 0.1 V. The source temperature was set at 120 °C and the desolvation temperature was set at 450 °C. The desolvation gas flow was 1,000 L/h and the cone gas flow was 50 L/h.

Quantification of the analytes was realized via multiple reaction monitoring in positive ion mode of the ion transitions of carbamazepine, carbamazepine-10,11-epoxide and the internal standard, lamotrigine. Following transitions were chosen as quantifier and qualifiers for carbamazepine *m/z* 237→194 (cone voltage 37 V, Ecollision 22 kV) and *m/z* 237→165 (cone voltage 37 V, Ecollision). For carbamazepine-10,11-epoxide the chosen transitions were *m/z* 253 →180 m/z (cone voltage 25 V, Ecollision 38 kV) and *m/z* 253→236 (cone voltage 25 V, Ecollision 12 kV). To finish, for the internal standard lamotrigine, the transitions followed were *m/z* 256→ 211 (cone voltage 25V, Ecollision 25 kV) and *m/z* 256→108 (cone voltage 56 V, Ecollision 35 kV).

The following results were obtained:
Carbamazepine was stable over the entire period of organogenesis (see Figure 6). Carbamazepine was metabolized by 25.3 ± 2.9% after 1 h incubation in our metabolic activation system, indicated by the remaining concentration of carbamazepine in the supernatant of the ultracentrifuged pre-incubation mixture (see Figure 7). The 1:10 dilution of this supernatant showed about a 10-fold lower carbamazepine concentration. For carbamazepine-10,11-epoxide, no metabolite was found in the control sample of carbamazepine (parent compound without pre-incubation), as it should be. In the ultracentrifuged and 1:10 ultracentrifuged samples of the pre-incubation mix, the concentration of carbamazepine-10,11-epoxide was about 150-fold lower than carbamazepine under these conditions, resulting in a concentration of about 0.1 µM carbamazepine-10,11-epoxide in the test solution (see Figure 7). As most developmental toxicants exert their effect in zebrafish embryos in the µM to mM range, 0.1 µM carbamazepine-10,11-epoxide is believed to be too low to further assess this test solution morphologically in our ZEDTA. We will first perform a range

finding study with different concentrations of carbamazepine-10,11-epoxide in order to determine the concentration that causes malformations in our ZEDTA. Depending on this outcome, we will increase the concentrations of carbamazepine in our metabolic activation system (up to substrate inhibition) first (as depicted in step 18 of our mZEDTA protocol) and when the ZEDTA remains negative, we will concentrate our test solution (see step 19 of the mZEDTA) by using a volatile solvent, such as methanol, followed by evaporation when higher concentrations of carbamazepine-10,11-epoxide are needed. This latter procedure has recently been proven successful for chemicals when using zebrafish embryos/larvae of 72 hpf [13].
So, in conclusion we developed a non-embryotoxic metabolic activation protocol that is generating the toxic metabolite. When necessary, the metabolite concentrations can be increased.

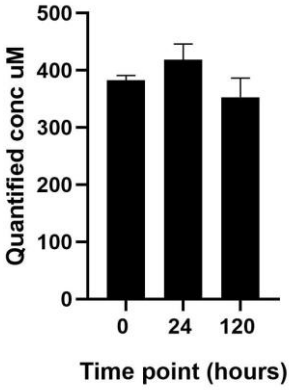


Figure 6. Quantified concentrations of carbamazepine (500 µM, 2 replicates) in embryo medium at 0, 24 and 120 h.

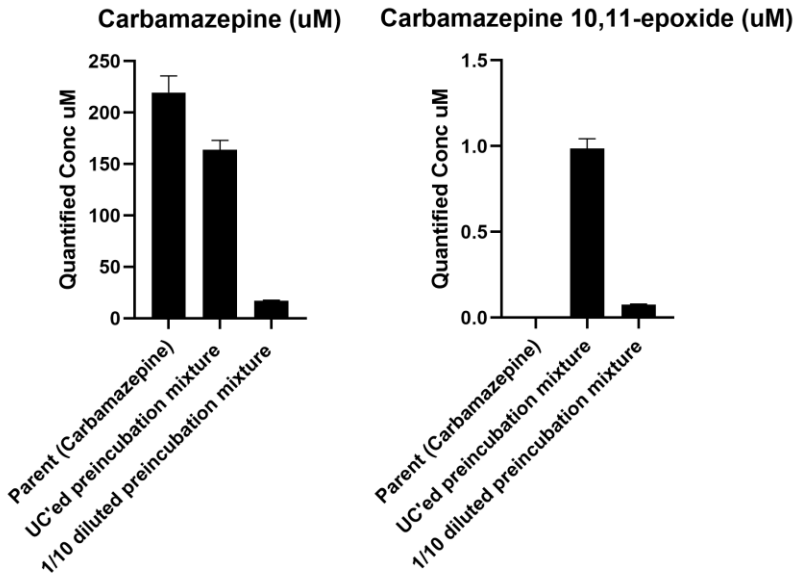


Figure 7. Quantified concentrations (µM (= uM); 2 replicates) of carbamazepine (left panel) and its toxic metabolite carbamazepine-10,11-epoxide (right panel) in the parent compound solution (without incubation), the undiluted supernatant of the ultracentrifuged (UC'ed) pre-incubation mix and the test solution, i.e., 1:10 diluted supernatant of the ultracentrifuged (UC'ed) pre-incubation mix.

3.3.3 sZEDTA

For the skeletal staining protocol, we used rosiglitazone (Merck Life Science UK Ltd, Dorset, UK) as test compound. Rosiglitazone is an anti-diabetic compound (PPAR gamma agonist) shown to inhibit osteoblast differentiation and activate osteoclast differentiation in several human *in vitro* and transgenic mice models. As data were lacking for the zebrafish embryo, we first performed a dose range finding study in order to find non-lethal concentrations of rosiglitazone. Concentrations of 15 µM and 20 µM caused lethality in zebrafish embryos, whereas 12.5 µM did not show any effects on survival nor gross morphology (data not shown). Therefore, a test solution of 12.5 µM rosiglitazone in 0.1% DMSO embryo medium was chosen for our sZEDTA protocol. A medium and solvent control were included. Statistical analysis was carried out with GraphPad Prism 8 (San Diego, CA, USA). Intensity data were analyzed by means of the Kruskal Wallis test with the Dunn's post hoc test. Results were considered significant at $p \leq 0.05$. There was no difference in intensity or shape for any of the bones between the solvent and medium controls. Rosiglitazone showed significant differences in bone intensities compared to the solvent control (see Figure 8 and Figure 9). The parasphenoid was less intensely stained, whereas the right utricular otolith and right circular otolith, the left and right entopterygoid, and the left and right branchiostegal rays 2 were more intensely stained. There was no effect on the shape of the bones. This skeletal staining will be further validated with proprietary and non-proprietary compounds in a consortium effort of the European Teratology Society.

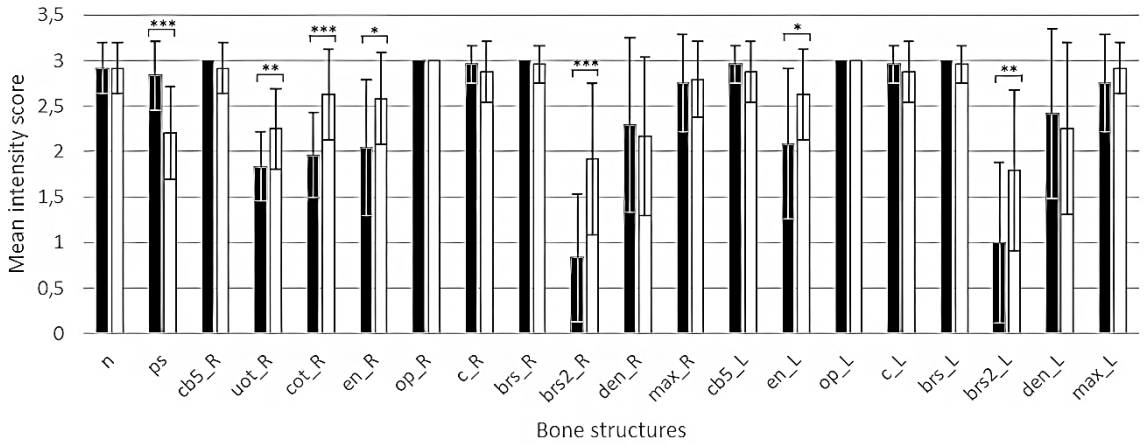


Figure 8. Mean intensity score (and SD) of each bone structure in 120 hpf zebrafish larvae (n = 20/group) for the solvent control group (DMSO; black bars) and for the group exposed to 12.5 µM rosiglitazone (white bars). For abbreviations of the bone structures, see Table 3. Left (L); right (R); *p < 0.05; **p < 0.01; ***p < 0.001; t_L, t_R, uot_L and cot_L could not be scored because they were hidden behind other bone structures.

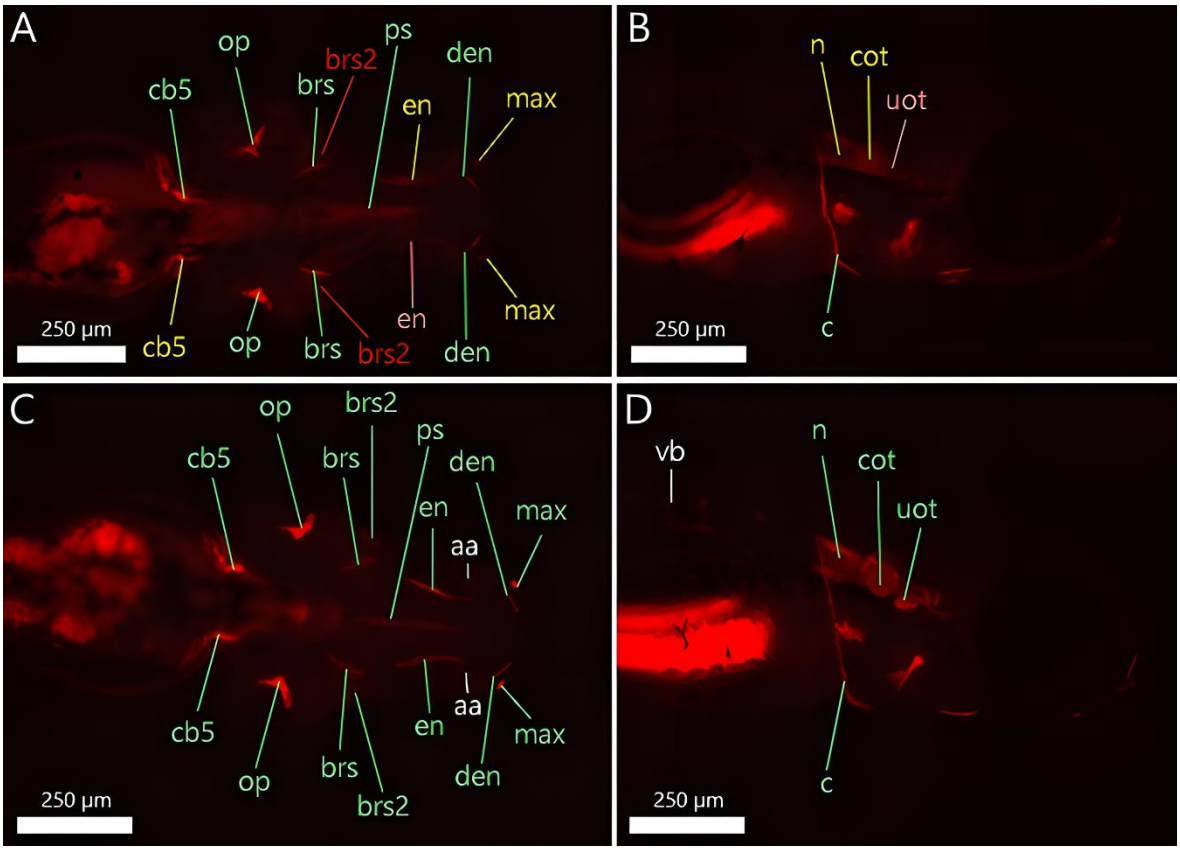
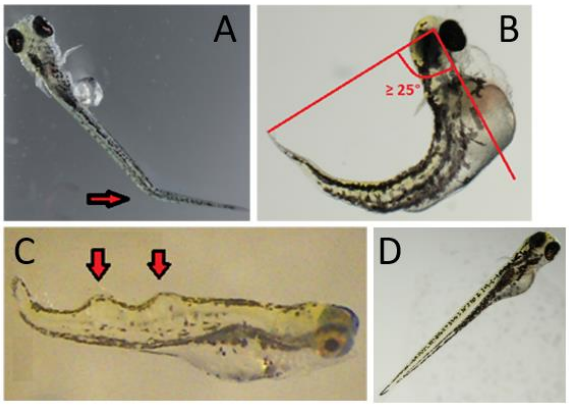
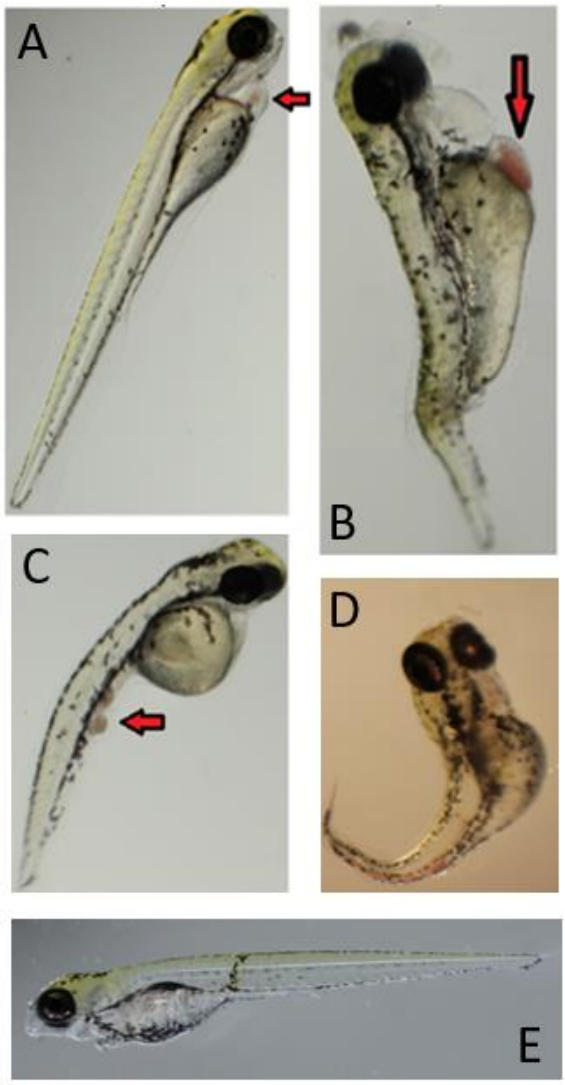


Figure 9. Solvent control (A-B) and 12.5 µM rosiglitazone exposed (C-D) zebrafish larvae at 120 hpf with bone structures that are stained with 0.005% alizarin red. Left panels (A-C) show a dorsal view. Right panels (B-D) show lateral view. The abbreviations are depicted in Table 3. The different colors of the abbreviated bone structures indicate different scores of intensities (green = score 3; yellow = score 2; pink = score 1; red = score 0). White-indicated structures (aa = anguloarticular bone; vb = vertebra) are structures that were stained, but expected at a later developmental stage.

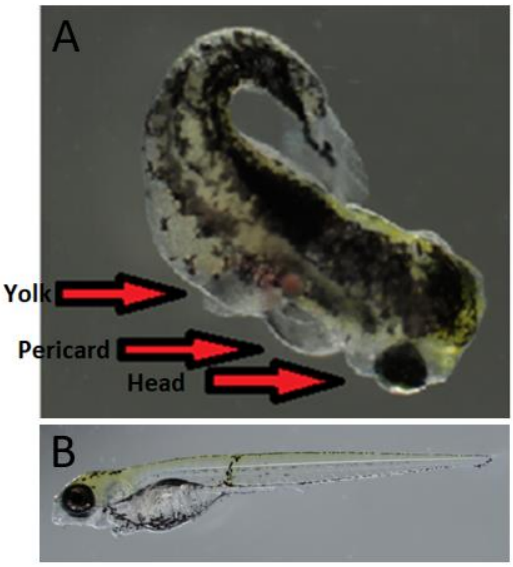
3.4 Supplementary figures



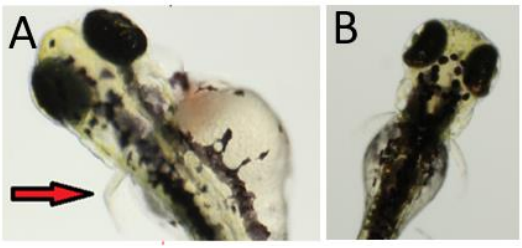
Supplementary Figure 1. Tail malformations in zebrafish larvae. A) Elbow, B) Curve (if curve ≥25°), C) Tissue deviation and D) No tail malformations.



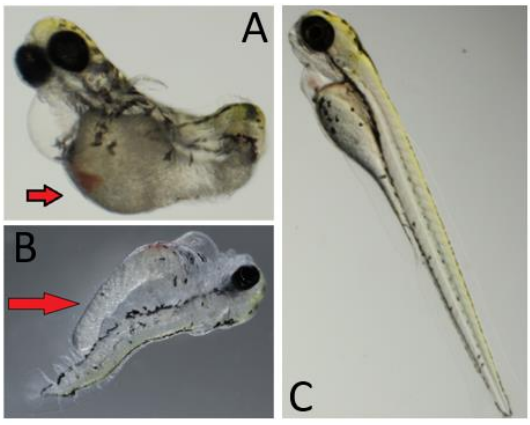
Supplementary Figure 3. Blood accumulation in zebrafish larvae in: A) the pericard, B) the yolk, C) the yolk extension and D) the tail. E) No blood accumulation.



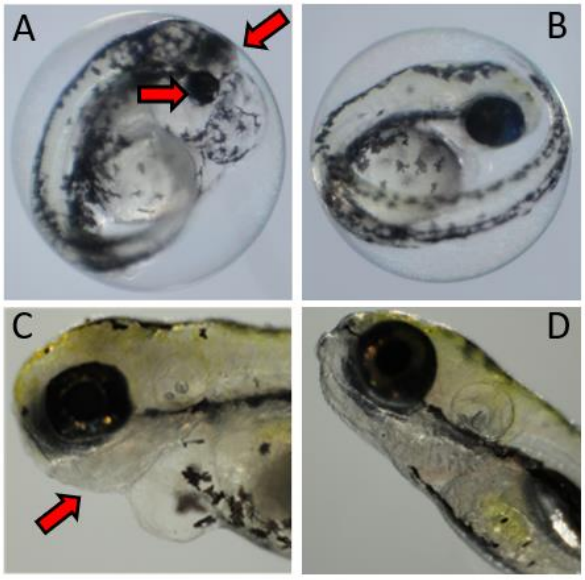
Supplementary Figure 2. Edema in zebrafish larvae. A) Yolk, pericard and head edema, B) No edema.



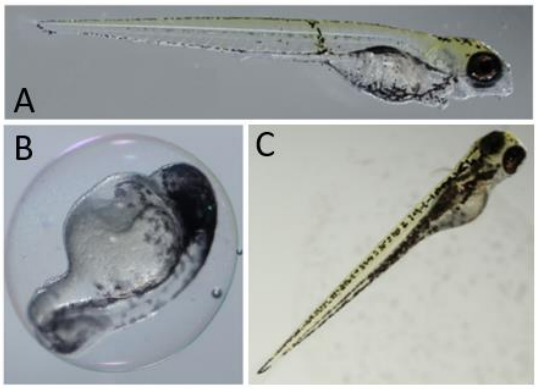
Supplementary Figure 4. Fin malformations in zebrafish larvae. A) Curved fin, B) Normal fins.



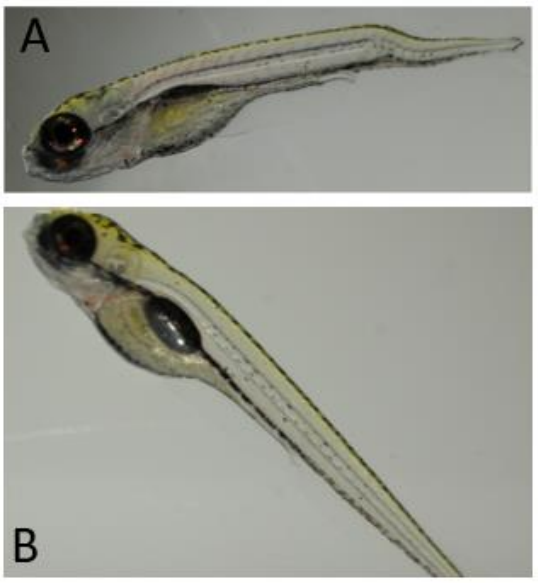
Supplementary Figure 5. Yolk malformations in zebrafish larvae. A&B) Larva with malformed yolk, C) No yolk malformations.



Supplementary Figure 6. Malformations of the head. A) Malformation of the eye and head shape, B&D) No head malformations, C) Malformation of the mouth.



Supplementary Figure 7. Deviating pigmentation. A&B) Larva with deviating pigmentation, C) Normal pigmentation.



Supplementary Figure 8. Deviating swim bladder. A) Swim bladder not inflated. B) Swim bladder inflated, normal.

3.5 References

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CHAPTER 4:
Determination of the maximal concentration of DMSO that can be safely used as a solvent

Adapted from: J. Hoyberghs, C. Bars, M. Ayuso, C. Van Ginneken, K. Foubert, S. Van Cruchten. DMSO Concentrations up to 1% are Safe to be Used in the Zebrafish Embryo Developmental Toxicity Assay. Front. Toxicol. 3 (2021) 804033. doi: 10.3389/ftox.2021.804033.

Abstract

Dimethyl sulfoxide (DMSO) is a popular solvent for developmental toxicity testing of chemicals and pharmaceuticals in zebrafish embryos. In general, it is recommended to keep the final DMSO concentration as low as possible for zebrafish embryos, preferably not exceeding 100 µL/L (0.01%). However, higher concentrations of DMSO are often required to dissolve compounds in an aqueous medium. The aim of this study was to determine the highest concentration of DMSO that can be safely used in our standardized Zebrafish Embryo Developmental Toxicity Assay (ZEDTA). In the first part of this study, zebrafish embryos were exposed to different concentrations (0%, 0.01%, 0.1%, 0.5%, 1%, and 2%) of DMSO. No increase in lethality or malformations was observed when using DMSO concentrations up to 1%. In a follow-up experiment, we assessed whether compounds that cause no developmental toxicity in the ZEDTA remain negative when dissolved in 1% DMSO, as false positive results due to physiological disturbances by DMSO should be avoided. To this end, zebrafish embryos were exposed to ascorbic acid and hydrochlorothiazide dissolved in 1% DMSO. Negative control groups were also included. No significant increase in malformations or lethality was observed in any of the groups. In conclusion, DMSO concentrations up to 1% can be safely used to dissolve compounds in the ZEDTA.

4.1 Introduction

Zebrafish embryos are gaining interest as an alternative to animal testing for developmental toxicity screening of candidate drugs and chemicals. Zebrafish embryo-based assays are therefore already used for this purpose by different research groups [1–10] and in our group we refer to this assay as the ZEDTA, i.e., Zebrafish Embryo Developmental Toxicity Assay [11–13]. This term will be further used throughout the manuscript for assays that are using zebrafish embryos for developmental toxicity screening of chemicals and pharmaceuticals. The ZEDTA is an *in vitro* test in which the morphological effects of pharmaceuticals and chemicals are assessed in a whole vertebrate organism during the period of organogenesis. Its use during screening has many advantages, as it reduces the use of laboratory animals, it is more cost-effective than the mammalian *in vivo* studies, results are obtained fast due to the short organogenesis period (5.25 hours post-fertilization (hpf) until 120 hpf) and only a small amount of test compound is needed due to the small size of the embryos. However, many xenobiotics are rather hydrophobic [1,14], and therefore organic solvents are needed to solubilize the compounds of interest for exposure experiments in zebrafish embryos [15].

Dimethyl sulfoxide (DMSO) is often used to dissolve compounds when using zebrafish embryos for toxicity screening, as it appears to be less toxic in the zebrafish embryo model than other well-known solvents [16]. High concentrations of DMSO, however, are toxic for zebrafish embryos and larvae [17–19]. Therefore, the Organization for Economic Cooperation and Development (OECD) recommends to keep the final solvent concentration as low as possible, preferably not exceeding 100 µL/L (0.01%), in the (zebra)Fish Embryo Acute Toxicity (zFET) Test (TG236) [20]. However, higher concentrations of DMSO are often required to dissolve compounds in an aqueous medium.

Indeed, for teratogenicity screening several research groups report the use of DMSO concentrations that are higher than 0.01% [1–4,6,7,9] and this is also true for other types of toxicity assessment, such as developmental neurotoxicity [21]. However, these DMSO concentrations vary and are scattered in literature, as most laboratories only report the concentration that was needed to dissolve their test compounds. As we recently standardized our ZEDTA [11] with defined exposure window, group size, and morphological endpoints to test several compounds, we need to know which concentrations of DMSO are safe to be used in our ZEDTA when dissolving these test compounds.

When reviewing literature, the threshold for morphological abnormalities caused by DMSO appears to vary between 1.0% and 2.5%, depending on the investigated endpoints, exposure duration and developmental stage of the zebrafish [17–19,22,23]. These upper limits cannot simply be implemented in our standardized ZEDTA, as there are quite some differences in the exposure window and morphological endpoints used in those studies when compared to our standardized protocol [11,17–19,22,23]. A first difference between our standardized protocol and other studies is the duration of the exposure and stage of the embryo/larva at termination of the study when morphological analysis is undertaken. In our standardized ZEDTA, exposure starts at 5.25 hpf and lasts until 120 hpf (i.e., the period of organogenesis). In several other studies and also in OECD guideline 236, the reference protocol for the zFET test, the exposure started at approximately the same developmental stage, but ended at 96 hpf [17,19,20,24,25]. As a result of this shorter exposure period, malformations that occur between 96 hpf and 120 hpf would be missed, and as such the toxic properties of DMSO may be underestimated. In some other studies, the exposure period lasted until after the organogenesis period [18,22]. In this way, effects that occur after the organogenesis period might overestimate the developmental toxicity of DMSO. Finally, effects of DMSO have also been reported for only a 24 h exposure period and starting from different developmental stages [23]. This study design is very informative to assess the susceptibility of different developmental stages to DMSO, but as the exposure period is short, effects that occur after a longer exposure period may be missed. A second difference between other studies and our standardized ZEDTA is the list of morphological endpoints that was evaluated, which is often rather limited in other studies. For some studies this can be explained by a focus on other endpoints than gross morphology [17,22], whereas for others the malformations were not specified and a rather general terminology was used (e.g. abnormal development, crooked body, etc.) [18,19,22]. As such, the toxic effects of DMSO may be underestimated. Finally, there are also other differences in study design between our standardized ZEDTA and other studies. The number of embryos per group, the number of replicates, the incubation temperature, the number of medium changes, and/or the evaluated timepoints, etc. are different from what we use in our standardized ZEDTA [11], and this might also influence the obtained results.

Based on the above, we decided to determine the maximum concentration of DMSO that can safely be used as solvent in our standardized ZEDTA. The results of this study will also benefit the broader scientific community when using this solvent for developmental toxicity testing of xenobiotics in the zebrafish embryo. In a first experiment, we used 2% DMSO as the highest concentration to be tested, as this appeared to be the maximum tolerated DMSO concentration in literature when

exposing the zebrafish embryos from 5.25 hpf until 96 hpf. In a second experiment, we evaluated two non-teratogens in combination with the maximum tolerated DMSO concentration of the first experiment, as very recently combined toxic effects of DMSO with chemicals that are non-toxic by themselves have been reported [25]. We opted for ascorbic acid and hydrochlorothiazide, as these compounds were tested in zebrafish embryos at high concentrations in combination with 0.5% DMSO and showed no developmental toxicity [3]. Furthermore, ascorbic acid is water soluble and DMSO is not strictly required, whereas hydrochlorothiazide requires DMSO to be solubilized but at concentrations lower than 1%.

4.2 Materials and methods

4.2.1 Chemicals and solutions

Embryo medium was made by dissolving 0.60 g of Instant Ocean® Sea Salt (Blacksburg, VA, United States) and 0.038 g of sodium bicarbonate (Sigma, Diegem, Belgium) in 2 L reverse osmosis (RO) water (pH 7.4 ± 0.3) (Barnstead™ Pacific™ RO Water Purification System, Thermo Scientific™, Waltham, MA, USA). The MS-222 solution (1 g/L) was made by dissolving methyl ethane sulfonate (i.e., MS-222) (Sigma) in embryo medium, and the pH was adjusted to 7.4 ± 0.3 with 1 M NaOH. For the first experiment, DMSO (Sigma) was added to embryo medium to obtain the different DMSO concentrations (0.01%, 0.1%, 0.5%, 1%, and 2%). For the second experiment, the following solutions were prepared: embryo medium (medium control), a 1% DMSO (Sigma) solution in embryo medium (solvent control), a 100 µM ascorbic acid (AA) (Sigma) solution in embryo medium, a 100 µM AA (Sigma) solution in embryo medium containing 1% DMSO, and a 1,000 µM hydrochlorothiazide (HCT) (Sigma) solution in embryo medium containing 1% DMSO.

4.2.2 Housing and egg collection

Experiments were conducted according to our standardized ZEDTA protocol [11]. In brief, adult zebrafish (*Danio rerio*) of the AB strain were used as breeding stock. The ratio of males to females was 50/50 and the fish density was <1 fish/L. The 60 L tanks that were used to house the adult fish were filled with reverse osmosis water (Barnstead™ Pacific™ RO Water Purification System, Thermo Scientific™) with Instant Ocean® Sea Salt (Blacksburg) and sodium bicarbonate (Merck, Darmstadt, Germany) to reach a pH of 7.5 ± 0.3 and a conductivity of 500 ± 40 µS/cm. The temperature was set at 28.5 ± 0.3 °C, and the tanks were enriched with plastic plants. Fish health and water parameters were checked daily. The limits for ammonia, nitrite and nitrate levels were <0.02 mg/L, <0.3 mg/L, and ≤12.5 mg/L, respectively. Adult fish were daily fed with thawed Artemia, Daphnia or red, black or white mosquito larvae (alternating; Ruto Frozen Fish food, Montford, The Netherlands). By means of an automated lighting system, fish were exposed to a cycle of 14/10 hours light/dark.

For embryo collection, ~30 adult fish were transferred into a spawning tank the evening before the planned egg collection. To avoid faeces and dirt in the spawning tank as much as possible, fish were fed at the latest at 9 a.m. in the morning on the day before collection. To prevent the fish from

eating their eggs, the spawning tank was equipped with two nets at the bottom where the eggs could pass through, but the fish could not. On the day of the collection, the fish were allowed to spawn and fertilize eggs for approximately 1 h after the lights turned on. The fish were transferred back to their normal tank, and eggs were collected from the bottom of the spawning tank. To remove the faeces and coagulated eggs, the embryos were washed two times in embryo medium. Then, the embryos were transferred into 48 well plates (Cellstar®, Greiner Bio-One, Frickenhausen, Germany), and only embryos with a normal cell division were selected using an Olympus CKX41 microscope (Olympus U-TV0.5XC-3 lighting; Olympus 4x/0.16 UplanAPO microscope objective) (Olympus Life Science, Shinjuku, Tokyo, Japan). The selected eggs were randomly transferred into new 48 well plates filled with embryo medium and kept at 28.5°C ± 0.3°C in a TIN-IN35 incubator (Phoenix instrument, Garbsen, Germany) with LED strips (LED02102-1, LEDStripXL, Deventer, The Netherlands) attached on the inside. Coagulated and malformed eggs were euthanized with 1 g/L tricaine methane sulfonate (MS-222), pH 7.4 (buffered).

4.2.3 Handling and exposure of zebrafish embryos

4.2.3.1 First experiment

The experiment consisted of a medium control group (embryo medium) and 5 test groups (0.01%, 0.1%, 0.5%, 1%, and 2% DMSO). Each experiment (n = 20/group) was replicated twice. 48-well plates with a total volume of 300 µL/well were used.

At the latest at 5.25 hpf, the embryos were exposed to the control and test solutions and placed in the incubator (28.5°C ± 0.3°C with a 14/10 h light/dark cycle). To avoid acidification and oxygen deprivation, the embryo medium or test solution was renewed every 48 h [12]. In addition, the pH of all test solutions was checked prior to exposing the embryos and after an incubation period of 48 h, to make sure a physiological pH was maintained throughout the experiment. A batch of eggs was considered to be valid for experimentation when a minimum of 80% of all eggs were fertilized and the rate of mortality and malformations of the controls was lower than, or equal to, 10% throughout the experiment [13].

4.2.3.2 Second experiment

The experiment consisted of a medium control group (embryo medium), a solvent control group (1% DMSO in embryo medium) and 3 test groups: 1) 100 µM ascorbic acid in embryo medium, 2) 100 µM ascorbic acid in 1% DMSO with embryo medium, and 3) 1,000 µM hydrochlorothiazide in 1% DMSO with embryo medium. The concentrations, 100 µM of ascorbic acid and 1,000 µM of hydrochlorothiazide, were based on Gustafson, et al. (2012) [3]. Each experiment (n = 20/group) was replicated twice. 48-well plates with a total volume of 300 µL/well were used. Exposure of embryos to control and test solutions was performed as described in 4.2.3.1.

4.2.4 Morphological evaluation

Zebrafish embryos were evaluated for several morphological endpoints (see Table 1) at 5.25, 10, 24, 48, 72, 96, and 120 hpf [11] using an Olympus CKX41 microscope (Olympus Life Science). The endpoints that were evaluated were: coagulation/lethality, no hatching, body parts indistinguishable or unrecognizable, deviations of the tail (curve, elbow and tissue), edema (head, pericard, yolk and yolk extension), blood accumulation (tail, head, heart, yolk and yolk extension), malformation of the pectoral fins (missing or curved), malformation of the cardiovascular system (malformation heart, heartbeat absent, no blood circulation in the tail, disturbed blood circulation in the tail), malformation of the head (deviating shape, deviation ear, deviation mouth, deviation eye), deviating pigmentation, malformation of the yolk, and non-detachment of the tail [11]. The 5.25 and 10 hpf timepoints were used as a last check-up to replace eggs that coagulated or started to show aberrations in development with spare eggs (also exposed at the latest at 5.25 hpf). From 24 hpf onwards, parameters were checked and scored 0 if they appeared to be normal and 1 if they were malformed. After the last gross morphology scoring at 120 hpf, the larvae were euthanized by means of an overdose of MS-222 (1 g/L in embryo medium) after which they were snap-frozen in liquid nitrogen to ensure death.

Table 1. General overview of morphological scoring of zebrafish embryos at different developmental stages in the ZEDTA. A detailed list of endpoints can be found in [11].

	Stage (hpf)						
	5.25	10	24	48	72	96	120
Coagulation/lethality	+	+	+	+	+	+	+
Hatching				+	+	+	+
Tail deviation			+	+	+	+	+
Edema			+	+	+	+	+
Blood accumulation			+	+	+	+	+
Malformation of the cardiovascular system			+	+	+	+	+
Malformation of the head			+	+	+	+	+
Malformation of the pectoral fins					+	+	+

hpf = hours post-fertilization

4.2.5 Statistical analysis

For the binary scoring data, a Fisher Exact test was used. P-values of ≤0.05 were considered to indicate statistically significant differences. All statistical analyses were performed using GraphPad Prism 8.4.0 or newer versions (GraphPad Software, Inc., San Diego, CA, USA).

4.3 Results

4.3.1 Exposure to a range of DMSO concentrations

Both replicates in our first experiment were valid, as ≥80% of all eggs were fertilized and the total number of malformed or dead larvae in the control groups (i.e., embryo solution) was ≤10% at the end of the experiment. The pH of all test solutions remained in a physiological range throughout

the experiment (i.e., pH 7.65 ± 0.10) (data not shown). For both replicates, no statistical differences were observed between any of the test groups and the control group (see Table 2 and Table 3). For all test groups, except for the 2% DMSO group, the total number of embryos/larvae that had at least one malformation or were dead at 120 hpf was less than, or equal to, 10% (i.e., 2/20). This cut-off of ≤10% is important, as the highest DMSO concentration will be used as a solvent control in future experiments.

In the 2% DMSO group, a total of 6 out of 20 larvae (30%) in the first replicate (see Table 2) and 5 out of 20 larvae (25%) in the second replicate (see Table 3) had at least one malformation or were dead. In the first replicate, four of these larvae (20%) were dead, while there was only one larva (5%) in the second replicate. The malformations that were observed in the 2% DMSO group of the first replicate were: tissue deviation of the tail and of the body, yolk edema, curved fin, and disturbed blood circulation in the tail. In the second replicate, tissue deviation of the tail and the body, yolk edema, pericardial edema, head edema, malformation of the yolk, no blood circulation in the tail, deviating shape of the head, and deviation of the eye were observed. In both replicates, tissue deviation of the tail or/and the body, which was observed as cell death in these areas, showed to be the most prominent malformation (see Figure 1) and was present in all of the alive, malformed larvae. As such, the 2% DMSO group has more than 10% malformed and/or dead larvae, which means that using 2% DMSO as a solvent control group makes the experiment invalid, and therefore cannot be used.

Additionally, when looking at the 2% DMSO group at different developmental stages, we noted that the total number of malformed/dead larvae was significantly increased at 120 hpf when compared to the start of the exposure (5.25 hpf), while there was no significant increase in the total number of malformed/dead larvae at 96 hpf (see Figure 2).

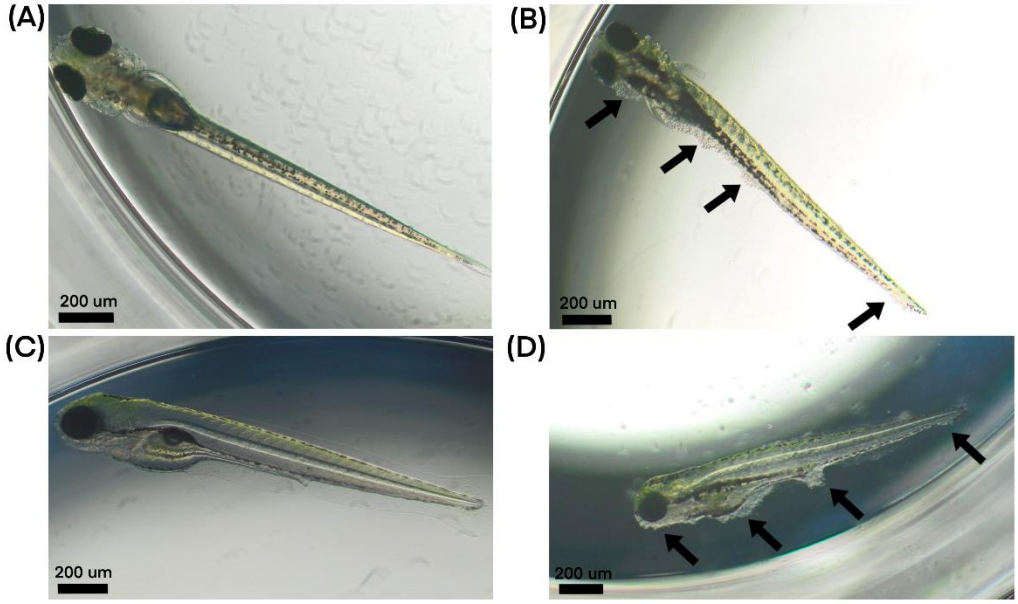

Figure 1. Larvae at 120 hpf. A-C) Normally developed control larvae. B-D) Larvae treated with 2% DMSO that developed tissue deviations of the tail and the body (i.e., areas of cell death) (arrows).

Table 2. Overview of lethality and malformations in the test groups at 120 hpf in replicate 1. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/lethality and total ≥1 malformations (incl. dead), this total number of larvae consisted only of larvae that were alive.

Parameter	Control	0.01% DMSO	0.1% DMSO	0.5% DMSO	1% DMSO	2% DMSO
Coagulation/lethality	0/20	**1/20**	**1/20**	0/20	0/20	**4/20**
Tot. ≥1 malf. (incl. dead)	**1/20**	**2/20**	**2/20**	**2/20**	0/20	**6/20**
Tot. ≥1 malf. (excl. dead)	**1/20**	**1/19**	**1/19**	**2/20**	0/20	**2/16**
BP indistinguishable	0/20	0/19	0/19	0/20	0/20	0/16
BP unrecognizable	0/20	0/19	0/19	0/20	0/20	0/16
No hatching	0/20	0/19	0/19	0/20	0/20	0/16
Elbow tail	0/20	0/19	0/19	0/20	0/20	0/16
Curved tail	0/20	0/19	0/19	**2/20**	0/20	0/16
Tissue deviation tail	**1/20**	**1/19**	0/19	0/20	0/20	**2/16**
Edema head	0/20	0/19	**1/19**	0/20	0/20	0/16
Edema pericard	0/20	0/19	**1/19**	0/20	0/20	0/16
Edema yolk	0/20	0/19	**1/19**	0/20	0/20	**1/16**
Edema yolk ext./tail	0/20	0/19	0/19	0/20	0/20	0/16
BA tail	0/20	0/19	0/19	0/20	0/20	0/16
BA head	0/20	0/19	0/19	0/20	0/20	0/16
BA heart	0/20	0/19	0/19	0/20	0/20	0/16
BA yolk	0/20	0/19	0/19	0/20	0/20	0/16
BA yolk extension	0/20	0/19	0/19	0/20	0/20	0/16
Missing fin left	0/20	0/19	0/19	0/20	0/20	0/16
Missing fin right	0/20	0/19	0/19	0/20	0/20	0/16
Curved fin left	0/20	0/19	0/19	0/20	0/20	**1/16**
Curved fin right	0/20	0/19	0/19	0/20	0/20	0/16
Malformation yolk	0/20	0/19	**1/19**	0/20	0/20	0/16
Malformation heart	0/20	0/19	0/19	0/20	0/20	0/16
No BC in tail	0/20	0/19	0/19	0/20	0/20	0/16
Disturbed BC in tail	0/20	0/19	0/19	0/20	0/20	**1/16**
Heartbeat absent	0/20	0/19	0/19	0/20	0/20	0/16
Deviating shape of head	0/20	0/19	0/19	0/20	0/20	0/16
Deviation ear	0/20	0/19	0/19	0/20	0/20	0/16
Deviation mouth	0/20	0/19	**1/19**	0/20	0/20	0/16
Deviation eye	0/20	0/19	0/19	0/20	0/20	0/16
Deviating pigmentation	0/20	0/19	0/19	0/20	0/20	0/16
Non-detachment tail	0/20	0/19	0/19	0/20	0/20	0/16

Abbreviations: blood accumulation (BA), blood circulation (BC), body parts (BP); Tot. ≥1 malf. (excl. dead): total number of embryos/larvae that were alive and had at least one malformation. Tot. ≥1 malf. (incl. dead): total number of embryos/larvae that had at least one malformation or were dead.

Table 3. Overview of lethality and malformations in the test groups at 120 hpf in replicate 2. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/lethality and total ≥1 malformations (incl. dead), this total number of larvae consisted only of larvae that were alive.

Parameter	Control	0.01% DMSO	0.1% DMSO	0.5% DMSO	1% DMSO	2% DMSO
Coagulation/lethality	0/20	0/20	0/20	0/20	**1/20**	**1/20**
Tot. ≥1 malf. (incl. dead)	**2/20**	0/20	**1/20**	**1/20**	**1/20**	**5/20**
Tot. ≥1 malf. (excl. dead)	**2/20**	0/20	**1/20**	**1/20**	0/19	**4/19**
BP indistinguishable	0/20	0/20	0/20	0/20	0/19	0/19
BP unrecognizable	0/20	0/20	0/20	0/20	0/19	0/19
No hatching	0/20	0/20	0/20	0/20	0/19	0/19
Elbow tail	0/20	0/20	0/20	0/20	0/19	0/19
Curved tail	**1/20**	0/20	0/20	0/20	0/19	0/19
Tissue deviation tail	**1/20**	0/20	**1/20**	**1/20**	0/19	**4/19**
Edema head	0/20	0/20	0/20	0/20	0/19	**1/19**
Edema pericard	0/20	0/20	0/20	0/20	0/19	**1/19**
Edema yolk	0/20	0/20	0/20	0/20	0/19	**1/19**
Edema yolk ext./tail	0/20	0/20	0/20	0/20	0/19	0/19
BA tail	0/20	0/20	0/20	0/20	0/19	0/19
BA head	0/20	0/20	0/20	0/20	0/19	0/19
BA heart	0/20	0/20	0/20	0/20	0/19	0/19
BA yolk	0/20	0/20	0/20	0/20	0/19	0/19
BA yolk extension	0/20	0/20	0/20	0/20	0/19	0/19
Missing fin left	0/20	0/20	0/20	0/20	0/19	0/19
Missing fin right	0/20	0/20	0/20	0/20	0/19	0/19
Curved fin left	0/20	0/20	0/20	0/20	0/19	0/19
Curved fin right	0/20	0/20	0/20	0/20	0/19	0/19
Malformation yolk	0/20	0/20	0/20	0/20	0/19	**1/19**
Malformation heart	0/20	0/20	0/20	0/20	0/19	0/19
No BC in tail	0/20	0/20	0/20	0/20	0/19	**1/19**
Disturbed BC in tail	0/20	0/20	0/20	0/20	0/19	0/19
Heartbeat absent	0/20	0/20	0/20	0/20	0/19	0/19
Deviating shape of head	0/20	0/20	0/20	0/20	0/19	**1/19**
Deviation ear	0/20	0/20	0/20	0/20	0/19	0/19
Deviation mouth	0/20	0/20	0/20	0/20	0/19	0/19
Deviation eye	0/20	0/20	0/20	0/20	0/19	**1/19**
Deviating pigmentation	0/20	0/20	0/20	0/20	0/19	0/19
Non-detachment tail	0/20	0/20	0/20	0/20	0/19	0/19

Abbreviations: blood accumulation (BA), blood circulation (BC), body parts (BP); Tot. ≥1 malf. (excl. dead): total number of embryos/larvae that were alive and had at least one malformation. Tot. ≥1 malf. (incl. dead): total number of embryos/larvae that had at least one malformation or were dead.

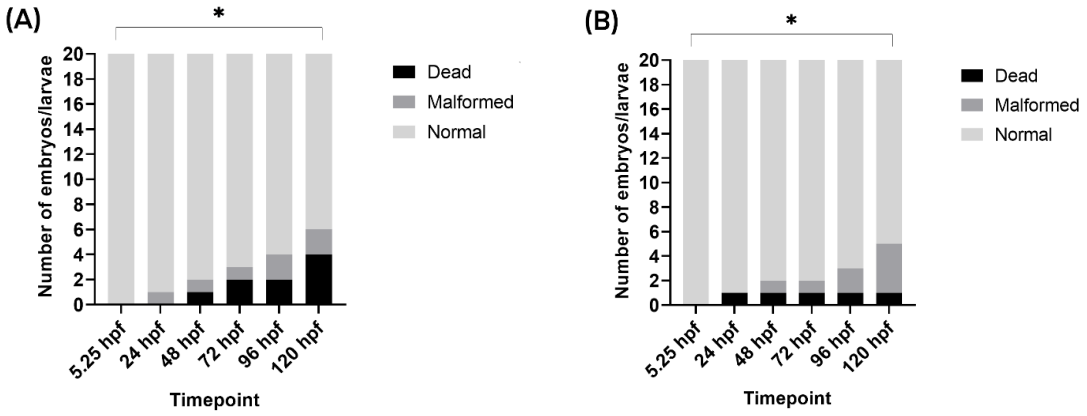

Figure 2. Overview of lethality and malformations for each timepoint after treatment with 2% DMSO. A) depicts the results of replicate 1, and B) depicts the results of replicate 2. In both replicates, the total number of dead/malformed larvae was significantly higher at 120 hpf when compared to 5.25 hpf (i.e., start of exposure). *p < 0.05

4.3.2 Exposure to a combination of DMSO and non-teratogenic compounds

Both replicates of the second experiment were valid, as ≥80% of all eggs were fertilized and the total number of malformed or dead larvae in the medium and solvent control groups (i.e., embryo solution and 1% DMSO) was ≤10% at the end of the experiment. The pH of all of the test solutions remained in a physiological range throughout the experiment (i.e., pH 7.60 ± 0.20) (data not shown). For both replicates, no statistical differences were observed between any of the test groups and the control groups (see Table 4 and Table 5).

In both replicates, there were a few more malformed larvae at 120 hpf after treatment with a combination of 100 µM of AA and 1% DMSO (3/20 or 15%), than after treatment with 100 µM AA alone (1/20 or 5%) or 1% DMSO alone (0/20 or 0% in replicate 1 and 2/20 or 10% in replicate 2). However, no statistical differences were noted. In both AA treated groups of the second replicate, only tissue deviations of the tail were observed (see Table 5). In the first replicate, a wider variety of malformations was observed (see Table 4).

For the second compound, HCT, similar results were obtained. There were no significant differences between the group treated with 1,000 µM of HCT with 1% DMSO and the solvent control (see Table 4 and Table 5). In the first replicate, there were only a few more malformations in the group treated with the combination of DMSO and HCT than in the solvent control. However, when the group treated with a combination of DMSO and HCT was compared with the medium control group, the total number of embryos/larvae that had at least one malformation was the same (i.e., 2/20 or 10%) (see Table 4). In the second replicate, the total number of embryos/larvae that had at least one malformation or were dead was even less than in the solvent control and medium control groups (see Table 5).

Table 4. Overview of lethality and malformations in the test groups at 120 hpf in replicate 1. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/lethality and total ≥1 malformations (incl. dead), this total number of larvae consisted only of larvae that were alive.

Parameter	Medium control	1% DMSO (SC)	AA (Esol)	AA (1% DMSO)	HCT (1% DMSO)
Coagulation/lethality	0/20	0/20	0/20	0/20	0/20
Tot. ≥1 malf. (incl. dead)	**2/20**	0/20	**1/20**	**3/20**	**2/20**
Tot. ≥1 malf. (excl. dead)	**2/20**	0/20	**1/20**	**3/20**	**2/20**
BP indistinguishable	0/20	0/20	0/20	0/20	0/20
BP unrecognizable	0/20	0/20	0/20	0/20	**2/20**
No hatching	0/20	0/20	0/20	0/20	**2/20**
Elbow tail	0/20	0/20	0/20	**1/20**	**2/20**
Curved tail	**1/20**	0/20	**1/20**	**2/20**	**1/20**
Tissue deviation tail	**1/20**	0/20	**1/20**	**1/20**	**2/20**
Edema head	0/20	0/20	0/20	0/20	0/20
Edema pericard	0/20	0/20	**1/20**	**1/20**	**2/20**
Edema yolk	0/20	0/20	0/20	**1/20**	0/20
Edema yolk ext./tail	0/20	0/20	0/20	0/20	0/20
BA tail	0/20	0/20	0/20	**1/20**	0/20
BA head	0/20	0/20	0/20	0/20	0/20
BA heart	0/20	0/20	0/20	0/20	0/20
BA yolk	0/20	0/20	0/20	0/20	0/20
BA yolk extension	0/20	0/20	0/20	0/20	0/20
Missing fin left	0/20	0/20	0/20	0/20	0/20
Missing fin right	0/20	0/20	0/20	0/20	0/20
Curved fin left	0/20	0/20	0/20	0/20	0/20
Curved fin right	0/20	0/20	0/20	0/20	0/20
Malformation yolk	0/20	0/20	0/20	**1/20**	**2/20**
Malformation heart	0/20	0/20	0/20	0/20	0/20
No BC in tail	0/20	0/20	0/20	**1/20**	**2/20**
Disturbed BC in tail	0/20	0/20	0/20	0/20	0/20
Heartbeat absent	0/20	0/20	0/20	0/20	0/20
Deviating shape of head	0/20	0/20	0/20	0/20	**2/20**
Deviation ear	0/20	0/20	0/20	0/20	**2/20**
Deviation mouth	0/20	0/20	0/20	0/20	**2/20**
Deviation eye	0/20	0/20	**1/20**	0/20	**2/20**
Deviating pigmentation	0/20	0/20	0/20	0/20	0/20
Non-detachment tail	0/20	0/20	0/20	0/20	0/20

Abbreviations: blood accumulation (BA), blood circulation (BC), body parts (BP); Tot. ≥1 malf. (excl. dead): total number of embryos/larvae that were alive and had at least one malformation. Tot. ≥1 malf. (incl. dead): total number of embryos/larvae that had at least one malformation or were dead.

Table 5. Overview of lethality and malformations in the test groups at 120 hpf in replicate 2. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/lethality and total ≥1 malformations (incl. dead), this total number of larvae consisted only of larvae that were alive.

Parameter	Medium control	1% DMSO (SC)	AA (Esol)	AA (1% DMSO)	HCT (1% DMSO)
Coagulation/lethality	0/20	**2/20**	0/20	0/20	0/20
Tot. ≥1 malf. (incl. dead)	**2/20**	**2/20**	**1/20**	**3/20**	**1/20**
Tot. ≥1 malf. (excl. dead)	**2/20**	0/18	**1/20**	**3/20**	**1/20**
BP indistinguishable	0/20	0/18	0/20	0/20	0/20
BP unrecognizable	0/20	0/18	0/20	0/20	0/20
No hatching	0/20	0/18	0/20	0/20	0/20
Elbow tail	0/20	0/18	0/20	0/20	0/20
Curved tail	0/20	0/18	0/20	0/20	0/20
Tissue deviation tail	**2/20**	0/18	**1/20**	**3/20**	**1/20**
Edema head	0/20	0/18	0/20	0/20	0/20
Edema pericard	0/20	0/18	0/20	0/20	0/20
Edema yolk	0/20	0/18	0/20	0/20	0/20
Edema yolk ext./tail	0/20	0/18	0/20	0/20	0/20
BA tail	0/20	0/18	0/20	0/20	0/20
BA head	0/20	0/18	0/20	0/20	0/20
BA heart	0/20	0/18	0/20	0/20	0/20
BA yolk	0/20	0/18	0/20	0/20	0/20
BA yolk extension	0/20	0/18	0/20	0/20	0/20
Missing fin left	0/20	0/18	0/20	0/20	0/20
Missing fin right	0/20	0/18	0/20	0/20	0/20
Curved fin left	0/20	0/18	0/20	0/20	0/20
Curved fin right	0/20	0/18	0/20	0/20	0/20
Malformation yolk	0/20	0/18	0/20	0/20	0/20
Malformation heart	0/20	0/18	0/20	0/20	0/20
No BC in tail	0/20	0/18	0/20	0/20	0/20
Disturbed BC in tail	0/20	0/18	0/20	0/20	0/20
Heartbeat absent	0/20	0/18	0/20	0/20	0/20
Deviating shape of head	0/20	0/18	0/20	0/20	0/20
Deviation ear	0/20	0/18	0/20	0/20	0/20
Deviation mouth	0/20	0/18	0/20	0/20	0/20
Deviation eye	0/20	0/18	0/20	0/20	0/20
Deviating pigmentation	0/20	0/18	0/20	0/20	0/20
Non-detachment tail	0/20	0/18	0/20	0/20	0/20

Abbreviations: blood accumulation (BA), blood circulation (BC), body parts (BP); Tot. ≥1 malf. (excl. dead): total number of embryos/larvae that were alive and had at least one malformation. Tot. ≥1 malf. (incl. dead): total number of embryos/larvae that had at least one malformation or were dead.

4.4 Discussion

Our study showed no statistical increase in lethality nor gross morphology malformations up to 120 hpf in all DMSO test groups when compared with the medium control group (0% DMSO). However, at 2% DMSO, more than 25% of the larvae had at least one malformation or died in both replicates, and one of the replicates showed 20% dead embryos at 120 hpf. As such, 2% DMSO cannot be used as solvent control group in future experiments with our standardized ZEDTA, as the total number of malformed and/or dead larvae in the (solvent) control group needs to be ≤10% in order to have a valid experiment. Furthermore, when comparing the number of malformed and dead embryos in the 2% DMSO group at the start of exposure (5.25 hpf) with the number of malformed and dead

embryos at the end of exposure (120 hpf), a significant increase was noted, which was absent at 96 hpf. These data show that extending the exposure in the ZEDTA to 120 hpf instead of 96 hpf makes the assay more sensitive. Also studies using shorter exposure periods indicate that extending the exposure window until 120 hf might be of added value [17,23]. Huang et al. (2018) showed that zebrafish mortality increased and LC50 values decreased in later developmental stages [17] and Maes et al. (2012) also found that later developmental stages were more sensitive than the earlier stages when exposing them for a 24 h period [23]. In many other studies, the exposure period ends at 96 hpf [17,19,24,25], but based upon the data above we recommend extending the exposure period in the ZEDTA to 120 hpf in any further experiments.

When looking more into detail to the malformations at 2% DMSO, several types of edema were observed, which was in line with other studies [18,19]. However, tissue deviation of the tail or/and the body (i.e., a collective term for all abnormalities that are visible in the tissue of the tail or/and the body) showed to be the most prominent malformations in our study, and manifested itself as cell death in these areas. These malformations have not been reported in any of the above studies, but they may have been missed, as this parameter was not included in their list of endpoints. Regarding lethality, Xiong et al. (2017) also reported an increase at 2% DMSO, as in our study, but at a later developmental stage, i.e., 7 dpf [18]. Hallare et al. (2006) found no effect on survival when exposing embryos/larvae to up to 2% [19], but their exposure period was 24 h shorter (i.e., up to 96 hpf) than in our study, confirming again the importance of extending the exposure period to 120 hpf in the ZEDTA.

Based on the data above, 1% DMSO appears to be the maximum tolerated concentration in our standardized ZEDTA. However, as other authors showed toxic effects when combining the transitional metal vanadium with 0.1% and 0.5% DMSO [25], which were absent when exposing the embryos solely to vanadium or 0.1 and 0.5% DMSO, we wanted to assess whether 1% DMSO does not cause developmental toxicity when combined with non-teratogens. Kim and Lee (2021) could relate the toxic effect to a significant decrease in pH when combining vanadium with DMSO. Large pH drops, i.e., from pH ~7 to pH ~4, were reported [25] and drastic changes in pH are well-known to have a negative impact on zebrafish development [26,27]. In our study, combining ascorbic acid and hydrochlorothiazide with 1% DMSO did not cause any developmental toxicity and the pH of the exposure medium remained within the physiological range (i.e., pH 7.60 ± 0.20).

In conclusion, we showed that 1% of DMSO can be safely used to dissolve chemicals in the ZEDTA. However, caution is needed for compounds that, with or without DMSO, change the pH of the exposure medium. We therefore recommend to check the pH of all test solutions, and adjust them to a physiological pH when needed. Furthermore, we only assessed the maximum tolerated concentration of DMSO in zebrafish embryos for developmental toxicity. When zebrafish embryos are used for other types of toxicity or when other endpoints than gross morphology are examined (e.g. hsp70 levels and behavioural responses), the DMSO concentrations may need to be further reduced, as already reported in other studies [18,19,22,24].

4.5 References

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CHAPTER 5:
Does implementation of a skeletal staining increase the sensitivity of the ZEDTA?

Adapted from: J. Hoyberghs, J. Ball, M. Trznadel, M. Beekhuijzen, M. Burbank, P. Wilhelmi, A. Muriana, N. Powles-Glover, A. Letamendia, S. Van Cruchten. Biological variability hampers the use of skeletal staining methods in zebrafish embryo developmental toxicity assays

In preparation

Abstract

Zebrafish embryo assays are used by pharmaceutical and chemical companies as new approach methodologies (NAMs) in developmental toxicity screening. Despite an overall high concordance of zebrafish embryo assays with *in vivo* mammalian studies for the classification of teratogens and non-teratogens, false negative and false positive results have been reported. False negative results in risk assessment models are of particular concern for human safety, as developmental anomalies may be missed. Interestingly, for several chemicals and drugs that were reported to be false negative in zebrafish embryo assays, skeletal findings were noted in the *in vivo* studies. As the number of skeletal endpoints assessed in zebrafish embryo developmental toxicity assays is very limited compared to the *in vivo* mammalian studies, the aim of this study was to investigate whether the sensitivity of this NAM could be increased by including a skeletal staining method. Three staining methods were tested on zebrafish embryos that were exposed to four mammalian teratogens, which were reported to cause skeletal anomalies in rats and/or rabbits and were false negative in zebrafish embryo assays. These methods included a fixed alizarin red-alcian blue staining, a calcein staining and a live alizarin red staining. The results indicated the presence of high variability in staining intensity of larvae exposed to mammalian skeletal teratogens, as well as variability between control larvae originating from the same clutch of zebrafish. Hence, biological variability in (onset of) bone development is clearly present in zebrafish embryos and larvae. As a result, this biological variability hampers the detection of (subtle) treatment-related bone effects that are not picked-up by gross morphology. In conclusion, the used skeletal staining methods could not increase the sensitivity of zebrafish embryo developmental toxicity assays.

5.1 Introduction

In recent years, new approach methodologies (NAMs) for hazard and risk assessment of xenobiotics have received a lot of attention [1]. At this moment, three NAMs for the assessment of developmental toxicity have been validated by the European Centre for the Validation of Alternative Methods (ECVAM): the rat Whole Embryo Culture test (rWEC), the mouse Embryonic Stem Cell Test (mEST) and the limb bud micromass test (MM) [2,3]. Although not validated yet, developmental toxicity assays using zebrafish embryos are currently also used for screening purposes by several pharmaceutical, (agro)chemical and cosmetic companies [4–15]. Its greatest advantage compared to the other three NAMs is that developmental effects can be assessed in a whole vertebrate organism during the main organogenesis period [16,17]. Moreover, in the European Union, studies on zebrafish embryos are not legally considered as animal experiments up to the free feeding stage (i.e., 5 dpf) (EU Directive 2010/63). Thus, the zebrafish embryo represents a holistic model that aligns well with the 3R principle for the development of NAMs [5,18].

To date, the use of NAMs in regulatory submissions for developmental and reproductive toxicology (DART) testing of pharmaceuticals has been, and is still very limited, although the third revision of the ICH S5 guideline on detection of toxicity to reproduction for human pharmaceuticals provides opportunities to do so. The guideline does not list any specific NAMs to be used, but says they

should be properly qualified [19]: *"If properly qualified, alternative assays have the potential to defer or replace (in certain circumstances) conventional in vivo studies. Approaches that incorporate alternative assays should provide a level of confidence for human safety assurance at least equivalent to that provided by the current testing paradigms."* From the above, it is clear that for zebrafish embryo assays to be considered for regulatory submissions, its potential to detect human teratogens should be well qualified.

Despite an overall high concordance (80-85% [9,20]) of zebrafish embryo assays with the *in vivo* mammalian studies, false negative and false positive results were reported [4,6–12,14,15,17,21]. In particular these false negative results hinder the use for regulatory purposes, as potential teratogens may be missed. For many chemicals and drugs that were reported to be false negative in zebrafish embryo assays, skeletal findings were noted in the *in vivo* studies. In contrast to the exhaustive list of skeletal endpoints assessed in rat and rabbit embryofetal development studies [22], in which skeletal staining is a standard procedure during examination, no skeletal endpoints are routinely assessed in zebrafish embryo developmental toxicity assays. As skeletal staining of zebrafish larvae is feasible and different methods have been described before (see Supplementary table 1) [23,24], the aim of this study was to investigate whether the sensitivity (i.e., the ability to detect true teratogens) of zebrafish embryo developmental toxicity assays can be increased by including an extended skeletal assessment. For this purpose, we selected four pharmaceutical compounds (i.e., levetiracetam and proprietary compounds 5, 9, and A) that showed skeletal malformations in rat and/or rabbit fetuses, but were false negative in zebrafish embryo assays [8,10]. As several staining methods for zebrafish embryos are reported in literature, each with different (dis)advantages (see Supplementary table 1), three (i.e., alizarin red (AR)-alcian blue (AB), calcein and alizarin red live) were tested to identify the most suitable staining method for increasing the sensitivity of zebrafish embryo assays.

5.2 Materials and Methods

5.2.1 Chemicals and solutions

Unless otherwise stated, all test chemicals were purchased from Sigma-Aldrich (Missouri, USA).

- Embryo medium (EM), a 0.3x Danieau's solution, was prepared from a 10x stock solution containing: 580 mM NaCl, 7.0 mM KCl, 4 mM $MgSO_4$, 6 mM $Ca(NO_3)_2$, and 50 mM HEPES (Invitrogen, Massachusetts, USA). The filtered (0.2 µM filter) 0.3x Danieau's solution was made from this 10x stock by adding ultrapure water, and the pH was adjusted to 7.3 ± 0.2 with 1 M NaOH (Thermo fisher, New Hampshire, USA).
- The MS-222 solutions (4 mg/mL and 0.2 mg/mL) were made by dissolving methyl ethane sulfonate in EM, and the pH was adjusted to 7.4 ± 0.3 with 1 M NaOH.
- Four mammalian skeletal teratogens that showed skeletal malformations in EFD studies in rat and/or rabbit but were false negative in zebrafish assays were selected to expose the zebrafish embryos to. Levetiracetam (L-8668-50MG, Sigma-Aldrich) (10, 100 and 1,000 µM), "proprietary compound 5" (10, 100 and 1,000 µM), "proprietary compound 9" (10,

100 and 1,000 μM) and “proprietary compound A” (1 μM, 10 μM and 100 μM) were used in the AR-AB staining experiments, and “proprietary compound 5” (100 and 1,000 μM) in the AR live staining experiments [8,10]. The test concentrations were prepared by dissolving the compound in EM containing 0.5% DMSO.

The chemicals and solutions needed for the staining protocol are described in section 5.2.6 Staining protocol.

5.2.2 Animal care and egg collection

Breeding stocks of healthy, unexposed adult zebrafish from the wild-type WIK strain (sourced from a historical line at Brixham AstraZeneca and outcrossed with WIK ZERC) were used to produce fertilized eggs. Water was maintained at pH 7.35 ± 0.65 and 28 ± 1°C. Fish were cultured in the aquarium facility with a 14 h light:10 h dark light cycle. Adult fish at a ratio of 2:1 females to males were placed into spawning tanks on the evening prior to the day of culture, and egg traps were positioned within each tank. Eggs were collected the next morning soon after spawning and incubated in system water as detailed in Paull et al. 2008 [25] at 28 (±1)°C for approximately 1–2 h, and then treated against fungal infection using a diluted Chloramine T bleaching solution (10 g/L) for 60 s with gentle periodic agitation. Following bleaching, the embryos were washed twice in rig water with constant agitation and then transferred into a Petri dish containing 0.3x Danieau’s solution. Between 100 and 200 fertilized eggs of the same developmental stage (i.e., a stage before 4 hpf) were transferred into a separate Petri dish containing 0.3x Danieau’s solution and maintained at 28 (±1)°C. Embryos were staged for development according to Kimmel et al. (1995) [26].

5.2.3 Uptake assessment

Uptake of the test solutions was assessed as detailed previously in Gustafson et al. (2012) [9]. Briefly, liquid chromatography–tandem mass spectrometry (LC–MS/MS) was used to determine the uptake of the compound into embryos of 1 dpf and larvae of 5 dpf. Uptake assessment in embryos and larvae was originally planned for 4 concentrations (i.e., 1, 10, 100 and 1,000 μM) of each test compound, and for a corresponding 0.5% DMSO solvent control (see 5.2.4 Exposure of zebrafish embryos). However, uptake at 1,000 μM was not assessed due to excessive precipitation of the test compounds in the solution (see results section for more details). After 24 h (30 hpf) or 5 days (120 hpf) of exposure the embryos/larvae were assessed for viability and transferred to a filter plate (3–4 embryos/larvae per well) in a final volume of 300 μL of EM, in triplicates. Embryos were washed using system water (containing 0.5 g/L tricaine at pH 7 (± 0.5)) under vacuum, before being transferred to a deep-well plate containing 300 μL of rig water and homogenized (Geno/grinder, SPEX Certiprep L.L.C., USA). 300 μL acetonitrile (HPLC grade Thermo Fisher, New Hampshire, USA) containing 50 nM of internal standard was added to each well followed by a second homogenization, and then addition of 900 μL (HPLC grade) water (Thermo Fisher, New Hampshire, USA). Samples were then mixed using a plate shaker and centrifuged for 30 min at 3,220 x g. An aliquot of each supernatant (700 μL) was then transferred to a separate deep-well plate for LC–MS/MS analysis. Compound calibration standards were prepared in 80:20 water:acetonitrile (HPLC

grade) and covered a concentration range over three orders of magnitude in semi-log steps. Standards and extracts were analyzed using reverse phase liquid chromatography-tandem mass spectrometry (TSQ Quantum Access, Thermo Fisher, USA), operated in positive electrospray ionization mode (ESI +).

Quantification was achieved by reference to calibration standards using an internal standard method. The measured per-embryo/larvae concentration was then expressed as a percentage of the nominal exposure (well plate) concentration. Stability of the exposure solution was determined by comparison of the day 0 solution concentration to the exposure solution concentration at 5 dpf. For this, samples were taken from the 10 µM concentrations by combining medium from multiple wells.

5.2.4 Exposure of zebrafish embryos

5.2.4.1 Compound administration to zebrafish embryos

At 4–6 hpf, zebrafish embryos were transferred individually into the wells of a 24-well plate (BD Falcon, NJ, USA) containing test compound solution, medium and/or vehicle (DMSO) controls in a final volume of 1 mL/embryo. In total, two replicates were used per staining experiment, and in each replicate 24 embryos/group were exposed. The solvent (DMSO) concentration was 0.5% (i.e., lower than the maximum final concentration of DMSO that is considered to be safe to be used in zebrafish assays [27]). If precipitation was observed, stock solutions were pH adjusted with 1 M NaOH or 1 M HCl within a range of pH 4-10 to facilitate dissolution with final adjustment to pH 6.4–8.4. Any precipitation in compound solutions and/or the well was recorded at the time of plating and at 5 dpf. All embryos were incubated at 28 (±1) °C for 5 days. More details about the exposure and chemicals that were used in the different experiments can be found in Table 1.

For the live staining experiments, the exposure was stopped at 5 dpf and the larvae were transferred to small crystalline dishes and were further reared in rig water until 9 dpf. Viability was checked daily. The crystalline dishes had a 50% water change daily with fresh rig water after the first feed of the day. The larvae were fed three times a day with 4 mg ZM (Zebrafish Management Ltd, Hampshire, UK) dry particle larval food (5-8 dpf ZM-000, 9 dpf 50:50 ZM-000:ZM-100).

Table 1. Overview of the different experiments. For each staining method the compound concentrations, control media tested, and the developmental stage at which the staining was performed are indicated. Abbreviations: alcian blue (AB), alizarin red (AR), days post-fertilization (dpf), not applicable (NA).

Staining method	Chemical	Concentration	Controls	Age
AR-AB (fixed)	Compound 5	10, 100 and 1,000 µM	Medium, DMSO	5 dpf
	Compound 9	10, 100 and 1,000 µM	Medium, DMSO	5 dpf
	Compound A	1, 10 and 100 µM	Medium, DMSO	5 dpf
	Levetiracetam	10, 100 and 1,000 µM	Medium, DMSO	5 dpf
Calcein (live)	NA	NA	Medium, DMSO	5, 9 dpf
AR (live)	Compound 5	100 and 1,000 µM	DMSO	5, 9 dpf

5.2.5 Viability, morphological evaluation, and length assessment

The viability of all larvae was assessed at 5 dpf, and also at 9 dpf for the live staining experiments. After the viability assessment on 5 dpf, larvae were anesthetized using tricaine (1 mM) and a morphological assessment was conducted for the same endpoints as used in Gustafson et al. (2012) [28] and Ball et al. (2014) [8]. For this assessment, a numerical system that has been previously described by Panzica-Kelly et al. was used [29]. The standard length (SL; in mm) at 5 and 9 dpf of all hatched larval zebrafish was determined using a Leica M205C stereomicroscope (Leica, UK). Images were captured using a Leica DMC4500 digital camera. Image analysis was conducted by applying Leica LAS X core and LAS X measurements® (see 5.2.8).

5.2.6 Staining protocol

5.2.6.1 Fixed tissue staining (alizarin red and alcian blue)

Two-color acid free staining of the zebrafish larvae was adapted from Walker and Kimmel (2007) [23]. In brief, after euthanasia in 4 mg/mL MS-222, half of the hatched larvae of each replicate (i.e., a maximum of 12/replicate) were fixed in 4% formalin (containing 0.1 M phosphate buffer) for 1 day at room temperature and then stored in 70% ethanol (Thermo Fisher, New Hampshire, USA) overnight at 4°C. Samples were then washed for 5 min in 50% methanol followed by 80% methanol. The larvae were then stained in alcian blue solution (0.02% w/v alcian blue (Sigma-Aldrich, Missouri, USA), 80 mM $MgCl_2$ (Sigma-Aldrich) in 73.5% ethanol) for 1 h. This was followed by a 5 min wash in 50% ethanol and two washes in water containing 0.2% v/v Triton ™ X-100 (Thermo Fisher). The larvae were bleached (0.8% KOH (Thermo Fisher) and 0.9% H_2O_2 (Sigma-Aldrich) in 0.2% Triton ™ X-100 water) for 30 min, while monitoring for pigmentation loss. This was followed by two 5 min washes in water containing 0.2% Triton ™ X-100. Stained larvae were immersed in 100% Borax (saturated sodium tetraborate (Thermo Fisher)) solution for 10 min followed by 1 h in digestion solution (1% trypsin (Sigma-Aldrich) in 60% borax solution with 0.08% Triton ™ X-100). Post digestion, larvae were stained for 3 h in alizarin red solution (0.003% alizarin red (w/v) (Sigma-Aldrich) in 1% KOH solution) and then 20 min in wash and clear solution (20% glycerol (Thermo Fisher), 0.8% KOH in 0.2% Triton ™ X-100). Samples were then stored in the dark at 4°C in 70% glycerol until imaging. Imaging was conducted for each batch within 72 h after staining. Transfer of larvae between different staining solutions was conducted using Netwell ™ permeable supports (15 mm insert with 74 μm polyester mesh (Corning, USA)).

5.2.6.2 Live calcein staining

Larvae were stained as detailed in Du et al. (2001) [24]. Calcein powder (C0875-5G, Sigma-Aldrich) was solubilized in deionized water at 2 mg/mL and adjusted to pH 7.4 ± 0.3 with 1 M NaOH (Thermo Fisher). Larvae were transferred to the calcein stain solution for 10 min, then washed in 3 volumes of rig water. The solution was replaced with 0.2 mg/mL MS-222 (pH 7.5) for 5 min. The larvae were embedded in 1% low melting point agarose and imaged immediately (see 5.2.8).

5.2.6.3 Live alizarin red staining

Alizarin red powder (A5533-25G, Sigma-Aldrich) was solubilized in 0.3x Danieau's solution at 0.5% w/v stock solution and stored in the dark. The alizarin red stain stock was diluted 1:100 in 0.3x Danieau's solution and pH adjusted with fresh 1M KOH (Thermo Fisher) to pH 7.4 ± 0.2 (i.e., the AR solution). Larvae were transferred to the AR solution for 1 h and then washed in a 3 times volume of rig water. The solution was then replaced with 0.2 mg/mL MS-222 (pH 7.5) for 5 min until loss of dorsoventral balance. The larvae were then embedded in 1% low melting point agarose and captured immediately (see below).

5.2.7 Embedding protocol

The fluorescent bone staining was performed in larvae at 5 and 9 dpf using the method of Parker et al. (2014) [30]. Each larva was anesthetized in MS-222 (0.2 mg/mL, pH 7.5) until the loss of dorsoventral balance. The larvae were then transferred into low melting point agarose (1 g/100 mL; containing 0.2 mg/mL MS-222) before being deposited in a total volume of 80 µL into a well created by a press-to-seal silicon isolator (Sigma-Aldrich) on a clear microscope slide. Each larva was then gently orientated onto its side with the head to the left, the agarose solidified by very brief (1-2 secs) exposure to a cooling plate (5°C), and two drops of MS-222 placed on top to minimize agarose shrinkage during imaging. Post imaging the larva was released from the agarose into 0.02 g/L MS-222 and then re-embedded in agarose (1 g/100 mL) with the dorsal side down and the head to the left. At 5 dpf, the larvae were then released into clean rig water and maintained till 9 dpf. The imaging of both the lateral and dorsoventral views was repeated at 9 dpf. At the end, the larvae were terminated in an overdose of anesthetic and secondary confirmation of termination was done by destruction of the brain tissue.

5.2.8 Image capture

Live larval zebrafish were imaged using a Leica M205C stereomicroscope (Leica, UK) combined with a Leica DMC4500 digital camera. Image analysis was conducted by Leica LAS X core and LAS X measurements®. Larvae were anesthetized in MS-222 (0.2 mg/mL, pH 7.5) until the loss of dorsoventral balance. After imaging, the larvae were returned to the exposure medium or euthanized with an overdose of anesthetic.

Live fluorescent bone staining was imaged using an Olympus SZX16 scope (Olympus, UK) at 6.3x magnification with Prior 200 Lumen illumination (100%). To do so, anesthetized larvae were embedded in 1 % low gelling temperature agar containing 0.2 g/L MS-222.

Alizarin red fluorescence was captured with a red fluorescent protein (RFP) light cube filter (620 nm with a bandwidth of 0 nm (excitation HQ545/30x, emission HQ620/60x)) (Chroma Technology Corporation. VT USA) and by using 100 ms exposure in Micromanager (v1.4). In total 8 images per stack were captured at a 3 second interval between the images using a Zyla 4.2 sCMOS camera (Andor, Oxford Instruments, UK).

Calcein (GFP) fluorescence was captured with a green fluorescent protein (GFP) light cube filter (460 nm with a bandwidth of 35 nm (excitation BP460 T2, emission BP495 T2)) (Chroma Technology Corporation. VT USA) and by using 20 or 9.84 ms exposure in Micromanager (v1.4). In total 8 images per stack were captured at a 3 second interval between the images using a Zyla 4.2 sCMOS camera (Andor, Oxford Instruments, UK).

Fixed, stained larval zebrafish larvae were imaged using a Leica M205C stereomicroscope (Leica, UK). Still images were captured using a Leica DMC4500 digital camera. Image analysis was performed using Leica LAS X core and LAS X measurements®.

5.2.9 Skeletal evaluation

5.2.9.1 Alizarin red and alcian blue stained larvae

Each bone and cartilage structure (see Table 2, Table 3, and Figure 1) of each larva was scored for staining intensity and shape by allocating a representative score (see Table 4). Each structure was scored in the position (i.e., lateral or dorsoventral) where it was most visible/stained. Moreover, the angle between the ceratohyal cartilages was measured. Previous studies showed that this angle may increase after exposure to xenobiotics, and therefore, can be an indication for xenobiotic toxicity [31].

Table 2. List of bone structures that can be scored in 5 dpf and 9 dpf zebrafish larvae when using an AR-AB fixed or an AR live staining. Bones that are likely to be present according to literature [32–35] and/or are clearly visible when stained are included in this list.

Bone structure	Fixed – 5 dpf	AR live – 5 dpf	AR live – 9 dpf
Notochord (n)	X	x	x
Parasphenoid (ps)	X	x	x
Vertebrae (vb)	/	/	x
Ceratobranchial 5 (cb5)	X	x	x
Pharyngeal teeth (t)	X	- [1]	- [1]
Utricular otolith (uot)	X	x/-	x/-
Circle saccular otolith (cot)	X	x/-	x/-
Entopterygoid (en)	X	x	x
Opercle (op)	X	x	x
Cleithrum (c)	X	x	x
Branchiostegal rays (brs)	X	x	x
Branchiostegal rays II (brs2)	/ [2]	/ [2]	x
Hyomandibular bone (hmb)	/ [2]	/	x
Ceratohyal bone (chb)	/ [2]	/	x
Dentary (den)	/ [2]	/	x
Maxilla (max)	/ [2]	/	x
Anguloarticular (aa)	/ [2]	/	x

Often present/scored (x), less likely to be present/scored if present (/), not present or visible/not scored (-), only one side could be scored (x/-). [1]Structure not distinguishable from cb5. [2] Structure was never visible at this age using this staining. Abbreviations: alcian blue (AB), alizarin red (AR), days post-fertilization (dpf).

Table 3. List of cartilage structures that can be scored in 5 dpf zebrafish larvae when using an alizarin red-alcian blue fixed staining. Cartilages that are present according to literature [34–37] and/or are clearly visible when stained are included in this list.

Cartilage structure	5 dpf
Ethmoid plate (eth)	X
Basihyal (bh)	X
Basibranchial (bb)	X
Meckel's cartilage (mk)	X
Palatoquadrate (qu)	X
Hyosymplectic (hys)	X
Ceratohyal (ch)	X
Ceratobranchials (cb)	X
Auditory capsule (aud)	X
Pectoral fin (pec fin)	X
Pterygoid process of the quadrate (pty)	X

Should be present/scored (x). Abbreviations: days post-fertilization (dpf).

Table 4. Intensity and shape scores and their meaning.

Intensity scoring		Shape scoring	
0	structure not stained/not present	0	structure is normal
1	structure is weakly stained	1	structure is malformed
2	structure is moderately stained		
3	structure is heavily stained		

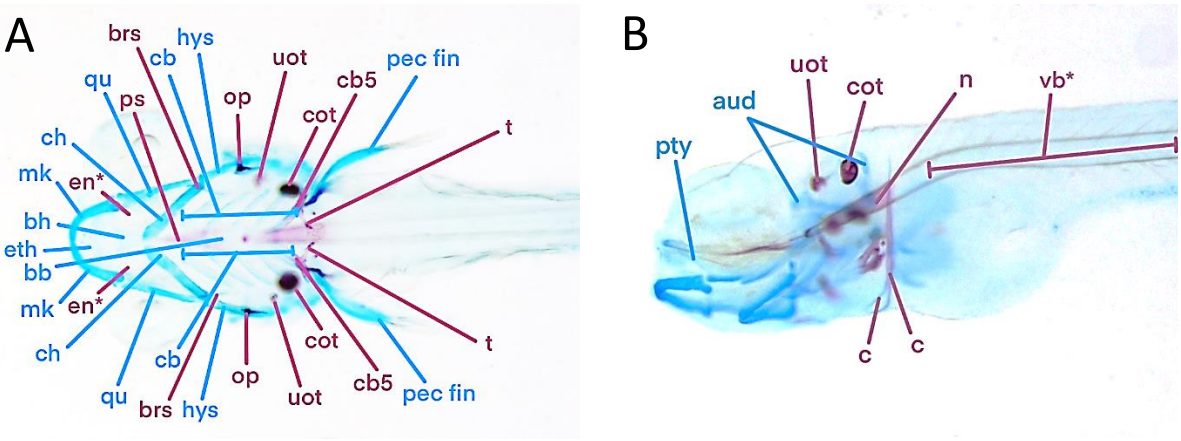


Figure 1. Zebrafish larvae at 5 dpf with bone (red) and cartilage (blue) structures that were stained with the alizarin red-alcian blue staining. Left panel (A) shows a ventral view. Right panel (B) shows a lateral view. Each structure (except for uot and cot) is indicated in only one of the orientations, namely in the orientation where it was scored. Uot and cot were scored by looking at both positions. The abbreviations are depicted in Table 2 and Table 3. The structures vb* and en* are not present in these images.

5.2.9.2 Calcein stained larvae

Six stacks of images (i.e., one stack of 8 images per position) for each orientation were taken for each larva. Two different aperture durations, i.e., 20 ms and 9.84 ms, with fluorescence intensity power at 100, 50 and 10% power were used to prevent overexposure of the bone tissue. However,

the larvae could not be scored for individual bone structures due to a high degree of variability in staining, as will be further explained in section 5.3.4.

5.2.9.3 Alizarin red live stained larvae

For each of the larvae, two stacks of images (i.e., one stack of 8 images per position) were processed in ImageJ and the intensity and shape of the bone structures (see Table 2, Figure 2 and Figure 3) of each of the larva were evaluated and a representative score was allocated (see Table 4) [16]. For each bone structure of each larva in each group, the image with the highest intensity score was selected to determine the final score of the bone.

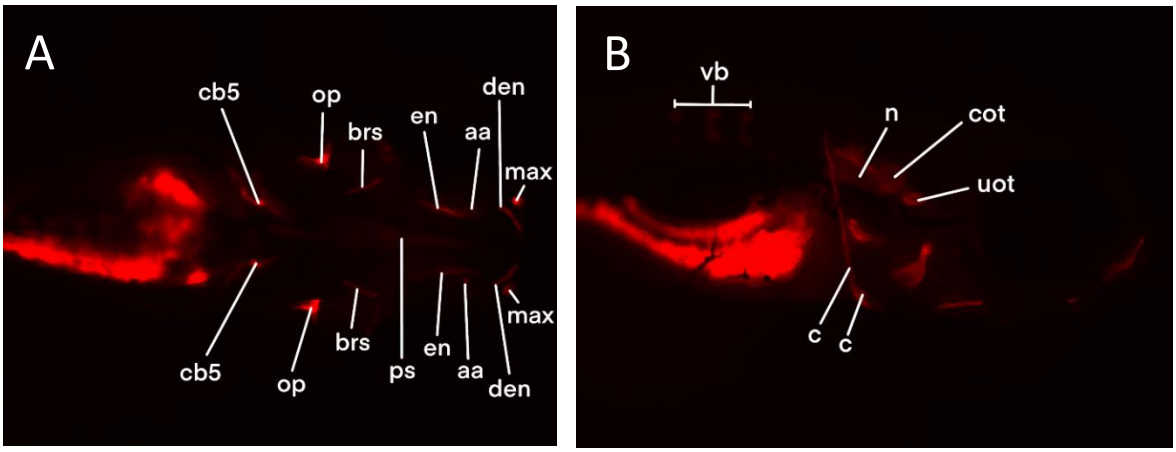


Figure 2. Zebrafish larvae at 5 dpf with bone structures that were stained with the alizarin red live staining. Left panel (A) shows a ventral view. Right panel (B) shows a lateral view. Each structure is indicated in only one of the orientations, namely in the orientation where it was scored. The abbreviations are depicted in Table 2.

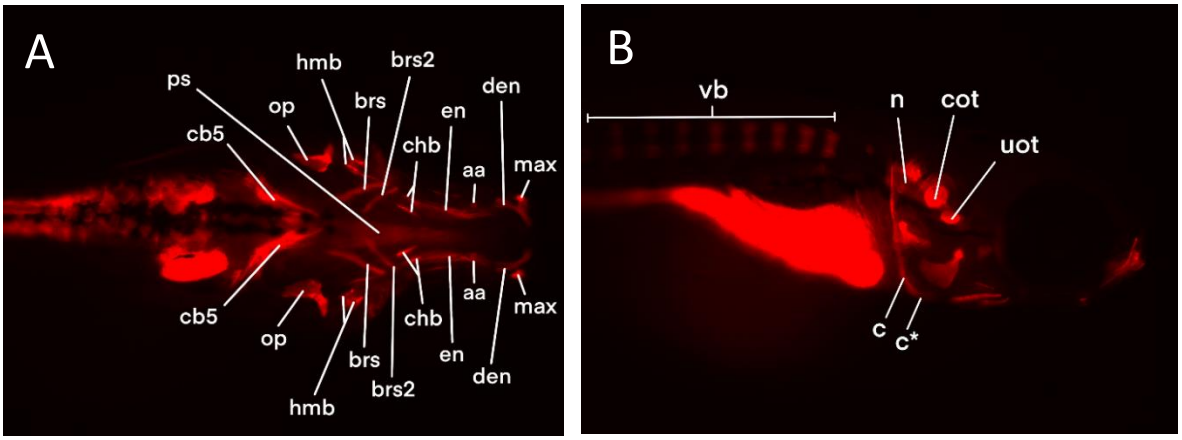


Figure 3. Zebrafish larvae at 9 dpf with bone structures that were stained with the alizarin red live staining. Left panel (A) shows a ventral view. Right panel (B) shows a lateral view. Each structure is indicated in only one of the orientations, namely in the orientation where it was scored. The abbreviations are depicted in Table 2. The left cleithrum (c*) is hidden behind the right cleithrum in this image.

5.2.10 Using solvent controls to investigate the cause of variability between replicates

Due to inconsistent results between replicates of compound-treated zebrafish larvae, additional experiments and/or analyses were performed to investigate the cause of this variability.

For the AR live staining experiments, the variability in the intensity of solvent controls was first checked by comparing the solvent controls of both replicates of compound 5 experiments at 5 dpf and 9 dpf. However, to make sure the time gap between the two replicates was not causing additional variability, new solvent control experiments were carried out. First, 0.5% DMSO treated (solvent control) larvae of two clutches of zebrafish (same spawning day, n=24) were compared. Second, the larvae of each of these clutches were divided into 3 groups of 8 fish (i.e., larva 1-8 in subgroup 1, larva 9-16 in subgroup 2, and larva 17-24 in subgroup 3) and these subgroups were compared to each other. In addition, the solvent control group of the second replicate of the compound 5 experiment (5 dpf) was also divided into 3 subgroups of 8 larvae each, so they could also be compared to each other.

For the fixed staining experiments, the variability in intensity and bone shape of the different medium control groups was checked by comparing the medium control groups of all previously conducted experiments (compound 5, 9, A, and levetiracetam; 2 replicates each). Thus, 8 control groups of maximum 12 zebrafish larvae were compared to each other.

5.2.11 Statistical analysis

Length measurements between different treatments were analyzed for statistical differences induced by compound treatment from the pooled experimental replicates. Minitab 21 Statistical software (computer software; www.minitab.com) was used. Normal distribution of the data was determined by Levene's and Bartlett's tests. If data were normally distributed, assessment of significant difference was performed using by Tukey ANOVA test. If no normal distribution was found, a Kruskal-Wallis test was applied and then an individual Mann-Whitney test was applied to each treatment group. For the binary data of the viability and the shape scoring a Fisher Exact test was performed and the Relative Risk was calculated. For the ordinal data (i.e., 0, 1, 2, or 3) of the intensity scoring a Kruskal-Wallis test with correction for multiple comparisons (Dunn's multiple comparisons test) was used to check if there was a significant difference between any of the test groups and the control group (exposure experiments) or between any of the control groups (variability experiments). Also, for the angle size data a Kruskal-Wallis test with correction for multiple comparisons (Dunn's test) was used. If there were only two groups, a Mann Whitney U test was used instead of the Kruskal-Wallis test. Except for length measurement analysis, all statistical analyses were performed using GraphPad 8.4.0 or newer versions.

5.3 Results

5.3.1 Uptake assessment

Uptake of all four test compounds was detected in zebrafish at the highest nominal exposure concentration of each compound between 1-31% of the nominal concentration at both 1 and 5 dpf (see Table 5). Precipitation was observed in the analysis solution of compound A at 100 µM nominal concentration at both 1 and 5 dpf. Therefore, to avoid excessive precipitation, the highest tested concentration used for exposure was 100 µM, and not higher (i.e., no 1,000 µM). The other compounds did not display any precipitation at 100 µM, and therefore, also 1,000 µM was included for evaluating the stainings. All compounds were stable in the medium over the 5 days (see Table 5). There was a reduction in the recorded uptake of all compounds between the 1 and 5 dpf sampling, except for 10 µM of compound A.

Table 5. Uptake by zebrafish at 1 dpf (A) and 5 dpf (B).

A) Exposure 0-1 dpf

Compound	Conc. in each Larva (µM)	Uptake as %[1]	Conc. in each Larva (µM)	Uptake as %[1]	Conc. in each Larva (µM)	Uptake as %[1]
	100 µM		10 µM		1 µM	
Compound A	0.667*	1*	0.953	10	<LOQ	
Levetiracetam	18.023	18	1.401	14	<LOQ	
Compound 9	30.651	31	3.114	31	0.458	46
Compound 5	28.967	29	2.463	24	0.326	33

B) Exposure 0-5 dpf

Compound	Conc. in each Larva (µM)	Uptake as %[1]	Conc. in each Larva (µM)	Uptake as %[1]	Conc. in each Larva (µM)	Uptake as %[1]	Stability
	100 µM		10 µM		1 µM		Day 5 as % of Day 0
Compound A	1.494*	1*	1.773	18	<LOQ		104
Levetiracetam	7.498	7	0.480	5	<LOQ		110
Compound 9	1.420	1	<LOQ		<LOQ		106
Compound 5	15.818	16	1.0548	11	<LOQ		105

* Precipitation observed at point of sampling of tissue for analysis.

[1] Uptake as % is the ratio of measured concentration in each larva (µM)/the nominal concentration *100 (e.g. 0.953 µM/10*100)

Uptake assessment at 1,000 µM was not performed due to excessive precipitation of the compounds in the solution, which would impact the background level of compound carryover in solution when assessing uptake.

Abbreviation: days post-fertilization (dpf).

5.3.2 Viability, morphological evaluation, and length assessment

5.3.2.1 5 dpf larvae exposed to compound 5, 9, A and levetiracetam (fixed experiments)

No significant difference in viability was observed between the test concentrations and the control groups (see Supplementary table 2).

Exposure to levetiracetam (LTC) up to 1,000 μM did not induce any malformations at 5 dpf (see Supplementary table 9). Levetiracetam also showed no effect on larval length.

At 5 dpf, no treatment related malformations were observed after exposure to compound A up to 100 μM (see Supplementary table 8). A significant reduction in length of the 1, 10 and 100 μM test groups was observed in one of the replicates for compound A compared to the DMSO control ($p = 0.000$ for 1 μM and 10 μM and $p = 0.014$ for 100 μM) (see Supplementary table 4), and the 1 μM and 10 μM test groups compared to the medium control ($p = 0.037$ for 1 μM and $p = 0.023$ for 10 μM).

Compound 5 induced treatment-related malformations in the jaw and neural tube, and pericardial edema and slow heart rate at 1,000 μM at 5 dpf (see Supplementary table 6). Also a significant reduction in length of the 1,000 μM exposed group compared to the medium and DMSO control groups ($p = 0.048$ to medium and $p = 0.031$ to DMSO in replicate 1; and $p = 0.000$ to DMSO in replicate 2) was observed (see Supplementary table 4).

Compound 9 did not induce any treatment-related malformations at 5 dpf compared to the medium and DMSO control larvae (see Supplementary table 7). The larval length at 1,000 μM of compound 9 was significantly reduced compared to the DMSO control in both replicates ($p = 0.000$ for replicate 1 and $p = 0.004$ for replicate 2), and the medium control in replicate 1 ($p = 0.002$) (see Supplementary table 4).

5.3.2.2 5 and 9 dpf larvae exposed to compound 5 (AR live experiments)

No significant difference in viability was observed between the test concentrations of compound 5 and the DMSO control group at 5 dpf (see Supplementary table 3). The larval length at 1,000 μM of compound 5, however, was significantly reduced compared to the DMSO control ($p = 0.001$), but only in the first replicate (see Supplementary table 5). At 9 dpf, the length was significantly reduced after exposure to 100 μM in the first replicate ($p = 0.030$) and after exposure to 1,000 μM in both replicates ($p = 0.011$ for replicate 1, $p = 0.003$ for replicate 2) (see Supplementary table 5). Also, significant differences in viability were found at 9 dpf between the 1,000 μM group and the control group ($p = 0.0001$ for replicate 1 and $p = 0.0496$ for replicate 2) (see Supplementary table 3).

5.3.3 AR-AB staining results

5.3.3.1 0.5% DMSO as a solvent

In each of the different experiments (see Figure 4 - Figure 15), the solvent control was compared to the medium control to check if the use of 0.5% DMSO as a solvent did affect the skeletal development or the AR-AB staining.

For all AR-AB staining experiments, no significant deviations in shape of bone and cartilage structures were observed in the medium and solvent controls (data not shown).

The angle between the ceratohyal cartilages was comparable between the medium and solvent control group of the different experiments (see Figure 6, Figure 9, Figure 12 and Figure 15). Exceptions were found in replicate 2 for compound 9 (see Figure 9, mean angle size was significantly larger in the solvent control group) and in replicate 1 of the levetiracetam experiment (see Figure 15; mean angle size was significantly larger in the medium control group). The other replicate of both experiments showed no significant differences in mean angle size between both control groups.

In both replicates of compound 5 (see Figure 4 and Figure 5) and levetiracetam (see Figure 13 and Figure 14), there were no significant differences in staining intensity between the solvent and medium control. Also, no differences in intensity of the different bone and cartilage structures were observed when comparing both controls in replicate 1 of the compound 9 experiment (see Figure 7 and Figure 8), and replicate 2 of the compound A experiment (see Figure 10 and Figure 11). However, in replicate 2 of the compound 9 experiment, the ceratobranchials 5 (left and right) were more intensely stained in the medium control group than in the solvent control group (see Figure 7B, indicated with a light green asterisk). In replicate 1 of the compound A experiment, the opercles (left and right) were more intensely stained in the solvent control group than in the medium control group (see Figure 10A, indicated by red asterisks). However, in all other replicates and experiments, the intensity of the opercles and ceratobranchials 5 were not significantly different between the solvent and medium controls.

Overall, the results of the intensity, shape and angle size showed that the use of 0.5% DMSO as a solvent did not have an impact on skeletal development nor on AR-AB staining. The results of the different test compound groups could therefore be compared to the solvent control.

5.3.3.2 Compound 5

No significant differences in shape of the bone and cartilage structures (data not shown) and ceratohyal angle size (see Figure 6) were observed when comparing the three test groups with the solvent control.

Compound 5 reduced the staining intensity in some bone structures (ps, t L&R, brs L&R) in replicate 2 at 1,000 µM (see Figure 4 and Supplementary figure 1). No significant effects were observed at

100 µM. Uot L was less intensely stained at 10 µM in replicate 2 (see Figure 4). In replicate 1, no significant effects of compound 5 on staining intensity were observed.

For the cartilage structures, cb (left and right) was less intensely stained in both replicates at 1,000 µM (see Figure 5 and Supplementary figure 1).

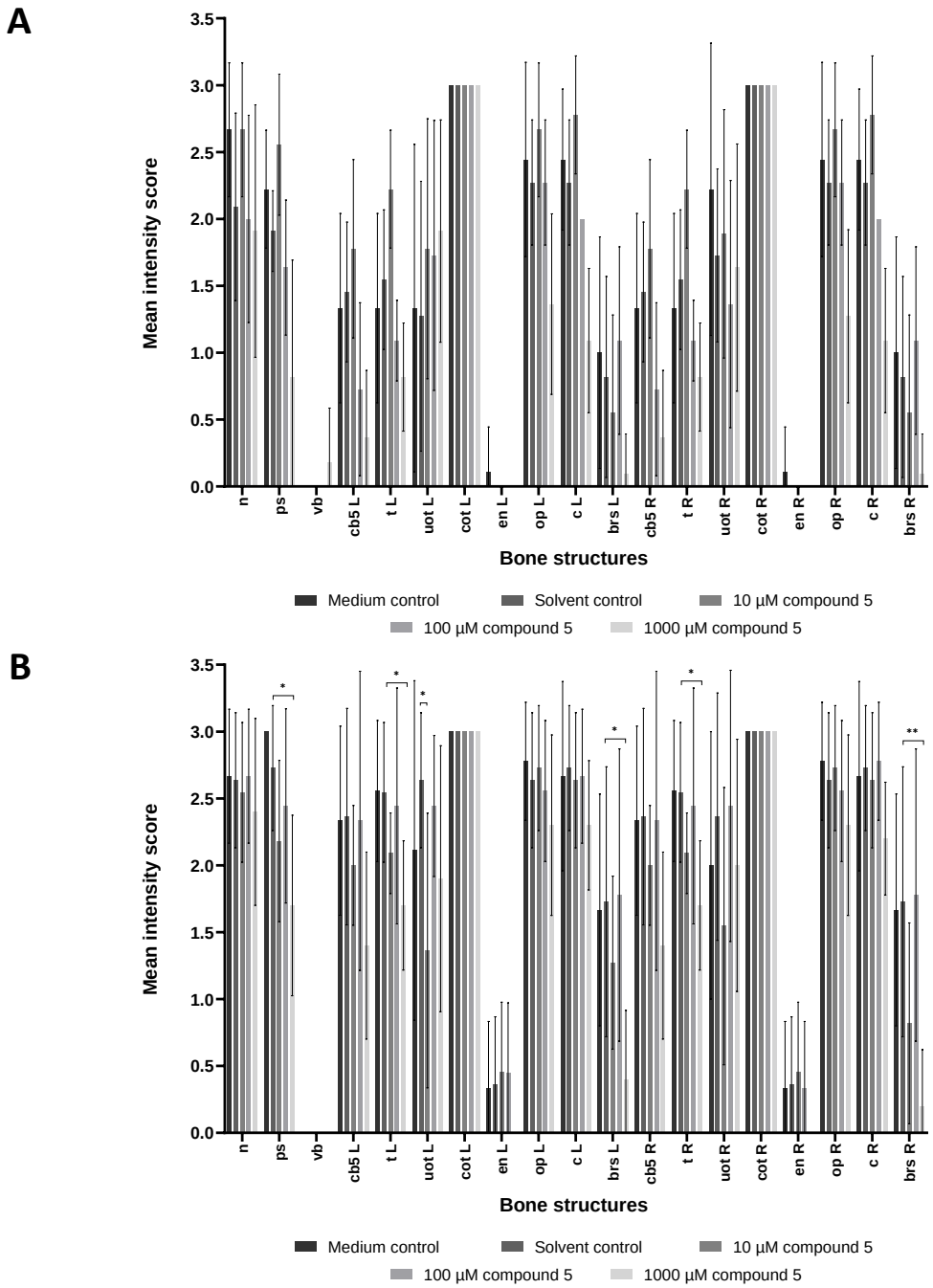


Figure 4. Mean intensity score of fixed AR-stained bones of 5 dpf larvae treated with proprietary compound 5. A) shows the results of replicate 1 and B) shows the results of replicate 2. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. $P \leq 0.05$ (*) and $p \leq 0.01$ (**).

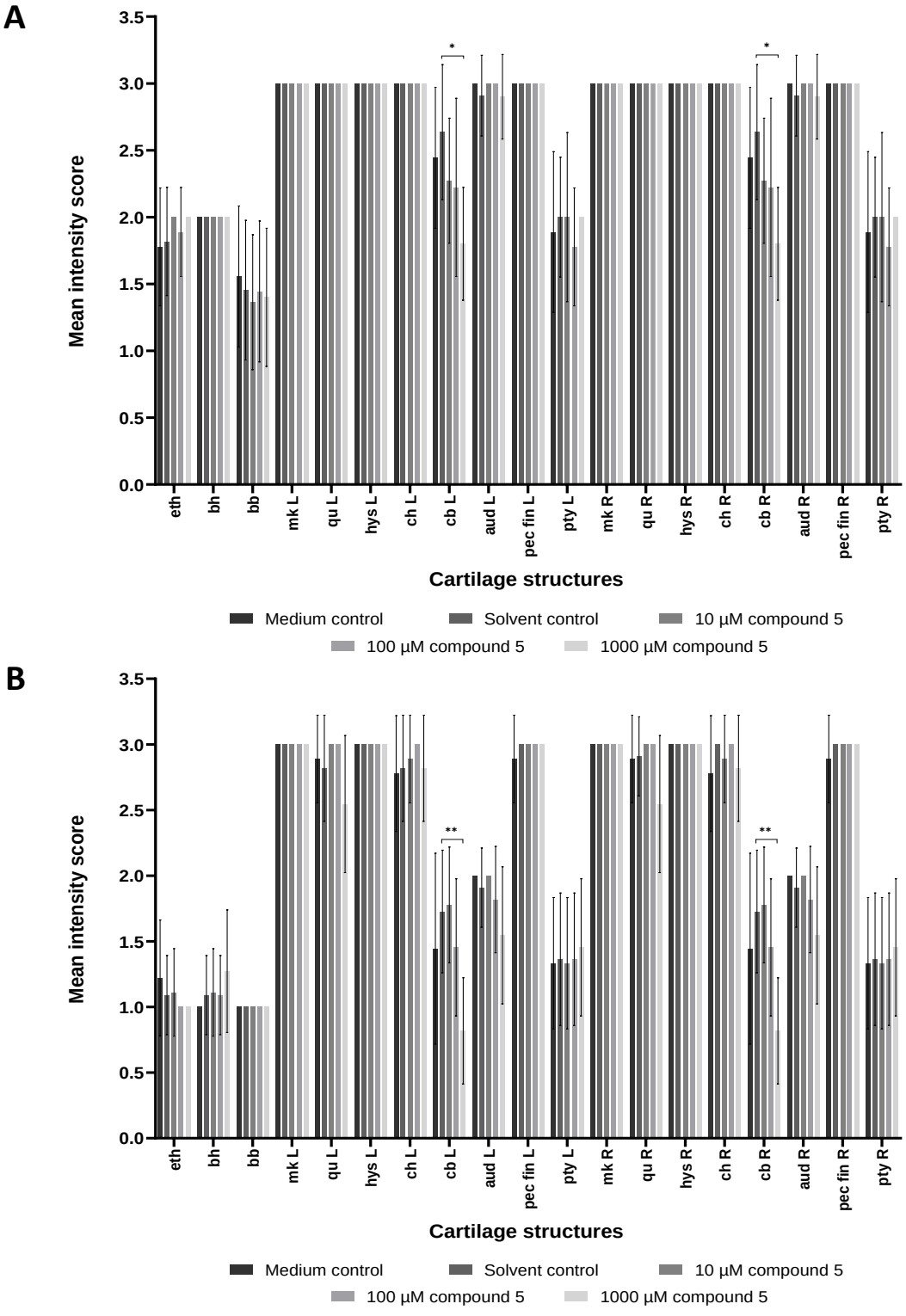


Figure 5. Mean intensity score of fixed AB-stained cartilages of 5 dpf larvae treated with proprietary compound 5. A) shows the results of replicate 1 and B) shows the results of replicate 2. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. $P \leq 0.05$ (*) and $p \leq 0.01$ (**).

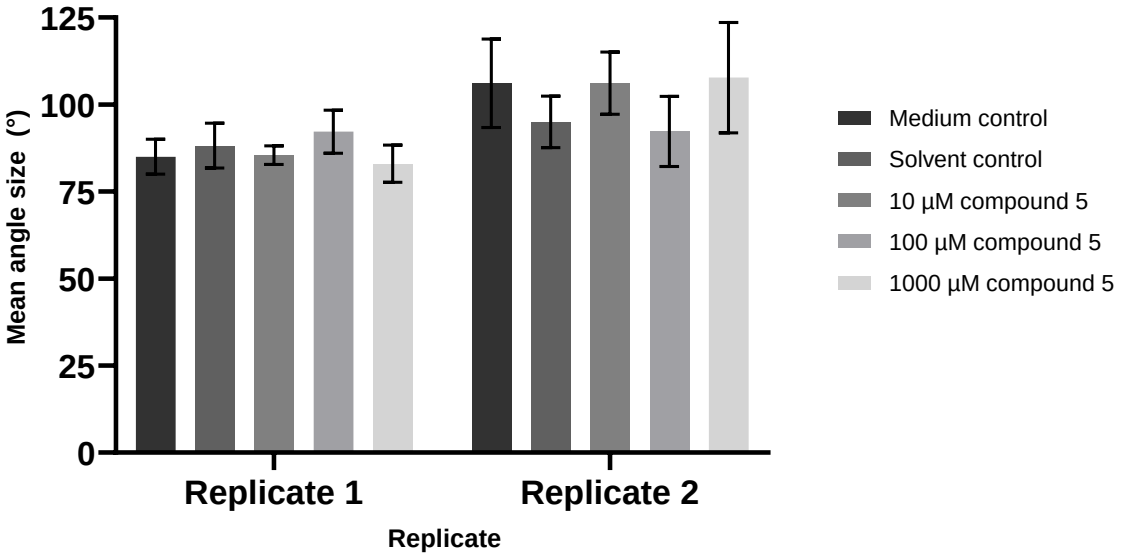


Figure 6. Mean angle sizes between ceratohyal cartilages in 5 dpf larvae treated with compound 5. All groups were compared to the solvent control group. No significant differences were observed between the 3 test groups or the medium control group and the solvent control group.

5.3.3.3 Compound 9

No differences in shape of the bone and cartilage structures were observed when comparing the three test groups to the solvent control (data not shown). Differences in mean angle size, however, were observed in replicate 2 (see Figure 9). The mean angle size of compound 9 was significantly smaller at 10 µM than in the control group. No effects on mean angle size were noted at the higher concentrations.

Compound 9 caused no significant differences in staining intensity at 1,000 µM when compared to the control group (see Figure 7). In replicate 2 at 100 µM, a significantly more intensely stained parasphenoid was noted than in the control group. An increase in staining intensity was also observed for the parasphenoid and the ceratobranchials 5 at 10 µM in replicate 2. For these structures, no increase in staining was observed at 1,000 µM (ps and cb5) or 100 µM (cb5).

For the cartilage structures, the basihyal (replicate 1), ceratohyals (replicate 2) and ceratobranchials (replicate 2) were less intensely stained in one of the replicates at 1,000 µM. No significant results were observed at 10 µM and 100 µM (see Figure 8).

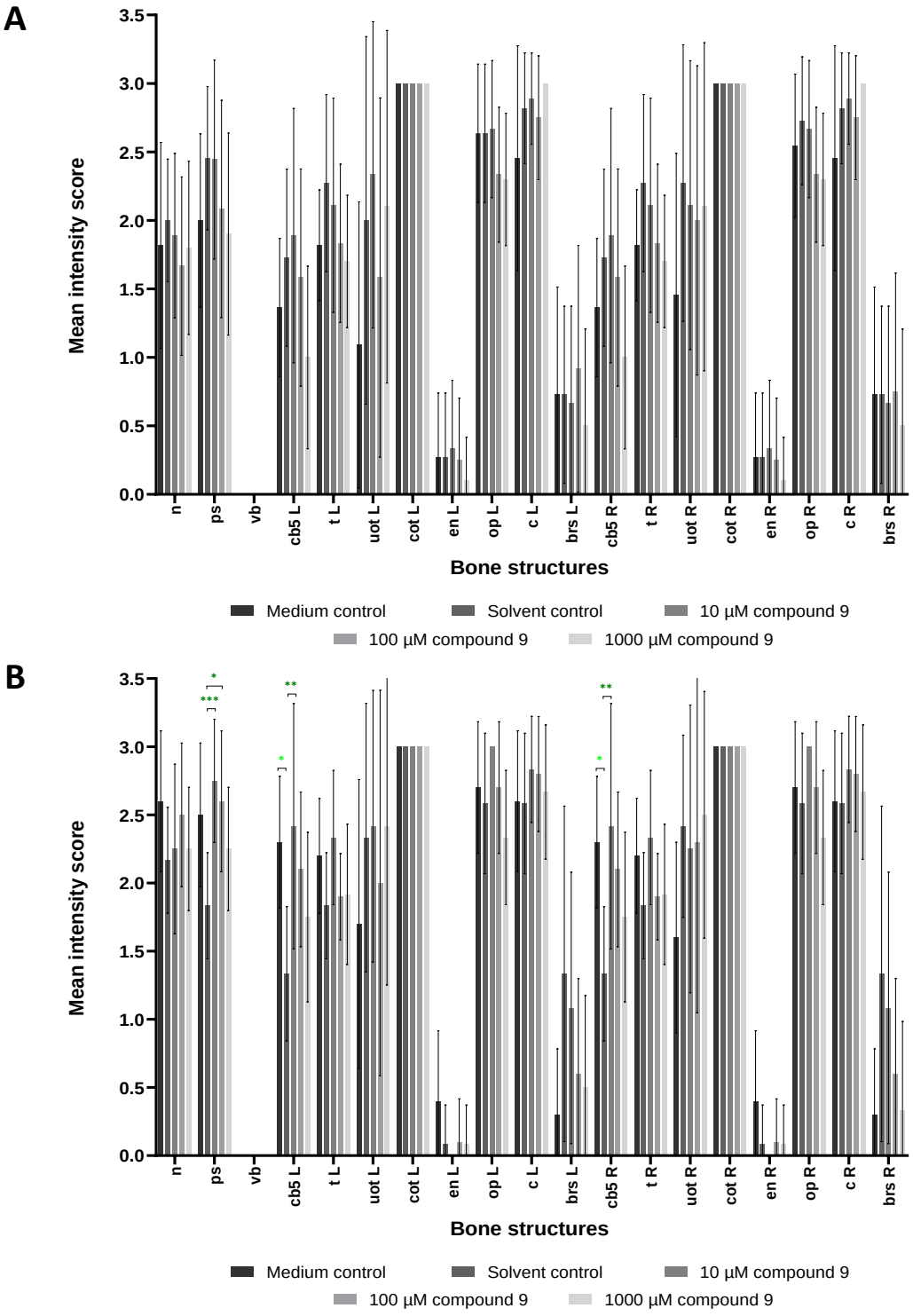


Figure 7. Mean intensity score of fixed AR-stained bones of 5 dpf larvae treated with proprietary compound 9. A) shows the results of replicate 1 and B) shows the results of replicate 2. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. P ≤ 0.05 (*), p ≤ 0.01 (**) and p ≤ 0.001 (***). The color of the asterisks indicate that the solvent control was significantly more (black) or less (green) intensely stained than the other group. The light green asterisks indicate that the solvent control was significantly less stained than the medium control.

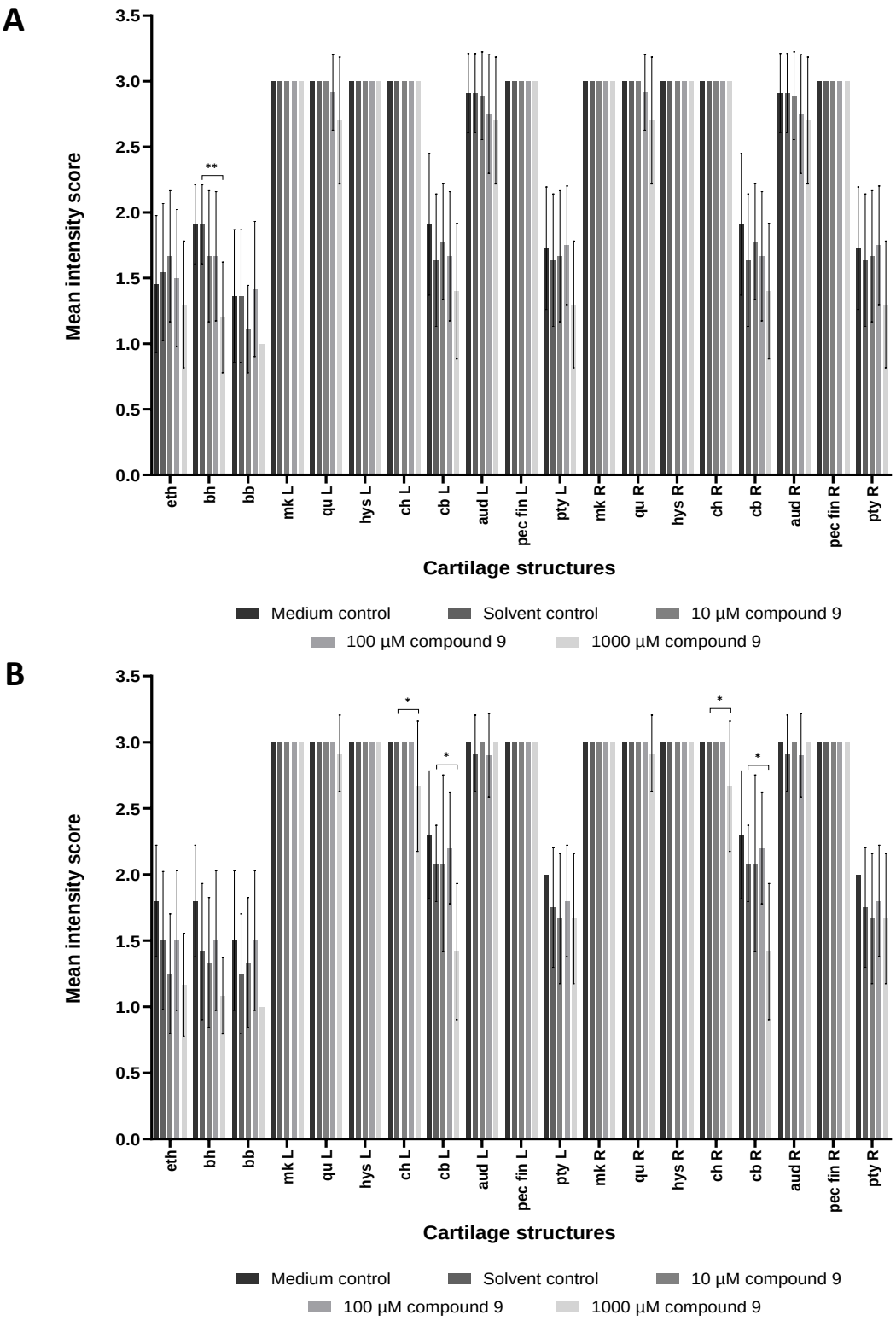


Figure 8. Mean intensity score of fixed AB-stained cartilages of 5 dpf larvae treated with proprietary compound 9. A) shows the results of replicate 1 and B) shows the results of replicate 2. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. $P \leq 0.05$ (*) and $p \leq 0.01$ (**).

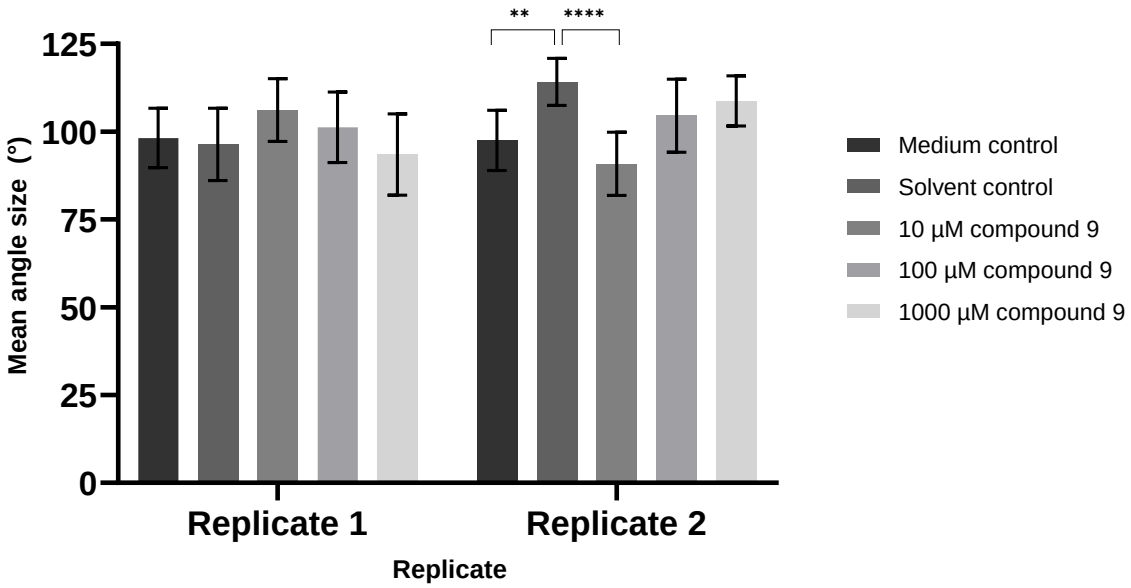


Figure 9. Mean angle sizes between ceratohyal cartilages in 5 dpf larvae treated with compound 9. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. P ≤ 0.01 (**) and p ≤ 0.0001 (****).

5.3.3.4 Compound A

No abnormalities in the shape of the bones and cartilages were observed after exposure to compound A (data not shown). The mean angle size was significantly increased at 100 µM in replicate 1 (see Figure 12 and Supplementary figure 2). The parasphenoid was less intensely stained at 100 µM in replicate 1 (see Figure 10 and Figure 11).

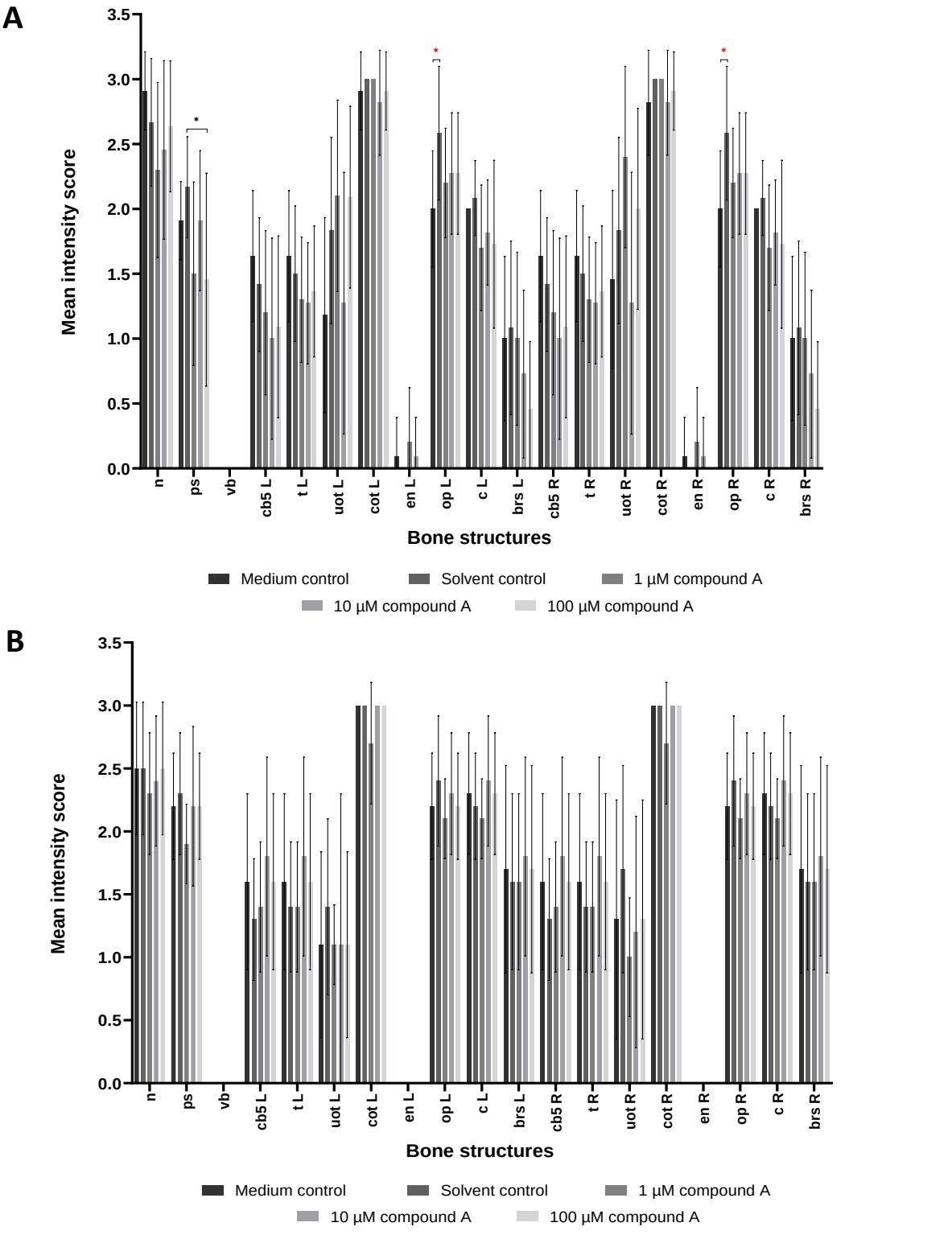


Figure 10. Mean intensity score of fixed AR-stained bones of 5 dpf larvae treated with proprietary compound A. A) shows the results of replicate 1 and B) shows the results of replicate 2. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. $P \leq 0.05$ (*). The black asterisks indicate that the solvent control was significantly more intensely stained than the other group. The red asterisks indicate that the solvent control was significantly more stained than the medium control.

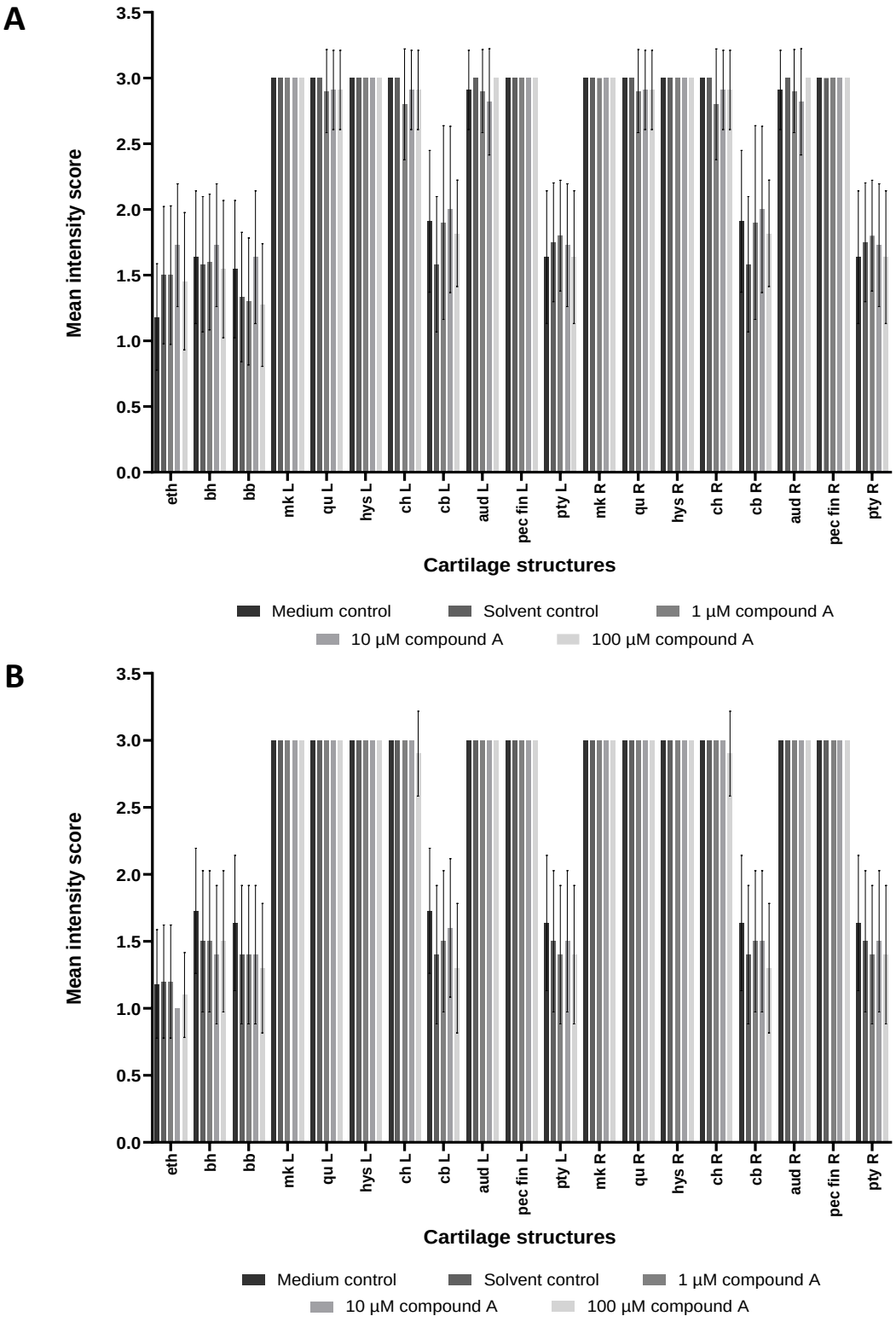


Figure 11. Mean intensity score of fixed AB-stained cartilages of 5 dpf larvae treated with proprietary compound A. A) shows the results of replicate 1 and B) shows the results of replicate 2. All groups were compared to the solvent control group. No significant differences were observed between the 3 test groups or the medium control group and the solvent control group.

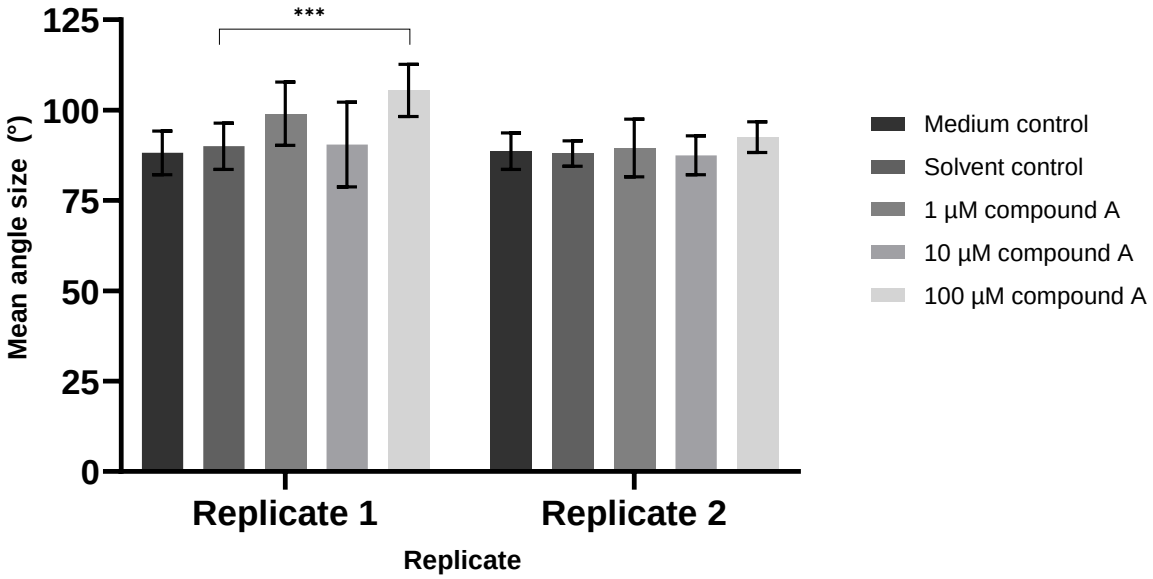


Figure 12. Mean angle sizes between ceratohyal cartilages in 5 dpf larvae treated with compound A. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. P ≤ 0.001 (***).

5.3.3.5 Levetiracetam

No significant differences in ceratohyal angle size (see Figure 15) or shape of the skeletal structures (data not shown) were observed when comparing the three test groups with the solvent control.

The staining intensity of skeletal structures after exposure to levetiracetam was very different in both replicates (see Figure 13 and Figure 14). This was especially different at 100 µM and 1,000 µM. A significant decrease in the intensity was observed for the ceratobranchials 5 (L&R) and the pharyngeal teeth (L&R) at 1,000 µM in replicate 1. The same structures were even less intensely stained, and thus more affected, at 100 µM in replicate 1. Moreover, also 3 other structures (i.e., opercle L&R, cleithrum L&R and parasphenoid) were less intensely stained at 100 µM. In contrast, the parasphenoid intensity significantly increased at 100 µM in replicate 2.

For cartilage intensity, only a significant decrease in the basihyal intensity was observed at 100 µM in replicate 1.

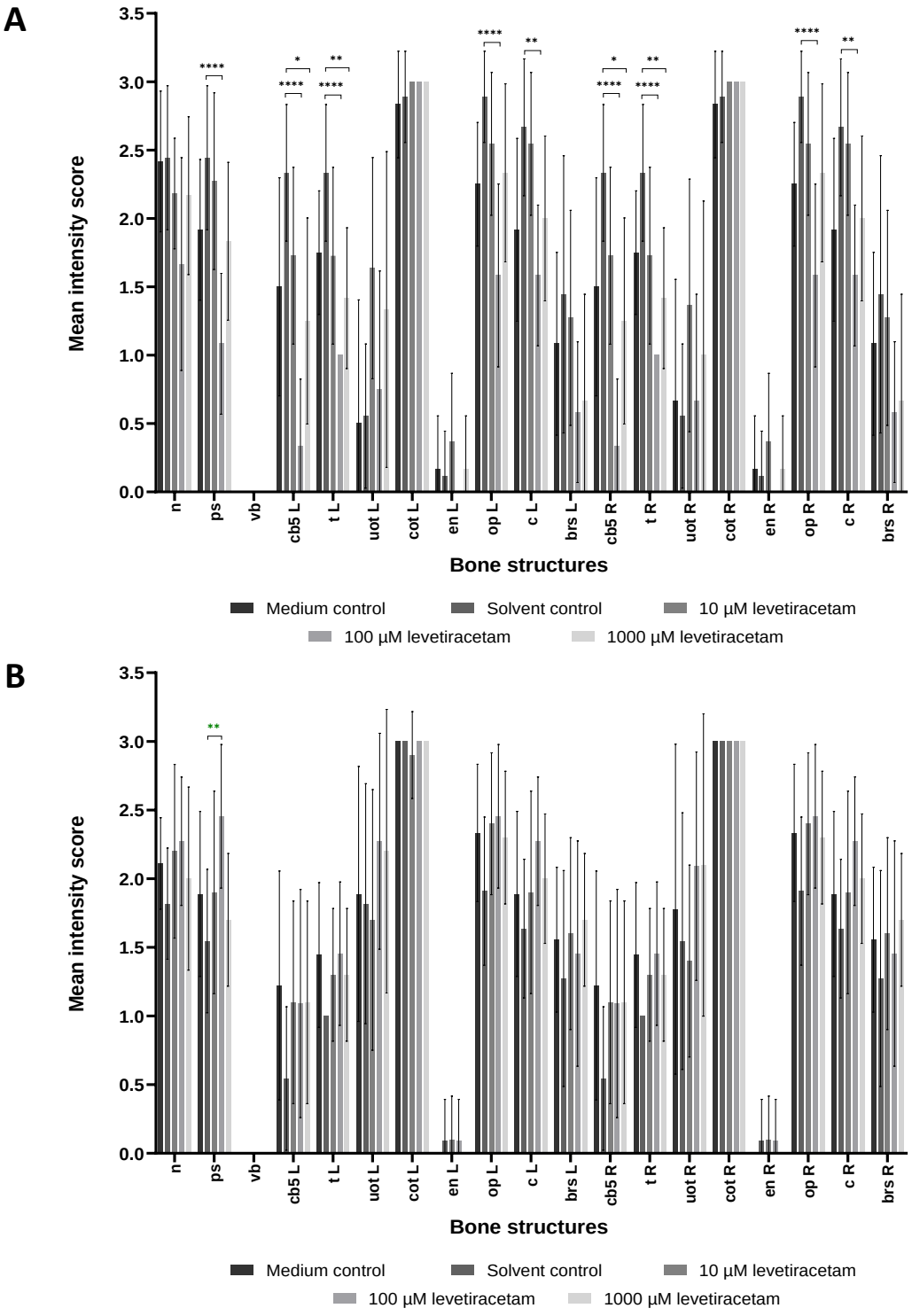


Figure 13. Mean intensity score of fixed AR-stained bones of 5 dpf larvae treated with levetiracetam. A) shows the results of replicate 1 and B) shows the results of replicate 2. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. P ≤ 0.05 (*), p ≤ 0.01 (**) and p ≤ 0.0001 (****). The color of the asterisks indicate that the solvent control was significantly more (black) or less (green) intensely stained than the other group.

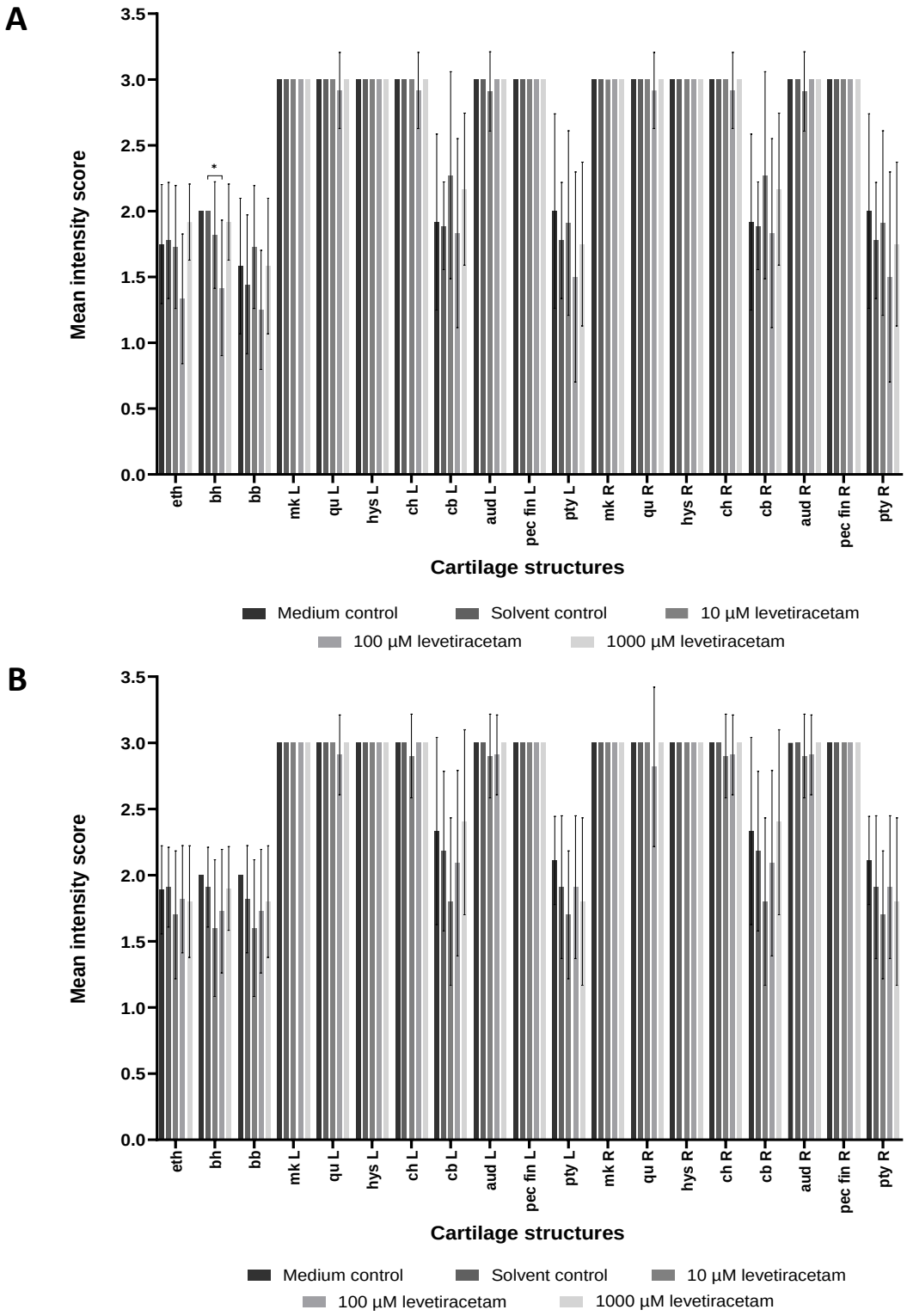


Figure 14. Mean intensity score of fixed AB-stained cartilages of 5 dpf larvae treated with levetiracetam. A) shows the results of replicate 1 and B) shows the results of replicate 2. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. $P \leq 0.05$ (*).

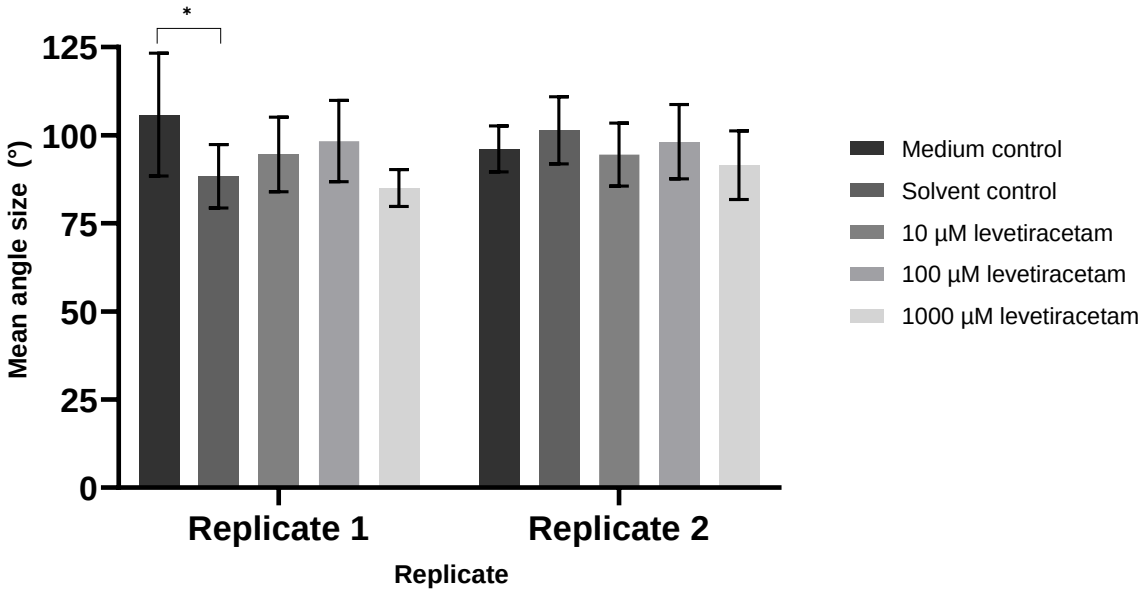


Figure 15. Mean angle sizes between ceratohyal cartilages in 5 dpf larvae treated with levetiracetam. All groups were compared to the solvent control group. Significant differences between the solvent control and any of the other groups are indicated. P ≤ 0.05 (*).

5.3.3.6 Conclusion on the AR-AB staining

A lot of variability in staining intensity was observed between the two replicates. Therefore, we decided to assess whether other staining methods may provide more consistent staining results.

5.3.4 Calcein live staining results

Assessment for the utility of calcein staining for determination of bone formation in the larval zebrafish was undertaken initially on control larvae of 5 dpf and 9 dpf (Figure 16A). Even before assessing the different bone structures more closely, the control larvae already demonstrated a lot of variability in staining (Figure 16B). The high degree of variability in fluorescence meant that, when imaging, adjustments in the exposure duration of the image capture had to be made for each larva to avoid overexposure of the image capture. There was also a large degree of autofluorescence (data not shown) within the tissues.

In conclusion, the high degree of variability in staining intensities between control individuals prevented the skeletal assessment. Therefore, calcein was not further used and a third staining method was explored.

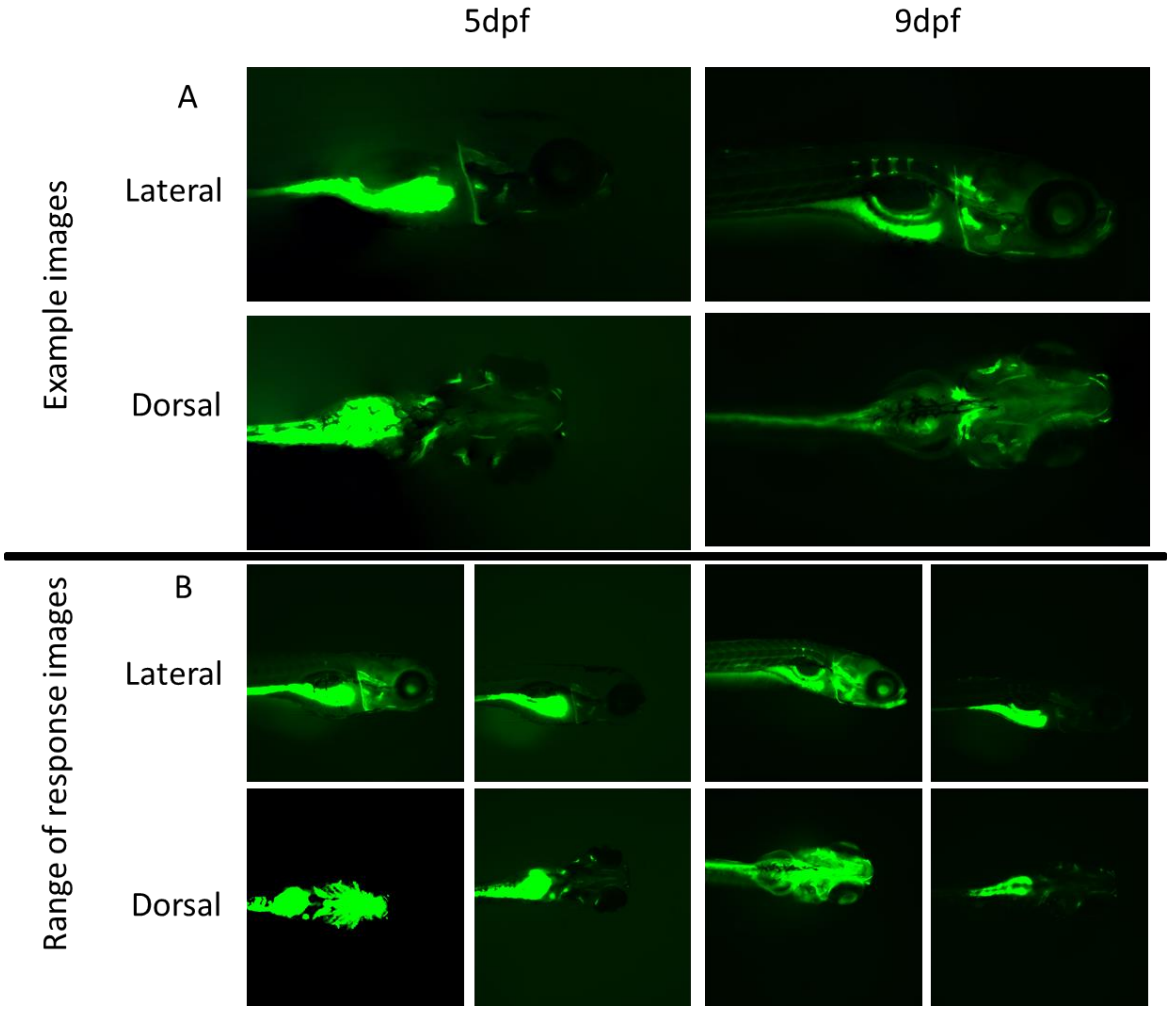


Figure 16. Calcein stained larval zebrafish at 5 and 9 dpf. Panel A shows the detail of bone formation detectable by calcein staining. Panel B demonstrates the variability between the same spawning group of individuals treated at the same time. Abbreviation: days post-fertilization (dpf).

5.3.5 AR live staining results

5.3.5.1 Compound 5

Based on the results of the AR-AB staining (see 5.3.3 and discussion), compound 5 was selected to expose zebrafish embryos to AR live, including a longer exposure window (9 dpf) (see discussion).

At 5 dpf, differences in shape of the parasphenoid were observed between both replicates (see Figure 17). In the first replicate, the parasphenoid was malformed at 1,000 µM ($p = 0.0199$, RR = 1.333). In the other replicate, no malformations were observed.

The staining intensity was significantly reduced in the right utricular otolith at 100 µM in the first replicate at 5 dpf (see Figure 18). In the second replicate, there was no significant effect on this structure, but an increase in staining intensity was observed in the ceratobranchials 5 L&R. At

1,000 µM, the staining intensity was significantly reduced in almost all the bone structures in the first replicate (see Figure 18). In the second replicate, however, the staining intensity was only decreased in the vertebrae and the anguloarticulars L&R, and increased in the ceratobranchials 5 L&R.

For both replicates at 9 dpf, no significant differences in shape of the bone structures were found when comparing the two treatment groups with the solvent control (data not shown).

At 9 dpf in the first replicate, several structures were significantly less stained at 100 µM and 1,000 µM when compared to the control group. In the second replicate, there was only a significant decrease in staining intensity of the left hyomandibular bone at 1,000 µM of compound 5 (see Figure 19).

5.3.5.2 Conclusion on the AR live staining

In conclusion, the AR live staining also showed variability between replicates. To assess whether biological variability in zebrafish skeletal development could be the cause of the variability between replicates, we performed a post-hoc analysis of all solvent control groups.

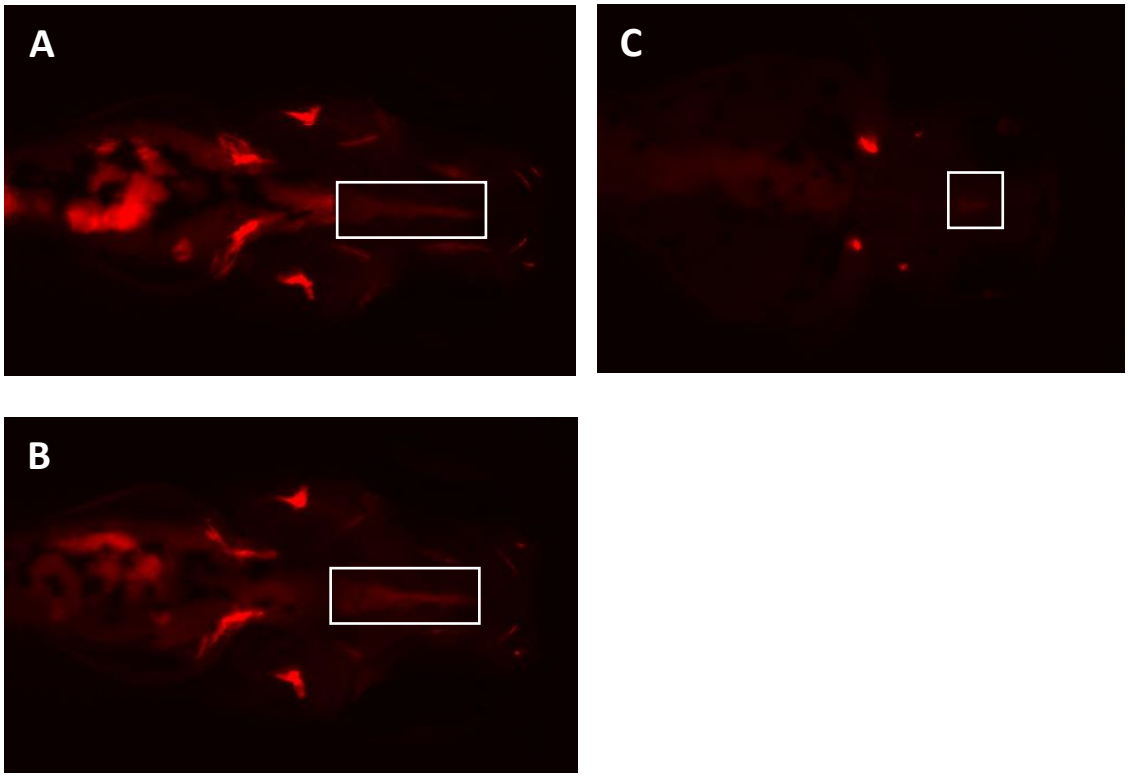


Figure 17. Images of AR live stained bones of 5 dpf larvae treated with A) the solvent control, B) 100 µM of compound 5 and C) 1,000 µM of compound 5. The parasphenoid, which is normal in A and B, and malformed in C, is indicated with a white box. All pictures are from larvae of the first replicate and show the larvae in dorsoventral position.

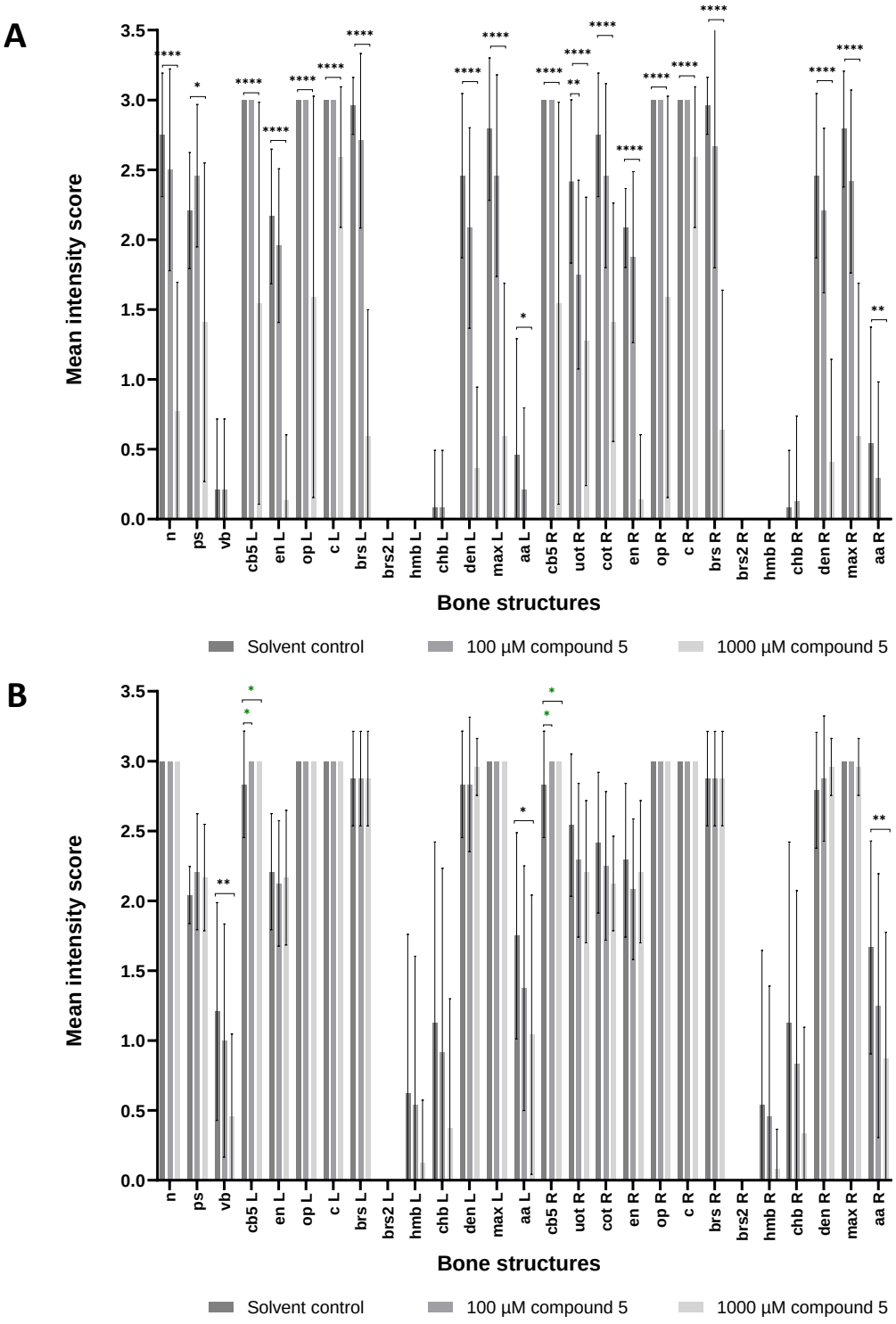


Figure 18. Mean intensity score of AR live stained bones of 5 dpf larvae treated with proprietary compound 5. A) shows the results of replicate 1 and B) shows the results of replicate 2. Both groups were compared to the solvent control group. Significant differences between the solvent control and the test groups are indicated. P ≤ 0.05 (*) and p ≤ 0.01 (**). The color of the asterisks indicate that the solvent control was significantly more (black) or less (green) intensely stained than the other group.

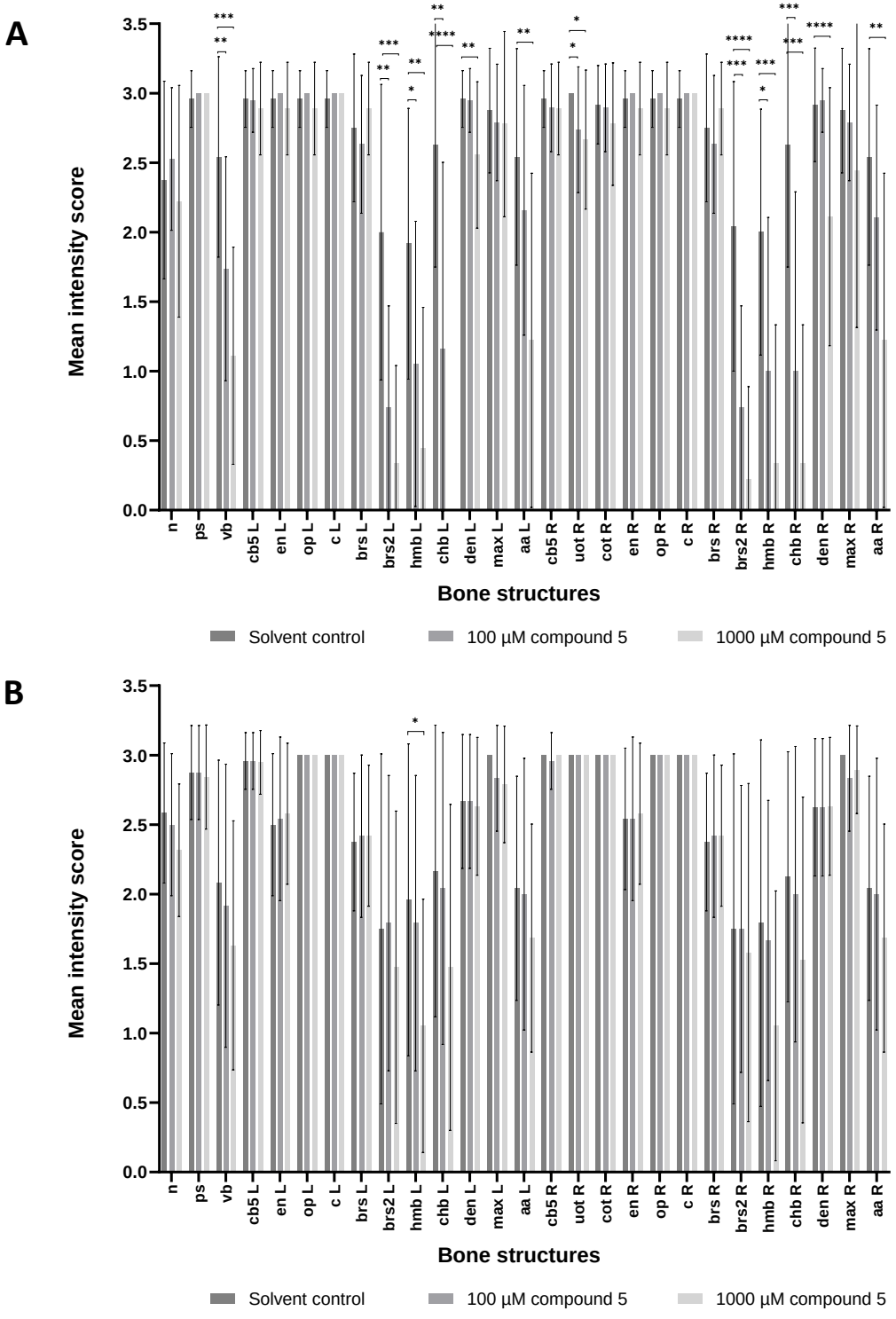


Figure 19. Mean intensity score of AR live stained bones of 9 dpf larvae treated with proprietary compound 5. A) shows the results of replicate 1 and B) shows the results of replicate 2. Both groups were compared to the solvent control group. Significant differences between the solvent control and the test groups are indicated. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) and p ≤ 0.0001 (****).

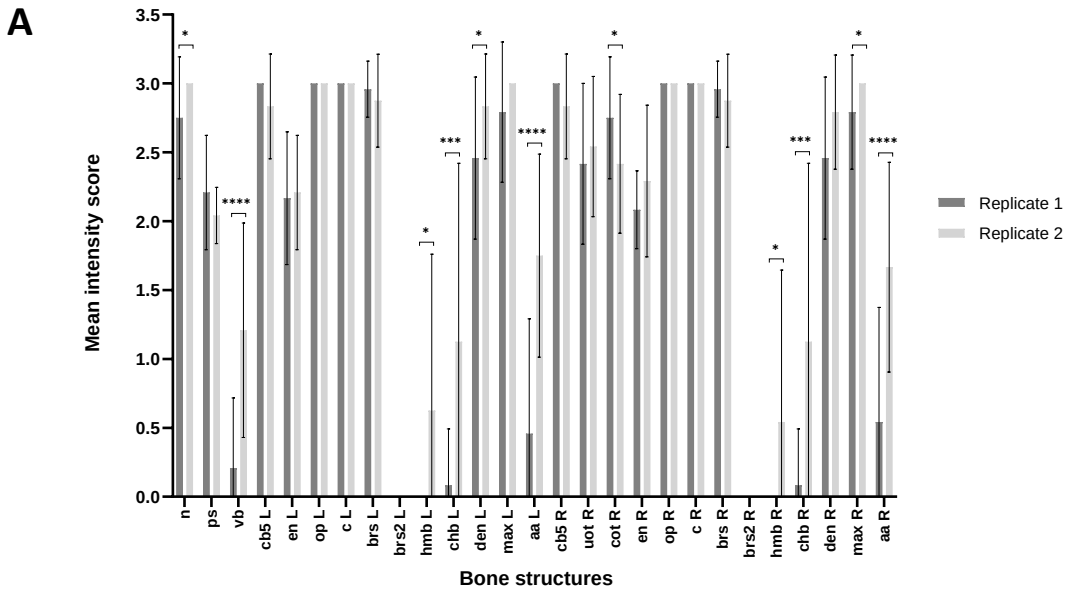
5.3.6 Post-hoc analysis: variability in AR live staining between control groups

When comparing the solvent controls of both replicates of the compound 5 experiment, many differences in bone staining intensity were found (see Figure 20). At 5 dpf, several bone structures were more intensely stained in the second than in the first replicate. Only the right circle saccular otolith was more intensely stained in the first replicate. In contrast, several structures were significantly more intensely stained in the first replicate at 9 dpf. This was even the case for structures that were observed to be less intensely stained in this first replicate at 5 dpf (i.e., chb R, den L, aa L, and aa R). As both replicates were conducted with 9 months of time in between, the use of different batches of adult fish and the use of freshly prepared staining solutions for both replicates may have contributed to this variability. Therefore, we decided to conduct an additional experiment to assess whether biological variability in (onset of) bone development or technical aspects caused the differences in bone staining intensity between replicates.

5.3.7 Variability experiments results

5.3.7.1 Variability between control larvae from 2 clutches of zebrafish (same spawning day)

In the first experiment, differences in bone staining intensity between control larvae from two clutches of fish (same spawning day) were checked (see Figure 21 and Supplementary figure 3). The results revealed that the left and right branchiostegal rays were more intensely stained in larvae that originated from the first clutch.



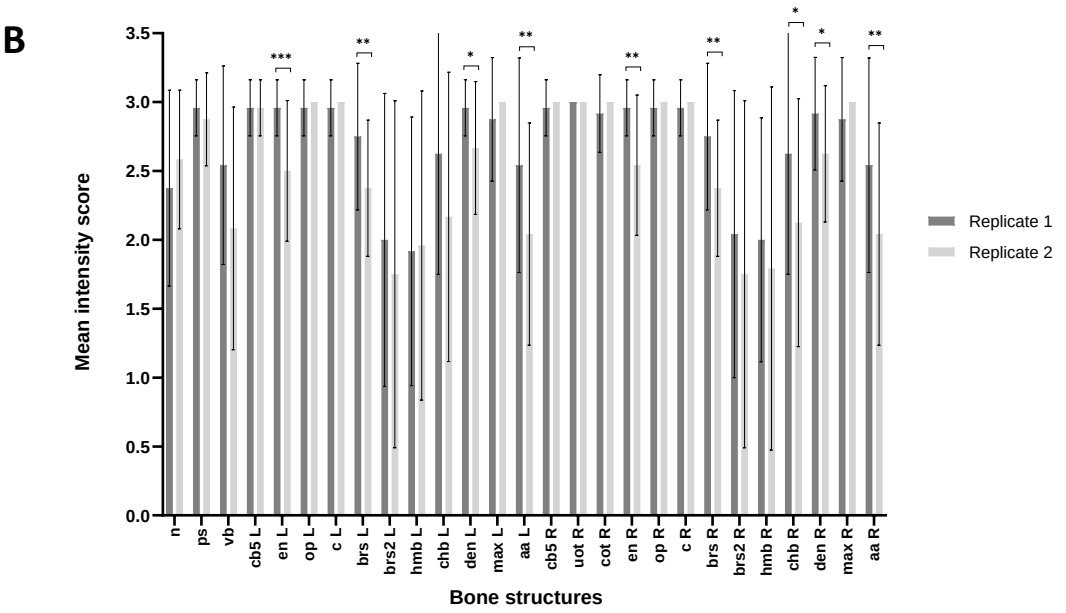


Figure 20. Variability in staining between control larvae of the two replicates of the AR live experiment. A) shows the results using 5 dpf larvae and B) shows the results using 9 dpf larvae. Significant differences between the two groups of solvent controls are indicated. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) and p ≤ 0.0001 (****).

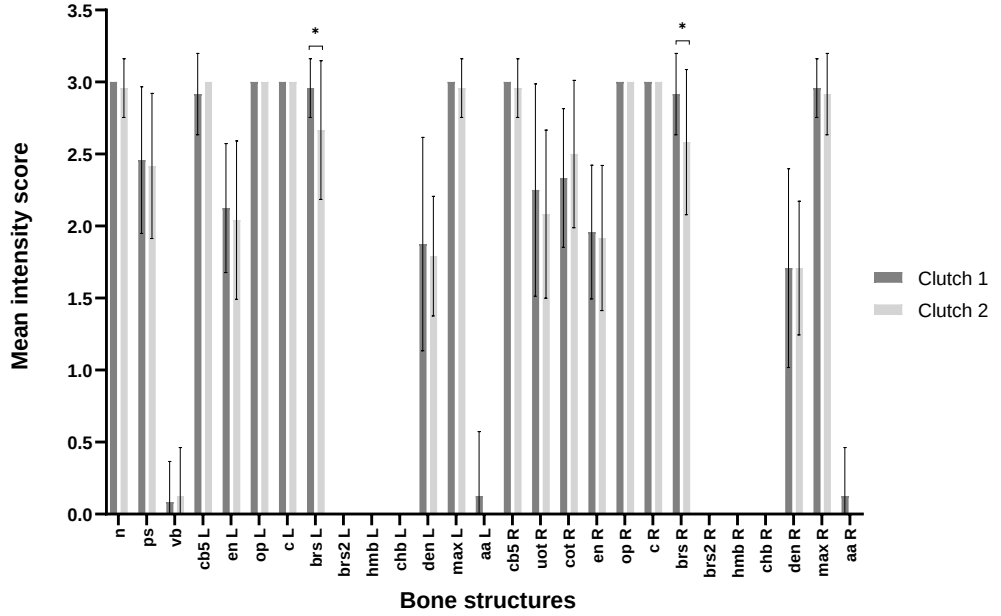


Figure 21. Variability in staining between two clutches of control larvae (same spawning day). Significant differences between the two clutches are indicated. P ≤ 0.05 (*).

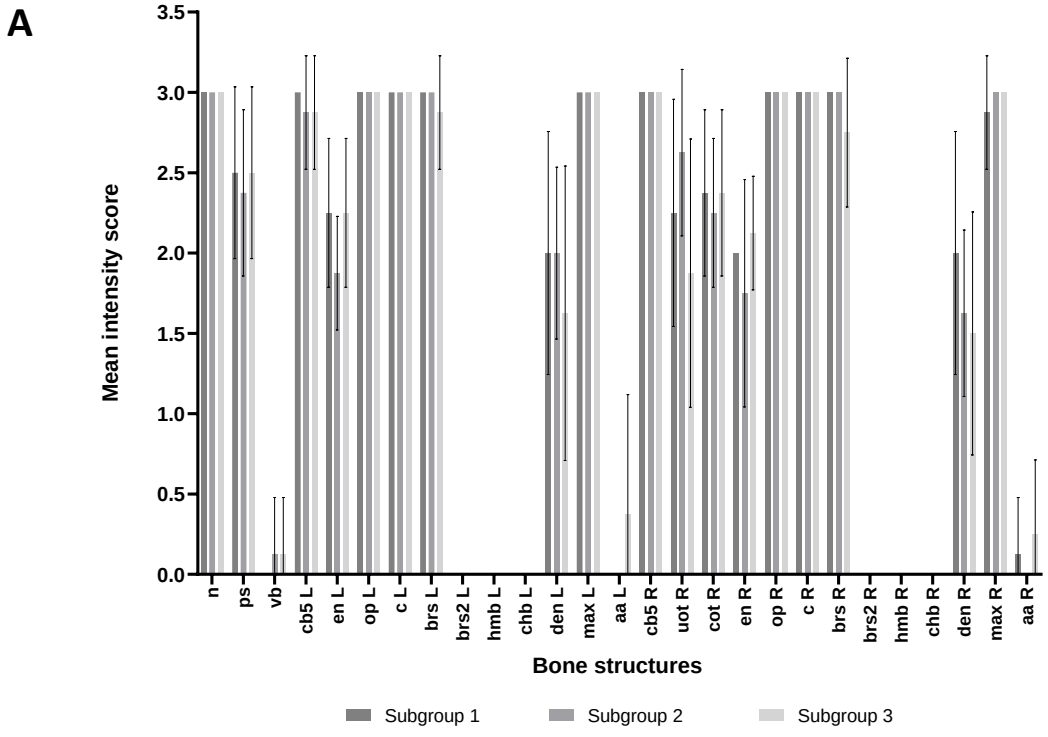
5.3.7.2 Variability between control larvae from the same clutch of zebrafish

In a second experiment, differences in staining intensities between larvae from the same clutch of zebrafish were assessed. For this, the larvae of each of the clutches (batch 1 and 2, respectively) of experiment 1 (see 5.3.7.1) and the solvent control group of the second replicate of the AR live compound 5 experiment (5 dpf) (batch 3) were randomly divided into 3 groups of 8 fish. These 3 subgroups were compared to each other.

For the first batch of experiment 1, no significant differences in staining intensity between the three subgroups were observed (see Figure 22A). For the other two batches, however, significant differences were observed. In the second batch, the right dentary was significantly less stained in the second subgroup than in the other subgroups (see Figure 22B). In the third batch, the right utricular otolith was significantly less stained in the third subgroup than in both other subgroups, and the left entopterygoid was significantly less stained than in the first subgroup (see Figure 22C).

5.3.7.3 Conclusion on the variability experiments

For the AR live staining, we found variability in staining intensities between larvae of two clutches of the same spawning day, as well as between larvae originating from the same clutch of fish. Thus, biological variability in (onset of) bone development is clearly present in zebrafish larvae.



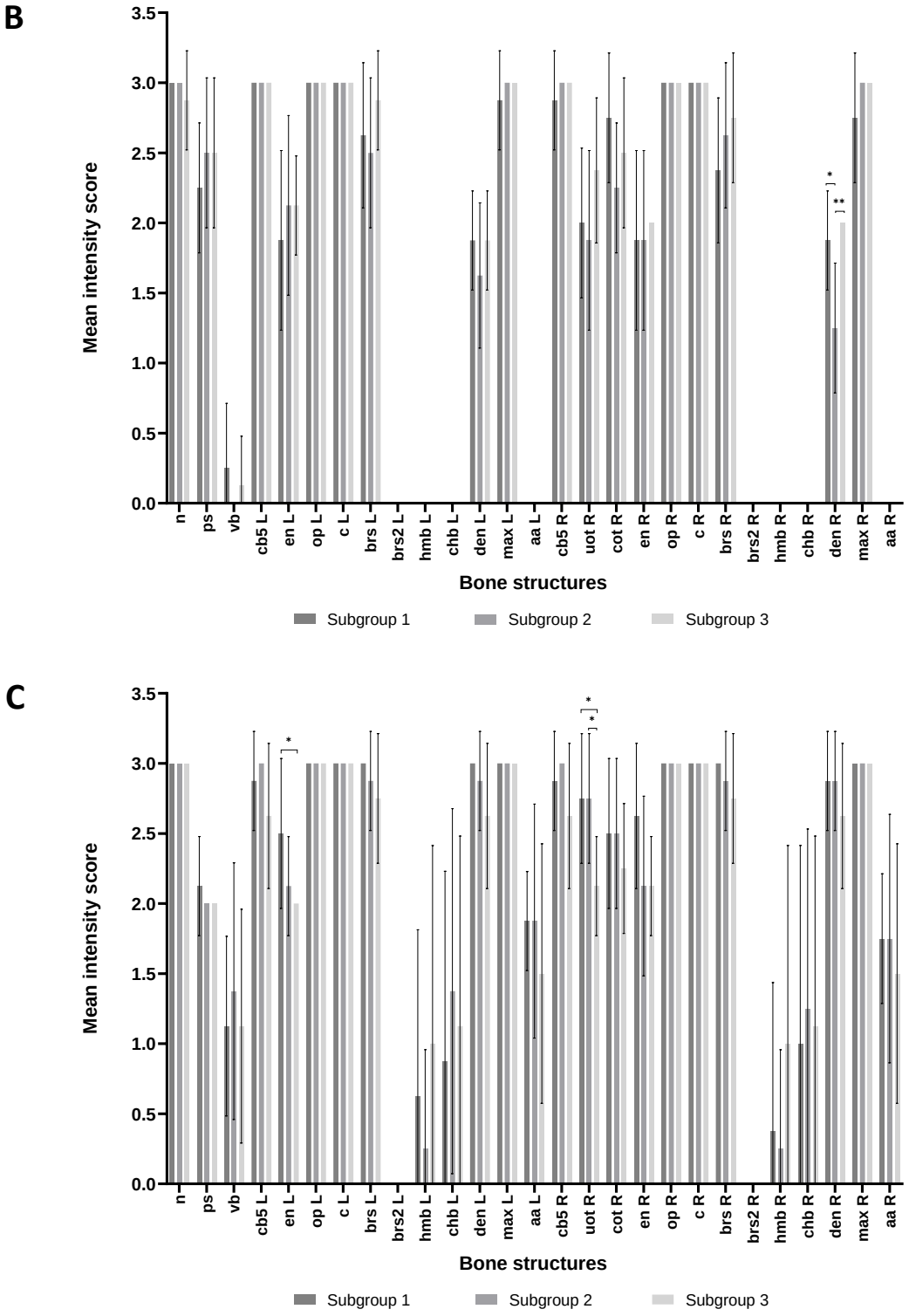


Figure 22. Variability in staining between subgroups of the same batch of control larvae. In A) the results of batch 1 are shown, in B) the results of batch 2 are shown and in C) the results of another batch (i.e., the solvent control group of the second replicate of the compound 5 experiment at 5 dpf) are shown. For each of these batches, the batch was divided into 3 subgroups of 8 fish. All the subgroups of a batch were compared to each other. Significant differences between the three subgroups are indicated. $P \leq 0.05$ (*) and $p \leq 0.01$ (**).

5.3.8 Post-hoc analysis: variability in AR-AB staining between control groups

As the results of the AR live staining indicated that biological variability in (onset of) bone development is clearly present in zebrafish larvae, we also performed a post-hoc analysis of the control groups of the AR-AB experiments to check whether the same staining variability can be observed.

The angle size, bone shape and skeletal structure intensity of eight medium control groups were compared to each other. No significant differences in shape were observed (data not shown). Two groups, C5.2 and LTC.1, had significantly increased angle sizes when compared with some other groups (i.e., C5.2 with C5.1, CA.1 and CA.2, and LTC.1 with C5.1) (see Figure 23). For staining intensity, significant results were observed for several structures (see Table 6). Especially the paraspenoid, branchiostegal rays, and the pharyngeal teeth (bone structures) and the basihyal, ceratohyal, ceratobranchials, and the auditory capsule (cartilage structures) were stained significantly different. So, also for the AR-AB experiments staining variability between different control groups was observed.

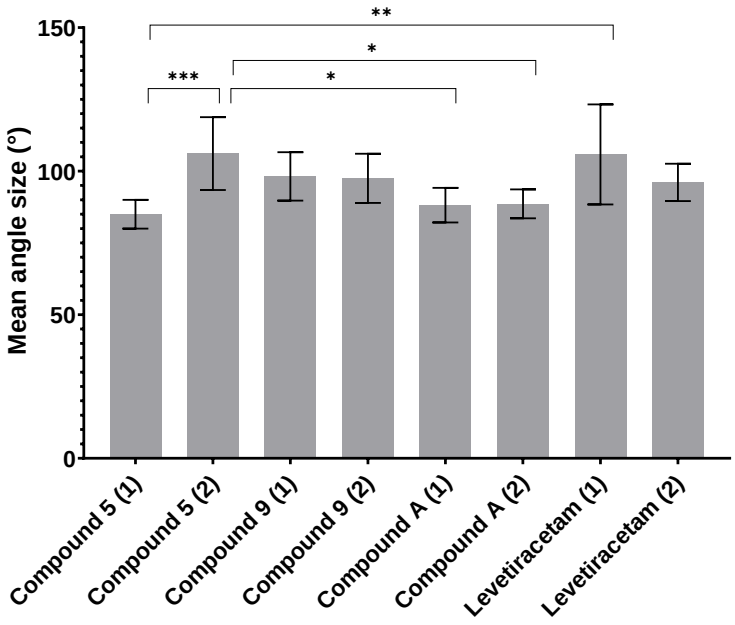


Figure 23. Differences in mean angle size between the medium controls of all fixed staining experiments. P ≤ 0.05 (*), p ≤ 0.01 (**) and p ≤ 0.001 (***).

Table 6. Differences in staining intensity between the medium controls of all fixed staining experiments. All medium control groups were compared to each other and all A) bone and B) cartilage structures that differed significantly are indicated. The asterisks represent the significance. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) and p ≤ 0.0001 (****). For vb, all scores were 0 (NA). If no left (L) or right (R) were indicated, the result of both sides was the same. Abbreviations: compound 5 (C5), compound 9 (C9), compound A (CA), levetiracetam (LTC), replicate 1 (.1) and replicate 2 (.2).

A

	n	ps	vb	cb5	t	uot L	uot R	cot	en	op	c	brs
C5.1 - C9.1		**	NA									
C5.1 - C9.2			NA									*
C5.1 - C5.2			NA		**							
C5.1 - LTC.1		***	NA			*						
C5.1 - CA.1		***	NA		*					*		
C5.1 - LTC.2		**	NA		**							
C5.1 - CA.2		*	NA									
C9.1 - C9.2			NA									
C9.1 - C5.2			NA									
C9.1 - LTC.1			NA									
C9.1 - CA.1	**		NA									
C9.1 - LTC.2			NA									
C9.1 - CA.2			NA									*
C9.2 - C5.2			NA									
C9.2 - LTC.1			NA									
C9.2 - CA.1			NA									
C9.2 - LTC.2			NA									*
C9.2 - CA.2			NA									***
C5.2 - LTC.1			NA				*					
C5.2 - CA.1			NA									
C5.2 - LTC.2			NA									
C5.2 - CA.2			NA									
LTC.1 - CA.1			NA									
LTC.1 - LTC.2			NA									
LTC.1 - CA.2			NA									
CA.1 - LTC.2	*		NA									
CA.1 - CA.2			NA									
LTC.2 - CA.2			NA									

B

	eth	bh	bb	mk	qu	hys	ch	cb	aud	pec fin	pty
C5.1 - C9.1								**			
C5.1 - C9.2											
C5.1 - C5.2		****						****	****		
C5.1 - LTC.1								**			
C5.1 - CA.1								**			
C5.1 - LTC.2											
C5.1 - CA.2								***			
C9.1 - C9.2											
C9.1 - C5.2		****					*		****		
C9.1 - LTC.1											
C9.1 - CA.1											
C9.1 - LTC.2											
C9.1 - CA.2											
C9.2 - C5.2		**							****		
C9.2 - LTC.1											
C9.2 - CA.1											
C9.2 - LTC.2											
C9.2 - CA.2											
C5.2 - LTC.1		****					*		****		
C5.2 - CA.1		*					*		****		
C5.2 - LTC.2		****	***						****		*
C5.2 - CA.2		**					*		****		
LTC.1 - CA.1											
LTC.1 - LTC.2											
LTC.1 - CA.2											
CA.1 - LTC.2	*										
CA.1 - CA.2											
LTC.2 - CA.2	*										

5.4 Discussion

The goal of this study was to investigate whether the sensitivity of zebrafish embryo developmental toxicity assays can be increased by including a skeletal staining. To avoid potential negative results due to the lack of compound uptake by zebrafish larvae, the first aim was to assess the uptake and stability of the four selected compounds. This assessment revealed that each compound was taken up by zebrafish embryos (1 dpf) and larvae (5 dpf) and remained stable in the medium over the tested period of five days.

Once uptake was confirmed, the viability and gross morphology of 5 dpf zebrafish larvae was assessed. Although all four compounds were previously reported to give false negative results [8,10], a re-assessment of the gross morphology in the present study allowed a comparison of the sensitivity with and without the use of skeletal staining methods. Also larval length was determined as an additional endpoint in this study, because it is known that exposure to xenobiotics can cause a reduced body length [38,39].

No significant differences in viability were observed between 5 dpf zebrafish larvae treated with any of the compounds and the control groups, which is in line with what was reported earlier [8].

The morphological assessment of 5 dpf larvae exposed to the highest concentration of compound 5, however, revealed malformations of the jaw, neural tube and heart, that were not observed earlier by Ball et al. (2014) [8]. The reason why these malformations were not observed before is unknown, as the compound was tested in several labs and the same morphological assessment method was used. Moreover, compounds 5 and 9 caused a shortened body after exposure to 1,000 µM at 5 dpf. Interestingly, one of the malformations that was detected in EFD studies for compound 9 was shorter long bones in rats [8]. Compound A showed a reduced body length in all concentrations in the first replicate (not dose-dependent), while the larvae in the second replicate were not affected. As larval length was not determined in the studies by Lee, et al. (2013) and Ball, et al. (2014), a comparison with those studies was not possible [8,10]. In our study, the larvae exposed to compound 5 were further reared until 9 dpf to see if skeletal assessment at a later age would make the assay more sensitive than at 5 dpf. At 9 dpf, the body length of larvae exposed to 1,000 µM (2 replicates) and 100 µM (1 replicate) of compound 5 was reduced. Moreover, the viability of the 9 dpf larvae exposed to 1,000 µM of compound 5 was significantly lower. Interestingly, this lethal effect only became visible at 9 dpf, so when the exposure had already been stopped.

Levetiracetam appeared to be non-teratogenic in our study, which is in agreement with the study by Lee, et al. (2013) [10]. The results of compound A are inconclusive due to the differences for larval length between both replicates. In contrast to what was found by Ball, et al. (2014), the results of the viability, length and morphological assessment in our study indicate that compound 5 might be teratogenic in zebrafish larvae. The reduced larval length observed for compound 9, which is a sign of growth retardation, is a first sign that this compound might have a negative impact on zebrafish development.

Despite the fact that negative effects were already found for compound 5 and 9 without skeletal staining, we still evaluated whether a skeletal assessment could make the assay more sensitive, i.e., reveal structural effects already at lower concentrations.

The AR-AB fixed staining was the first staining that was tested, since this method allowed visualization of most skeletal structures as both bone and cartilage structures are stained. However, for all four compounds the results were inconsistent due to the variability between replicates. This variability was not found with the gross morphology, length, and viability assessments, except for the length of larvae exposed to compound A. Especially the staining intensity of skeletal structures showed to be very different between the replicates of all four compounds. In contrast, there were hardly any differences in shape and almost no significant differences in angle size. The mean angle size was only significantly increased at 100 µM of compound A in replicate 1. In addition to the differences between replicates, the results of the intensity staining revealed often more pronounced effects after exposure to lower, instead of higher, concentrations of LTC (i.e., not dose-dependent). For compound 9, increased, instead of the expected decreased, staining intensities were observed for some structures (i.e., cb5 and ps). However, this might be due to the fact that the solvent control was significantly less stained for cb5 and ps when compared the medium control group. Therefore, the increase in intensity is likely due to a decreased staining in the control group.

Due to the high variability between replicates and inconsistent results, it was difficult to determine whether the AR-AB staining could pick up malformations that were not found by using only a gross morphological, length and viability assessment. In contrast to the use of AR-AB as a skeletal staining, the length assessment was consistent between the replicates of three of the compounds. Therefore, including larval length as a standard endpoint in zebrafish embryo assays could be considered. As a staining method should be able to provide a consistent staining, and especially variability in staining intensity was found to be the most prominent cause of differences between replicates, we decided to explore other staining methods. As most significant differences were found in bones and not in cartilages, the use of skeletal staining methods that only visualize bone might be sufficient to increase the sensitivity. The observed malformations of the jaw, the reduced length, and the reduced intensity of ceratobranchial cartilages after exposure to compound 5 may suggest that, of all compounds, an impact on bone development is the most likely for compound 5. Therefore, this compound was used to test the other staining methods.

The calcein live staining caused a high degree of variability in fluorescence between control larvae, which prevented the assessment of the different bone structures. Hence, our findings indicated that calcein cannot be used for bone assessment in developing zebrafish embryos. This is in contrast to what was found in the study by Du, et al. (2001) [24]. They reported that calcein was a more sensitive staining method when compared to AR-AB staining. In their study, calcein revealed most skeletal structures, whereas there was almost no staining from alizarin red and only a subset of the structures could be visualized using alcian blue. However, they did not use an acid-free AR-AB staining method. Walker and Kimmel (2007) reported that the combination of alizarin red and alcian blue is problematic if acidic conditions are used to differentiate the tissue in the alcian blue staining. This acid demineralizes bone, which affects the alizarin red staining as this depends on mineralization of the bone matrix [23]. Hence, this might explain why Du et. al barely found alizarin red stained structures, and therefore reported a higher sensitivity for the calcein staining. Moreover, as alcian blue is a cartilage staining (i.e., it stains the extracellular matrix that is associated with chondrocytes [40]) and not a bone staining, it is reasonable that more calcified skeletal structures were stained with calcein, as the latter specifically binds to calcified skeletal structures. Due to the variability in fluorescence in the controls, calcein was not further used in our study and a third staining, the AR live staining, was explored.

Since an AR live staining allows staining of living larvae, the until 5 dpf exposed larvae could be reared until 9 dpf and checked again for skeletal malformations. The aim was to investigate whether a skeletal assessment at a later age (i.e., 9 dpf) would increase the sensitivity of the assay compared to an assessment at 5 dpf. Although the AR live staining looked promising during an initial test with a test compound using only one replicate in a previous study [16], the variability in staining intensity between replicates of compound 5 appeared to be a problem for this staining as well. This variability was not detected in Hoyberghs, et al. (2020), as only one replicate was used [16]. In our present study, the staining intensity of almost all affected structures was only reduced in one of the replicates at both concentrations at 5 and 9 dpf. In addition, the results indicated that a bone staining is not more sensitive at 9 dpf, as already a reduced body length and increased mortality was observed at this age. In the group treated with the highest concentration of compound 5, only

9 fish survived. At 5 dpf, the AR live staining was not more sensitive in the 1,000 µM group, as gross morphological abnormalities were present. Interestingly, a reduced staining intensity of the dentary and maxilla was observed in replicate 1. This is in line with the observed jaw malformations of the gross morphological assessment. Moreover, in this replicate also a malformation in the shape (i.e., reduced length) of the parasphenoid was found, which is in line with the reduced larval length. Only in the 100 µM group a bone staining at 5 dpf showed to be more sensitive than gross morphology. However, due to the variability in staining between replicates, it is not clear if including an AR live bone staining visualizes malformations at lower concentrations. Nevertheless, the results indicate that a bone staining is not more sensitive at 9 dpf than at 5 dpf.

Due to inconsistent results between replicates of compound-treated zebrafish larvae, additional analyses on AR live and AR-AB-stained control larvae were performed to check whether replicates of control larvae also showed this variability. For both staining methods, many differences in bone staining intensity were found between replicates of control larvae. A reliable staining method should provide consistent results in independent experiments with control larvae. For the AR live staining at 5 and 9 dpf, a lot of differences in bone staining were present between two groups of control larvae. However, as both replicates were conducted with 9 months of time in between, the use of different batches of adult fish and the use of freshly prepared staining solution for both replicates may have caused additional variability. Therefore, additional AR live staining experiments on control larvae were conducted to assess whether biological variability in (onset of) bone development or technical aspects caused the differences in bone staining intensity between replicates. In these experiments, variability in staining between larvae of two clutches of the same spawning day, as well as variability in staining between larvae originating from the same clutch of zebrafish were found. These findings are in line with the study by Cubbage and Mabee (1996) that specifically looked at the development of the zebrafish cranium and paired fins. Using a smaller number of zebrafish larvae per age, inter-individual variability in the onset of ossification was also clearly observed in their study. Moreover, the degree of variability was dependent on the bone structure [32].

In conclusion, we found that biological variability in (onset of) bone development is clearly present in zebrafish larvae. This biological variability hampers the detection of (subtle) treatment-related bone effects that are not picked-up by a gross morphological assessment.

5.5 Supplementary tables and figures

Supplementary table 1. Overview of different skeletal staining methods for zebrafish larvae. Abbreviations: alizarin red (AR), alizarin red-alcian blue (AR-AB).

Method	**Acid-free AR-AB staining** [23]	**Calcein staining** [24]	**AR live staining**[1]
Principle	AR stains bone by binding specifically to calcified skeletal structures, and AB stains cartilage via an electrostatic interaction with negatively charged acidic mucopolysaccharides. Magnesium chloride is used instead of acid to prevent decalcification of the bone matrix.	A fluorescent chromophore that stains bone by penetrating into zebrafish embryos and specifically binding to calcified skeletal structures	A fluorescent chromophore that stains bone by penetrating into zebrafish embryos and specifically binding to calcified skeletal structures
Advantages	- Staining of both bone and cartilage structures [23]	- Live staining: monitor development over time [41] - More inclusive and sensitive method for visualizing skeletal structures in zebrafish larvae than AR or AB [24]	- Live staining: monitor development over time [41] - Fluorescent spectrum not similar to autofluorescence spectrum of zebrafish tissue [41]
Disadvantages	- Fixed staining: monitor development over time not possible - Relatively long staining procedure [24]	- Fluorescent spectrum similar to autofluorescence spectrum of zebrafish tissue [41] - No cartilage staining	- No cartilage staining

[1] Personal communication from Dr. C. Hammond (Bristol University, UK) to the authors.

Supplementary table 2. Viability of AR-AB fixed stained zebrafish larvae at 5 dpf after exposure to the different test solutions. No significant differences in viability were observed between any of the test groups and the control groups (medium and DMSO).

Compound	Replicate	Conc. (µM)	Viability (#alive/#exposed)
Compound 5	1	Medium	24/24
		DMSO	24/24
		10	24/24
		100	24/24
		1,000	24/24
	2	Medium	23/24
		DMSO	24/24
		10	24/24
		100	24/24
		1,000	24/24
Compound 9	1	Medium	24/24
		DMSO	24/24
		10	23/24
		100	23/24
		1,000	23/24
	2	Medium	24/24
		DMSO	24/24
		10	24/24
		100	24/24
		1,000	24/24
Compound A	1	Medium	24/24
		DMSO	24/24
		1	24/24
		10	24/24
		100	24/24
	2	Medium	24/24
		DMSO	24/24
		1	24/24
		10	24/24
		100	24/24
LTC	1	Medium	24/24
		DMSO	22/24
		10	22/24
		100	24/24
		1,000	24/24
	2	Medium	24/24
		DMSO	24/24
		10	24/24
		100	24/24
		1,000	24/24

Supplementary table 3. Viability of AR live stained zebrafish larvae at 5 and 9 dpf after exposure to compound 5. P ≤ 0.05 (*) and p ≤ 0.0001 (****). [1]One larva was lost during the staining process at day 5.

Age (dpf)	Replicate	Conc. (µM)	Viability (#alive/#exposed)	Viability p-value
5	1	DMSO	24/24	
		100	24/24	ns
		1,000	22/24	ns
	2	DMSO	24/24	
		100	24/24	ns
		1,000	24/24	ns
9	1	DMSO	24/24	
		100	23/24	ns
		1,000	**9/24****	**p = 0.0001***
	2	DMSO	24/24	
		100	24/24	ns
		1,000	**19/24*[1]**	**p = 0.0496**

Supplementary table 4. Length of AR-AB fixed stained zebrafish larvae at 5 dpf after exposure to the different test solutions. P ≤ 0.05 (*), p ≤ 0.01 (**) and p ≤ 0.001 (***).

Compound	Replicate	Conc. (µM)	N	Length (mm) mean ± SD	Length p-value
Compound 5	1	Medium	22	3.8464 ± 0.1233	
		DMSO	24	3.8375 ± 0.1137	
		10	24	3.8604 ± 0.1030	**ns**
		100	24	3.7871 ± 0.1236	**ns**
		1,000	22	3.7568 ± 0.1457	**p = 0.048* (medium), p = 0.031* (DMSO)**
	2	Medium	20	3.7660 ± 0.1910	
		DMSO	23	3.8591 ± 0.0916	
		10	23	3.8557 ± 0.0803	**ns**
		100	24	3.8800 ± 0.0611	**ns**
		1,000	23	3.7270 ± 0.1040	**p = 0.000*** (DMSO)**
Compound 9	1	Medium	24	3.9121 ± 0.0857	
		DMSO	24	3.9304 ± 0.0679	
		10	23	3.9470 ± 0.0532	ns
		100	23	3.9299 ± 0.0612	ns
		1,000	23	3.8396 ± 0.0610	**p = 0.002** (medium), p = 0.000*** (DMSO)**
	2	Medium	23	3.8696 ± 0.0813	
		DMSO	24	3.8954 ± 0.1006	
		10	24	3.8883 ± 0.0813	ns
		100	24	3.9021 ± 0.0647	ns
		1,000	24	3.8188 ± 0.0941	**p = 0.004** (DMSO)**
Compound A	1	Medium	24	3.7671 ± 0.1004	
		DMSO	24	3.8237 ± 0.0858	
		1	23	3.7252 ± 0.0797	**p = 0.037* (medium), p = 0.000*** (DMSO)**
		10	24	3.7158 ± 0.1112	**p = 0.023* (medium), p = 0.000*** (DMSO)**
		100	24	3.7529 ± 0.1011	**p = 0.014* (DMSO)**
	2	Medium	24	3.8258 ± 0.1402	
		DMSO	23	3.8017 ± 0.1300	
		1	24	3.8442 ± 0.1073	ns
		10	22	3.8473 ± 0.1233	ns
		100	20	3.7605 ± 0.1326	ns
LTC	1	Medium	24	3.7487 ± 0.0821	
		DMSO	22	3.7759 ± 0.0436	
		10	22	3.7882 ± 0.0652	ns
		100	24	3.7837 ± 0.0641	ns
		1,000	24	3.7800 ± 0.0425	ns
	2	Medium	20	3.8905 ± 0.0871	
		DMSO	23	3.8722 ± 0.1321	
		10	22	3.8845 ± 0.0790	ns
		100	23	3.8822 ± 0.1302	ns
		1,000	22	3.8777 ± 0.1061	ns

Supplementary table 5. Length of AR live stained zebrafish larvae at 5 and 9 dpf after exposure to compound 5. P ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) and p ≤ 0.0001 (****)

Age (dpf)	Replicate	Conc. (µM)	N	Length (mm) mean ± SD	Length p-value
5	1	DMSO	24	3.6092 ± 0.1429	
		100	16	3.5931 ± 0.1946	ns
		1,000	22	3.3509 ± 0.2709	**p = 0.001*** **
	2	DMSO	24	3.9838 ± 0.1003	ns
		100	24	3.9850 ± 0.1271	ns
		1,000	24	4.0071 ± 0.1015	ns
9	1	DMSO	24	3.8579 ± 0.1614	
		100	19	3.7295 ± 0.1428	**p = 0.030***
		1,000	9	3.6567 ± 0.1849	**p = 0.011***
	2	DMSO	24	4.3921 ± 0.1210	
		100	24	4.3417 ± 0.1906	ns
		1,000	19	4.2826 ± 0.1148	**p = 0.003****

Supplementary table 6. Morphological evaluation of 5 dpf larvae exposed to compound 5.

Compound 5 - Replicate 1																													
									% Abnormal of individual category																		% Other Types of Abnormalities			
Compound	Concentration (µM)	# Larvae Cultured	# Larvae Viable	% Larvae Lethality	# Larvae Unhatched	% Larvae Unhatched	# Larvae Abnormal	% Larvae Abnormal	Body Shape Malformed	Somites Malformed	Notochord Malformed	Tail Malformed	Fins Malformed	Heart Malformed	Face Malformed	Neural Tube Malformed	Arches/ Jaws Malformed	# Scored	Swim bladder Not well-inflated	Swim bladder Not evident	Poor Pigmentation	Excess Pigmentation	Abnormal Pigmentation	Yolk ball - Remnant excessive	Yolk ball - Remnant, dark	Stomach, Not Evident	Liver - Not Evident	Liver - Enlarged	Abdomen distended or yolk sac edema
Medium	N/A	24	24	0.00	1	4.17	1	5%	4.5%	0.0%	0.0%	4.5%	0.0%	0.0%	0.0%	0.0%	0.0%	22	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
DMSO	0.5%	24	24	0.00	0	0.00	2	8%	4.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.2%	24	0.0%	4.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C5	10.0	24	24	0.00	0	0.00	2	8%	4.2%	0.0%	0.0%	4.2%	4.2%	0.0%	0.0%	0.0%	0.0%	24	4.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C5	100.0	24	24	0.00	1	4.17	1	4%	0.0%	0.0%	0.0%	0.0%	4.2%	0.0%	0.0%	0.0%	0.0%	24	20.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C5	1000.0	24	24	0.00	2	8.33	13	59%	4.5%	4.5%	0.0%	0.0%	31.8%	59.1%	18.2%	40.9%	40.9%	22	90.9%	27.3%	0.0%	0.0%	0.0%	0.0%	0.0%	4.5%	0.0%	4.5%	18.2%
Compound 5 - Replicate 2																													
									% Abnormal of individual category																		% Other Types of Abnormalities			
Compound	Concentration (µM)	# Larvae Cultured	# Larvae Viable	% Larvae Lethality	# Larvae Unhatched	% Larvae Unhatched	# Larvae Abnormal	% Larvae Abnormal	Body Shape Malformed	Somites Malformed	Notochord Malformed	Tail Malformed	Fins Malformed	Heart Malformed	Face Malformed	Neural Tube Malformed	Arches/ Jaws Malformed	# Scored	Swim bladder Not well-inflated	Swim bladder Not evident	Poor Pigmentation	Excess Pigmentation	Abnormal Pigmentation	Yolk ball - Remnant excessive	Yolk ball - Remnant, dark	Stomach, Not Evident	Liver - Not Evident	Liver - Enlarged	Abdomen distended or yolk sac edema
Medium	N/A	24	23	4.17	4	16.67	4	20%	5.0%	0.0%	0.0%	5.0%	5.0%	10.0%	5.0%	10.0%	20.0%	20	10.0%	5.0%	0.0%	0.0%	0.0%	5.0%	0.0%	0.0%	0.0%	0.0%	0.0%
DMSO	0.5%	24	24	0.00	1	4.17	1	4%	4.3%	4.3%	4.3%	0.0%	4.3%	4.3%	4.3%	4.3%	4.3%	23	17.4%	4.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.3%
C5	10.0	24	24	0.00	1	4.17	1	4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.3%	23	13.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C5	100.0	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	58.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C5	1000.0	24	24	0.00	1	4.17	23	100%	0.0%	30.4%	0.0%	0.0%	65.2%	100.0%	73.9%	82.6%	95.7%	23	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	30.4%

Supplementary table 7. Morphological evaluation of 5 dpf larvae exposed to compound 9.

Compound 9 - Replicate 1																												
									% Abnormal of individual category																	% Other Types of Abnormalities			
Compound	Concentration (µM)	# Larvae Cultured	# Larvae Viable	% Larvae Lethality	# Larvae Unhatched	% Larvae Unhatched	# Larvae Abnormal	% Larvae Abnormal	Body Shape Malformed	Somites Malformed	Notochord Malformed	Tail Malformed	Fins Malformed	Heart Malformed	Face Malformed	Neural Tube Malformed	Arches/ Jaws Malformed	# Scored	Swim bladder Not well-inflated	Swim bladder Not evident	Poor Pigmentation	Excess Pigmentation	Abnormal Pigmentation	Yolk ball - Remnant excessive	Yolk ball - Remnant, dark	Stomach, Not Evident	Liver - Not Evident	Liver - Enlarged	Abdomen distended or yolk sac edema
Medium	N/A	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
DMSO	0.5%	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C9	10.0	23	23	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	23	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C9	100.0	23	23	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	23	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C9	1000.0	23	23	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	23	95.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Compound 9 - Replicate 2																												
									% Abnormal of individual category																	% Other Types of Abnormalities			
Compound	Concentration (µM)	# Larvae Cultured	# Larvae Viable	% Larvae Lethality	# Larvae Unhatched	% Larvae Unhatched	# Larvae Abnormal	% Larvae Abnormal	Body Shape Malformed	Somites Malformed	Notochord Malformed	Tail Malformed	Fins Malformed	Heart Malformed	Face Malformed	Neural Tube Malformed	Arches/ Jaws Malformed	# Scored	Swim bladder Not well-inflated	Swim bladder Not evident	Poor Pigmentation	Excess Pigmentation	Abnormal Pigmentation	Yolk ball - Remnant excessive	Yolk ball - Remnant, dark	Stomach, Not Evident	Liver - Not Evident	Liver - Enlarged	Abdomen distended or yolk sac edema
Medium	N/A	24	24	0.00	1	4.17	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	23	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
DMSO	0.5%	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C9	10.0	24	24	0.00	0	0.00	1	4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.2%	4.2%	24	4.2%	4.2%	0.0%	0.0%	0.0%	4.2%	0.0%	0.0%	0.0%	0.0%	0.0%
C9	100.0	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	12.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
C9	1000.0	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	95.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

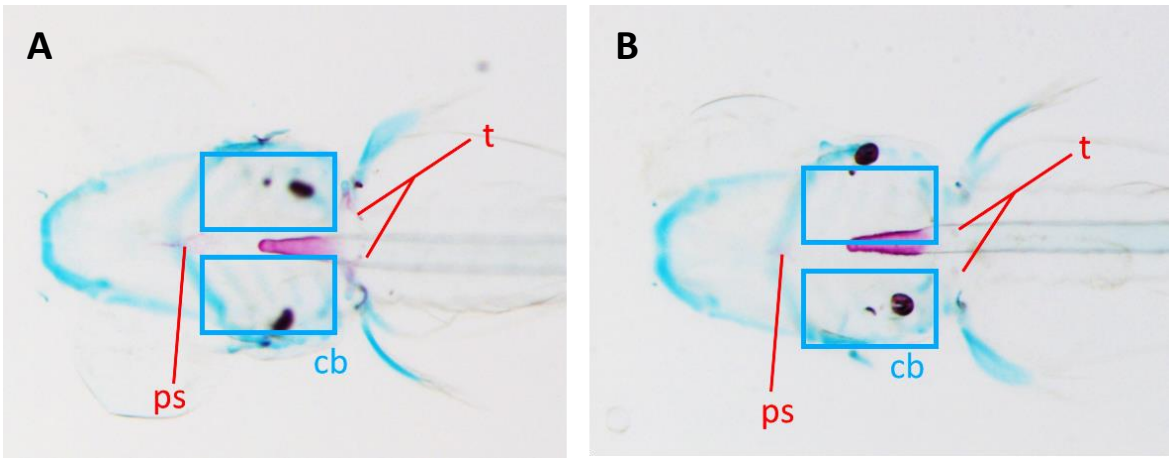
Supplementary table 8. Morphological evaluation of 5 dpf larvae exposed to compound A.

Compound A - Replicate 1																													
									% Abnormal of individual category																		% Other Types of Abnormalities			
Compound	Concentration (µM)	# Larvae Cultured	# Larvae Viable	% Larvae Lethality	# Larvae Unhatched	% Larvae Unhatched	# Larvae Abnormal	% Larvae Abnormal	Body Shape Malformed	Somites Malformed	Notochord Malformed	Tail Malformed	Fins Malformed	Heart Malformed	Face Malformed	Neural Tube Malformed	Arches/ Jaws Malformed	# Scored	Swim bladder Not well-inflated	Swim bladder Not evident	Poor Pigmentation	Excess Pigmentation	Abnormal Pigmentation	Yolk ball - Remnant excessive	Yolk ball - Remnant, dark	Stomach, Not Evident	Liver - Not Evident	Liver -Enlarged	Abdomen distended or yolk sac edema
Medium	N/A	24	24	0.00	0	0.00	1	4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.2%	24	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
DMSO	0.5%	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
CA	1.0	24	24	0.00	1	4.17	1	4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.3%	23	8.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
CA	10.0	24	24	0.00	0	0.00	2	8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	8.3%	24	8.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
CA	100.0	24	24	0.00	0	0.00	2	8%	0.0%	0.0%	0.0%	0.0%	0.0%	4.2%	0.0%	0.0%	4.2%	24	8.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Compound A - Replicate 2																													
									% Abnormal of individual category																		% Other Types of Abnormalities			
Compound	Concentration (µM)	# Larvae Cultured	# Larvae Viable	% Larvae Lethality	# Larvae Unhatched	% Larvae Unhatched	# Larvae Abnormal	% Larvae Abnormal	Body Shape Malformed	Somites Malformed	Notochord Malformed	Tail Malformed	Fins Malformed	Heart Malformed	Face Malformed	Neural Tube Malformed	Arches/ Jaws Malformed	# Scored	Swim bladder Not well-inflated	Swim bladder Not evident	Poor Pigmentation	Excess Pigmentation	Abnormal Pigmentation	Yolk ball - Remnant excessive	Yolk ball - Remnant, dark	Stomach, Not Evident	Liver - Not Evident	Liver -Enlarged	Abdomen distended or yolk sac edema
Medium	N/A	24	24	0.00	0	0.00	1	4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.2%	24	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
DMSO	0.5%	24	24	0.00	1	4.17	1	4%	0.0%	0.0%	0.0%	4.3%	4.3%	0.0%	0.0%	0.0%	0.0%	23	4.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
CA	1.0	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	8.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
CA	10.0	24	24	0.00	2	8.33	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	22	18.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
CA	100.0	24	24	0.00	4	16.67	2	10%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	5.0%	10.0%	20	25.0%	0.0%	0.0%	0.0%	0.0%	5.0%	0.0%	0.0%	0.0%	0.0%	0.0%

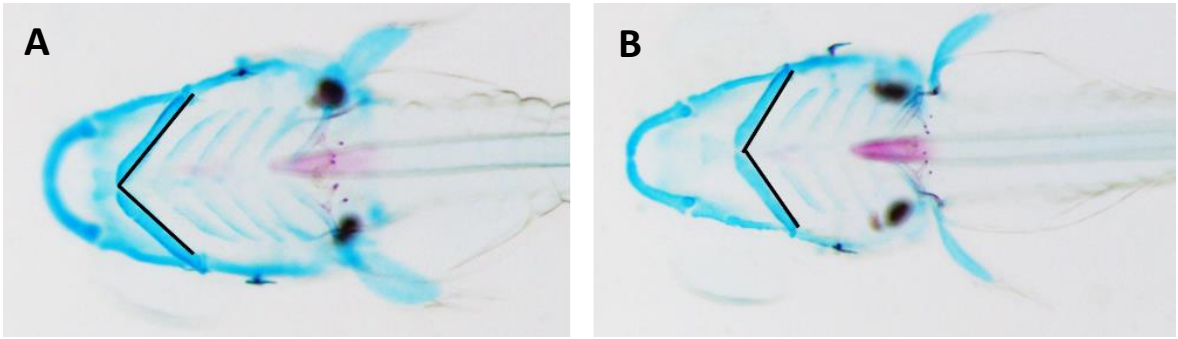
Supplementary table 9. Morphological evaluation of 5 dpf larvae exposed to levetiracetam.

Levetiracetam - Replicate 1																														
									% Abnormal of individual category											% Other Types of Abnormalities										
Compound	Concentration (µM)	# Larvae Cultured	# Larvae Viable	% Larvae Lethality	# Larvae Unhatched	% Larvae Unhatched	# Larvae Abnormal	% Larvae Abnormal	Body Shape Malformed	Somites Malformed	Notochord Malformed	Tail Malformed	Fins Malformed	Heart Malformed	Face Malformed	Neural Tube Malformed	Arches/ Jaws Malformed	# Scored	Swim bladder Not well-inflated	Swim bladder Not evident	Poor Pigmentation	Excess Pigmentation	Abnormal Pigmentation	Yolk ball - Remnant excessive	Yolk ball - Remnant, dark	Stomach, Not Evident	Liver - Not Evident	Liver -Enlarged	Abdomen distended or yolk sac edema
Medium	N/A	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
DMSO	0.5%	24	22	8.33	2	8.33	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	22	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
LTC	10.0	24	22	8.33	2	8.33	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	22	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
LTC	100.0	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
LTC	1000.0	24	24	0.00	0	0.00	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	24	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

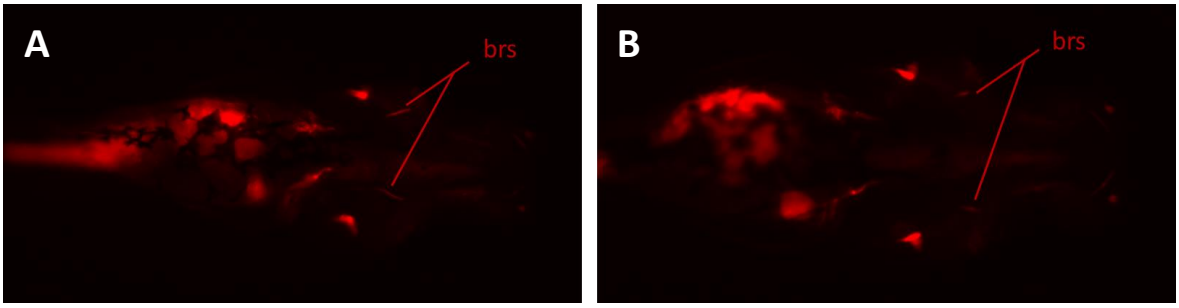
Levetiracetam - Replicate 2																														
									% Abnormal of individual category											% Other Types of Abnormalities										
Compound	Concentration (µM)	# Larvae Cultured	# Larvae Viable	% Larvae Lethality	# Larvae Unhatched	% Larvae Unhatched	# Larvae Abnormal	% Larvae Abnormal	Body Shape Malformed	Somites Malformed	Notochord Malformed	Tail Malformed	Fins Malformed	Heart Malformed	Face Malformed	Neural Tube Malformed	Arches/ Jaws Malformed	# Scored	Swim bladder Not well-inflated	Swim bladder Not evident	Poor Pigmentation	Excess Pigmentation	Abnormal Pigmentation	Yolk ball - Remnant excessive	Yolk ball - Remnant, dark	Stomach, Not Evident	Liver - Not Evident	Liver -Enlarged	Abdomen distended or yolk sac edema
Medium	N/A	24	24	0.00	4	16.67	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	20	5.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
DMSO	0.5%	24	24	0.00	1	4.17	2	9%	0.0%	0.0%	0.0%	0.0%	4.3%	0.0%	0.0%	0.0%	4.3%	23	4.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
LTC	10.0	24	24	0.00	22	91.67	1	5%	0.0%	4.5%	0.0%	0.0%	4.5%	4.5%	4.5%	4.5%	4.5%	22	9.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.5%
LTC	100.0	24	24	0.00	23	95.83	0	0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	23	4.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
LTC	1000.0	24	24	0.00	22	91.67	1	5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.5%	22	18.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%



Supplementary figure 1. Images of AR-AB stained structures of 5 dpf larvae treated with A) the solvent control and B) 1,000 µM of compound 5. The parasphenoid (ps), pharyngeal teeth (t), branchiostegal rays (brs, not present/stained in these pictures), and ceratobranchials (cb) are less intensely stained in the group treated with 1,000 µM compound 5 in the 2nd replicate.



Supplementary figure 2. Images of AR-AB stained 5 dpf larvae treated with A) the solvent control and B) 100 µM of compound A. The angle size (indicated) between the ceratohyal cartilages is significantly increased in the first replicate of 100 µM compound A when compared to the solvent control.



Supplementary figure 3. Variability between 5 dpf control larvae from two clutches (same spawning day). Image A) is a larva from clutch 1 and B) is a larva from clutch 2. The branchiostegal rays (brs) are more intensely stained in larvae from clutch 1.

5.6 References

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CHAPTER 6:
Assessing developmental toxicity and non-CYP mediated biotransformation of two anti-epileptics and their human metabolites in zebrafish embryos and larvae

Adapted from: J. Hoyberghs, A. Coppens, C. Bars, C. Van Ginneken, K. Foubert, S. Van Cruchten. Assessing developmental toxicity and non-CYP mediated biotransformation of two anti-epileptics and their human metabolites in zebrafish embryos and larvae

In preparation

Abstract

Zebrafish embryo-based assays are a promising alternative for animal testing to screen new compounds for developmental toxicity, as morphological effects of xenobiotics can be assessed in a whole vertebrate organism during the main organogenesis period. However, recent studies in zebrafish embryos showed an immature intrinsic cytochrome P450 (CYP)-mediated biotransformation capacity, as most CYPs were only active at the end of the organogenesis period. Data on other phase I enzymes involved in the biotransformation of xenobiotics in zebrafish embryos is limited. This information is pivotal for proteratogens needing bioactivation to exert their teratogenic potential. Therefore, this study aimed to investigate whether carbamazepine (CBZ) and levetiracetam (LTC), two anti-epileptic drugs that require bioactivation to exert their teratogenic potential, are biotransformed into non-CYP mediated metabolites in the zebrafish embryo and whether one or more of these metabolites cause developmental toxicity in this species. The human metabolites of LTC and CBZ, etiracetam carboxylic acid (ECA) and 9-acridine carboxaldehyde (9ACA), acridine (AI), and acridone (AO), respectively, were selected as they were reported to be teratogenic or toxic in other species. In the first step, zebrafish embryos were exposed to 100-10,000 µM LTC and 250-500 µM CBZ and their non-CYP mediated human metabolites, 1-500 µM ECA and 3-60 µM 9ACA, 3-300 µM AI and 3-60 µM AO, respectively, from 5.25 to 120 hpf and morphologically evaluated. Next, the uptake of all compounds and the formation of ECA and 9ACA, AI, and AO were assessed using LC-MS methods. Moreover, also the formation of carbamazepine-10,11-epoxide (E-CBZ) and iminostilbene (IM) was assessed, as they are intermediate metabolites in an alternative metabolization pathway of CBZ. As LTC and ECA were, respectively, poorly or not taken up by zebrafish larvae during the exposure experiments, we could not determine if LTC and ECA are teratogenic. However, biotransformation of LTC into ECA was observed at 24 hpf and 120 hpf, which indicates that the special type of B-esterase is already active at 24 hpf. CBZ and its three metabolites were teratogenic, as a significant increase in malformed embryos was observed for all of them. All three metabolites were more potent teratogens than CBZ, with AI being the most potent, followed by 9ACA and AO. The myeloperoxidase (MPO) homologue is already active at 24 hpf, as CBZ was biotransformed into 9ACA and AO in 24 hpf zebrafish embryos, and into 9ACA in 120 hpf larvae. Moreover, 9ACA was also found to be biotransformed into AI and AO, and AI into AO. As such, one or more of these metabolites probably contribute to the teratogenic effects observed in zebrafish larvae after exposure to CBZ.

6.1 Introduction

In recent years, new approach methodologies (NAMs) for hazard and risk assessment of xenobiotics have received a lot of attention. Several pharmaceutical, agrochemical, and cosmetic companies are currently using the zebrafish embryo as an alternative for animal testing to screen new compounds for developmental toxicity [1–4]. Zebrafish embryo-based assays, such as the ZEDTA [5–8], are a promising alternative as morphological effects of xenobiotics can be assessed in a whole vertebrate organism during the main organogenesis period, which is in contrast to other alterative assays, such as the rat whole embryo culture and mouse embryonic stem cell test [9]. Moreover,

using zebrafish embryo assays as a screening tool confers various other advantages as well. Zebrafish are cost-effective and need only a small amount of test compound due to the small size of the embryos [8,10]. They are easy to maintain and to breed due to their high reproductive capacity and fertility, show a rapid *ex utero* development, and have a short organogenesis period from 5.25 hours post-fertilization (hpf) until 120 hpf [8,11]. In addition, zebrafish embryos have a transparent chorion and embryonic tissue during early development which facilitates detailed observation of morphological changes [11,12]. Despite the advantages, the assay suffers from some drawbacks, and further standardization and optimization are needed [5,8]. The false negative results in the ZEDTA are especially of concern for safety purposes. These can be caused by, among others, the low biotransformation capacity of zebrafish during organogenesis, resulting in the non-detection of compounds that require bioactivation to form their teratogenic metabolite(s), i.e., proteratogens.

Cytochrome P450 (CYP) enzymes are the major superfamily of phase I metabolizing enzymes involved in the biotransformation and bioactivation of xenobiotics. These membrane-bound enzymes are mainly present in the endoplasmic reticulum of liver cells, followed by the small intestine, and function by enhancing the polarity of xenobiotics by catalyzing their oxidation [13]. It is already known that CYP-mediated biotransformation is immature during mammalian and human embryofetal development [14]. However, because human pregnancy takes place *in utero*, human embryos can still be exposed to CYP-mediated metabolites originating from the mother. Recent studies also showed an immature intrinsic CYP-mediated biotransformation capacity in zebrafish embryos [7,15,16], as most CYPs appear to be only active at the end of the organogenesis period, i.e., around 72-96 hpf when the liver becomes functional [17]. However, as zebrafish embryos develop externally, the embryos will not be exposed to maternal metabolization products during a significant part of the organogenesis period. Therefore, possible teratogenic effects may be missed in this NAM. To circumvent the immature intrinsic CYP-mediated biotransformation capacity, several research groups have been exploring using an exogenous Metabolic Activating System (MAS) based upon liver microsomes [5,18–21]. By adding an exogenous MAS as a modular system for the zebrafish embryo assay, zebrafish embryos can be exposed to metabolically activated proteratogens during the main organogenesis period. Although CYP-mediated biotransformation in zebrafish embryos has been reasonably well characterized, there is still a knowledge gap in this species for other phase I enzymes involved in the biotransformation of xenobiotics. This information is pivotal for proteratogens needing bioactivation to exert their teratogenic potential. This study aimed to investigate whether carbamazepine (CBZ) and levetiracetam (LTC), two anti-epileptic drugs (AEDs) that require bioactivation to exert their teratogenic potential, are biotransformed into non-CYP mediated metabolites in the zebrafish embryo and whether these metabolites cause developmental toxicity in this species. CBZ and LTC were selected in this study as the literature suggested that they might be biotransformed into toxic non-CYP mediated metabolites [7,22–26].

The first AED, CBZ, and its primary CYP-mediated metabolite, carbamazepine-10,11-epoxide (E-CBZ), have been previously exposed to zebrafish embryos to explore their teratogenic potential in this model. Although E-CBZ is known to cause developmental toxicity in man, no teratogenic effects

were observed after exposing zebrafish embryos to this metabolite [7]. Interestingly, zebrafish larvae showed malformations after CBZ exposure from 5.25-120 hpf in a dose-dependent manner [7]. Therefore, it was suggested that CBZ might be teratogenic and did not require bioactivation in zebrafish or that CBZ might be biotransformed into other metabolites, such as acridine, that could be responsible for the developmental toxicity in this species [7]. This latter suggestion looks pretty plausible as different research groups have already shown that some of its metabolites are more toxic than CBZ itself [22–24]. Acridine (AI), a transformation product (TP) of CBZ generated by electrochemistry, appeared to be the most toxic product in the Zebrafish Embryo Acute Toxicity Test (zFET), and was also found to be more toxic than CBZ [24]. In another study, both AI and acridone (AO), two TPs formed during ultraviolet photolysis, were reported to be significantly more toxic than CBZ in three acute toxicity assays representing algae, bacteria, and crustaceans. Of both TPs, AI was the most toxic across all three assays [23]. Moreover, incubation of human lymphocytes with 9-acridine carboxaldehyde (9ACA), a reactive metabolite of CBZ, resulted in 40% cell death, while no effect of viability was seen after incubation with CBZ [22].

Interestingly, all three previously mentioned metabolites of CBZ are found to be formed in man by myeloperoxidase (MPO) enzymes. MPOs are phase I enzymes present in monocytes, neutrophils, and neutrophil precursors in human bone marrow. After activation, these leukocytes interact with hydrogen peroxide and chloride to form hypochlorous acid, which oxidizes CBZ to form the following metabolites: an intermediate aldehyde, 9ACA, AI, AO, and chloroacridones (see Figure 1) [22,27]. Besides the metabolization of CBZ to these metabolites in humans, one of these metabolites, AI, has already been detected in extracts of 96 hpf old zebrafish after direct exposure of the embryo to CBZ [28]. In addition, the expression and activity of MPO enzymes have already been reported in zebrafish embryos at 18 hpf and 33 hpf, respectively [29]. Therefore, we decided to examine whether 9ACA, AI, and AO cause developmental toxicity in zebrafish embryos and whether they are formed in this species after exposure to CBZ.

The teratogenic potential of LTC has also already been examined in both mammalian and zebrafish studies. While malformations such as skeletal abnormalities [26,30], growth retardation [26,30,31], internal organ abnormalities [30,31] and fetal mortality [26] were reported in mammalian studies, no teratogenic effects were found when exposing zebrafish embryos to LTC [32,33]. Hence, false negative results for LTC are obtained in assays using zebrafish embryos. However, etiracetam carboxylic acid (ECA or UCB L057), which is a human metabolite of LTC (i.e., 24% of the administered dose [34]) was found to cause growth retardation and several skeletal abnormalities in mice [26]. Although the specific enzymes responsible for the biotransformation are unknown, it was proposed that ECA is formed by a special type of B-esterases, which are distinct from classical carboxylesterases and cholinesterases, via enzymatic hydrolysis of the acetamide group in the blood [26,35]. To our knowledge, no studies exposing zebrafish embryos to ECA have been reported yet. Therefore, we investigated whether ECA can cause teratogenic effects in zebrafish embryos, and if not, whether this metabolite might not be formed (yet) in zebrafish embryos and young zebrafish larvae exposed to LTC, explaining the false negative result.

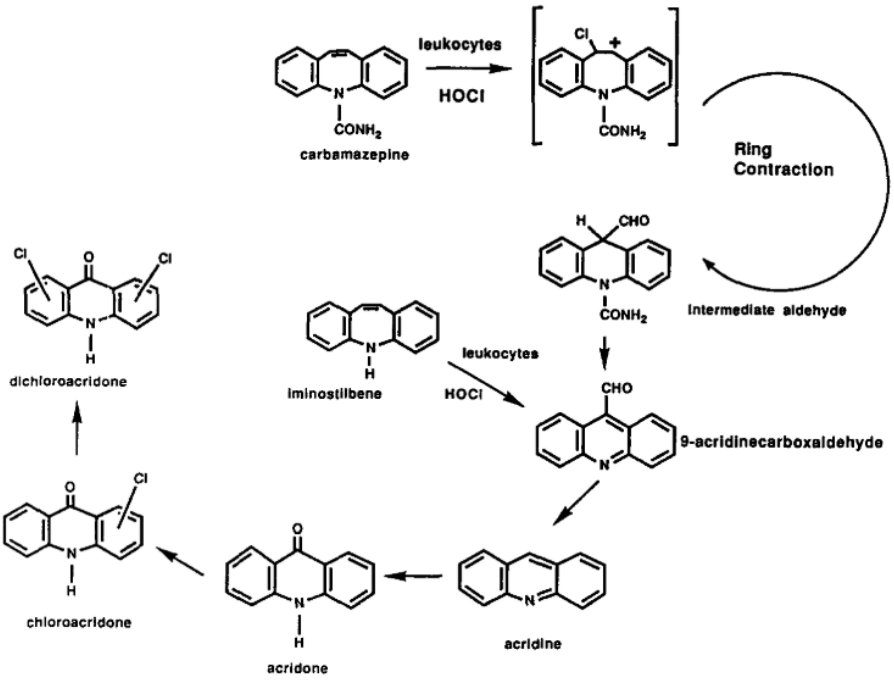

Figure 1. Proposed pathway of carbamazepine in humans by myeloperoxidases in activated leukocytes [27].

6.2 Materials and methods

6.2.1 Chemicals and solutions

- Embryo medium was made by dissolving 0.60 g of Instant Ocean® Sea Salt (Blacksburg, VA, United States) and 0.038 g of sodium bicarbonate (Sigma, Diegem, Belgium) in 2 L reverse osmosis (RO) water (pH 7.4 ± 0.3 and conductivity 500 ± 40 µS/cm) (Barnstead™ Pacific™ RO Water Purification System, Thermo Scientific™, Waltham, MA, USA).
- The tricaine methane sulfonate (MS-222) solution (1 g/L) was made by dissolving methyl ethane sulfonate (i.e., MS-222) (Sigma) in embryo medium (pH adjusted to 7.4 ± 0.3 with 1 M NaOH).
- LTC concentrations of 100 µM, 1,000 µM, 5,000 µM, and 10,000 µM were made by dissolving LTC (≥98% purity, CAS 102767-28-2, Sigma) in embryo medium. These concentrations were based on concentrations previously used in zebrafish research [32,33].
- For the metabolite of LTC, ECA (or UCB L057), concentrations of 1 µM, 10 µM, 100 µM, 250 µM, and 500 µM were made by dissolving UCB L057 (CAS 67118-31-4, Sigma) in embryo medium. These concentrations are selected as they are in the range of 1/10th of the parent compound concentrations, as metabolites that are formed at this rate or higher need to be further investigated in toxicity studies.
- For CBZ, concentrations of 250 µM and 500 µM were made by dissolving CBZ (≥99.0% purity, CAS 298-46-4, Sigma), which is poorly water-soluble, in DMSO (Sigma) and then in embryo medium, so a final non-toxic percentage of 1% DMSO could be obtained [8]. These concentrations were based on Bars et al. (2021) and Weigt et al. (2011), which used 250 µM CBZ and both 250 µM and 500 µM of CBZ, respectively [7,36]. The solvent control was made by dissolving 1% DMSO in embryo medium.

- For the three metabolites of CBZ, concentrations of 3 µM, 30 µM and 300 µM for AI and 3 µM, 30 µM and 60 µM for AO and 9ACA were made by dissolving AI (97% purity, CAS 260-94-6, Sigma), AO (99% purity, CAS 578-95-0, Sigma) and 9ACA (97% purity, CAS 885-23-4, Sigma) in DMSO (Sigma) and then in embryo medium, so a final percentage of maximum 1% DMSO could be obtained [8]. The lowest and medium concentrations of the metabolites were based on Zhu et al. (2015), which tested AI concentrations ranging from 0.625 to 10 mg/L (i.e., 3.4 – 55.8 µM) in zebrafish embryos [24]. For the highest concentration, we tested the 10-fold concentration (i.e., 300 µM) of our medium concentrations for AI, and for 9ACA and AO 60 µM was tested as higher concentrations could not be reached due to precipitation of the compounds.
- The pH of all test solutions was checked, and if needed adjusted, before exposure to make sure a physiological pH was maintained throughout the experiments.
- To set up calibration curves, mixes containing 0.0005 - 1 µg/mL of CBZ (≥99.0% purity, CAS 298-46-4, Sigma), 9ACA (97% purity, CAS 885-23-4, Sigma), AI (97% purity, CAS 260-94-6, Sigma), AO (99% purity, CAS 578-95-0, Sigma), iminostilbene (IM) (CAS 256-96-2, Sigma), and E-CBZ (≥98.0% purity, CAS 36507-30-9, Sigma) dissolved in acetonitrile (purity ≥99,9%, Merck) for the CBZ experiment and 0.001 – 1 µg/mL of LTC (≥98% purity, CAS 102767-28-2, Sigma) and UCB L057 (CAS 67118-31-4, Sigma) dissolved in acetonitrile for the LTC experiment were prepared.

6.2.2 Adult zebrafish housing and egg collection

Experiments were conducted according to our standardized ZEDTA protocol [5]. In brief, adult zebrafish (*Danio rerio*) of the wild-type AB strain were used as breeding stock. Glass aquaria of approximately 60 L, filled with reverse osmosis water (Barnstead™ Pacific™ RO Water Purification System, Thermo Scientific™) with Instant Ocean® Sea Salt (Blacksburg) and sodium bicarbonate (Merck, Darmstadt, Germany) (pH 7.5 ± 0.3, conductivity 500 ± 40 µS/cm and temperature 28.5 ± 0.3°C) were used to house the adult fish. The ratio of males to females was 50/50 and the fish density was <1 fish/L. For enrichment, plastic plants were added to the tank. An automated lighting system with a 14/10 hour light/dark cycle was applied. The health of the zebrafish and water parameters were checked daily. The limits for ammonia, nitrite and nitrate levels were <0.02 mg/L, <0.3 mg/L and ≤12.5 mg/L, respectively. The adult fish were daily fed with thawed Artemia, Daphnia or red, black, or white mosquito larvae (alternating; Ruto Frozen Fish food, Montford, The Netherlands).

To allow embryo collection, approximately 20 adult fish (ratio males to females 50/50) were transferred into a spawning tank the evening before egg collection. To prevent the fish from eating eggs, the spawning tank was equipped with two nets at the bottom where the eggs could pass through. The fish were fed at the latest at 9 a.m. in the morning on the day before collection to avoid faeces and dirt in the spawning tank as much as possible. On the day of the collection, the fish were allowed to spawn and fertilize the eggs for approximately 1 h after the lights turned on. Afterwards, the fish were transferred to their normal tank, and eggs were collected from the bottom of the spawning tank by siphoning them out with a tube. Then, the embryos were washed twice in embryo medium (i.e., to remove faeces and coagulated eggs) and transferred to 48 well plates (Cellstar®, Greiner Bio-One, Frickenhausen, Germany). At approximately 3 hpf, embryos with

a normal cell division were selected using an Olympus SZX16 microscope (Olympus Life Science, Shinjuku, Tokyo, Japan) and randomly transferred to new 48 well plates filled with embryo medium. The selected eggs were kept at 28.5°C ± 0.3°C in a TIN-IN35 incubator (Phoenix instrument, Garbsen, Germany) with LED strips (LED02102-1, LEDStripXL, Deventer, The Netherlands), with a 14/10 hour light/dark cycle, attached on the inside. Coagulated eggs and malformed embryos were euthanized with buffered 1 g/L tricaine methane sulfonate (MS-222; pH 7.4).

6.2.3 Exposure of zebrafish embryos to parent and metabolite concentrations

Each experiment (n = 20 embryos/group) was performed in two biological replicates and consisted of an embryo medium control group (for LTC experiments) or a solvent control group (i.e., 1% DMSO in embryo medium; for CBZ experiments) and test groups exposed to different concentrations of the parent compounds and their metabolites. The following test groups were used in the LTC experiments: 100, 1,000, 5,000, and 10,000 µM LTC; and 1, 10, 100, 250, and 500 µM ECA. For the CBZ experiments, the following test groups were used: 250 and 500 µM CBZ; 3, 30, and 300 µM AI; and 3, 30, and 60 µM AO and 9ACA. 48-well plates with a total volume of 300 µL/well were used. At the latest at 5.25 hpf, the embryos were exposed to the control and test solutions and placed in the incubator (28.5°C ± 0.3°C with a 14/10 hour light/dark cycle). To avoid acidification and oxygen deprivation, the embryo medium or test solution was renewed every 48 h [6]. A batch of eggs was considered to be valid for experimentation when a minimum of 80% of all eggs were fertilized, and the controls' mortality and malformations rate was lower than, or equal to, 10% throughout the experiment [7].

6.2.4 Morphological evaluation of the exposed zebrafish embryos and larvae

The zebrafish embryos and larvae were morphologically evaluated at different developmental stages (i.e., 5.25, 10, 24, 48, 72, 96, and 120 hpf [5]) using an Olympus SZX16 microscope (Olympus Life Science). The 5.25 and 10 hpf time points were used as a last check-up to replace eggs that coagulated or started to show aberrations in development with spare eggs (also exposed at the latest at 5.25 hpf). At 24 and 48 hpf, a morphological scoring of coagulation/mortality, indistinguishable or unrecognizable body parts, tail deviations (i.e., curved, elbow, and tissue deviations), edema (in the head, pericard, yolk, and yolk extension), blood accumulation (in the tail, head, heart, yolk, and yolk extension), malformation of the yolk, malformation of the cardiovascular system, (i.e., malformation heart, deviating heartbeat, no blood circulation in the tail, disturbed blood circulation in the tail), malformation of the head (i.e., deviating shape, deviation ear, deviation mouth, deviation eye), deviating pigmentation and non-detachment of the tail was conducted. At 72, 96, and 120 hpf, all previously mentioned endpoints were scored with two additional parameters: hatching and malformations of the pectoral fins (i.e., missing or curved) [5]. A parameter was scored 0 if normal and 1 in case of malformation. After scoring at 120 hpf, the zebrafish larvae were euthanized using an MS-222 solution (1 g/L) and snap-frozen in liquid

nitrogen to ensure death, except for larvae exposed to the parent compounds or their metabolites in the highest concentration as they are needed for further analytical investigation with ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (see 6.2.5).

6.2.5 Extraction of the parent and metabolite compounds present in zebrafish embryos and larvae

To assess the uptake of the compounds and possible formation of the metabolites of interest in 24 hpf embryos and 120 hpf larvae with UPLC-MS/MS, an extraction protocol based on Bars et al. (2021) was performed [7] at the zebrafish embryos/larvae exposed to 500 µM CBZ and 10,000 µM LTC (for exposure method see 6.2.3). In addition, larvae exposed to the highest concentration of the metabolites, ECA (500 µM), AI (30 µM; not 300 µM as there was 100% lethality), AO (60 µM) and 9ACA (60 µM) were also collected at 120 hpf to verify the uptake and, in case of 9ACA and AI, biotransformation to other metabolites.

During the extraction protocol, the collected embryos and larvae that were exposed to the same conditions were pooled and rinsed three times with cold embryo medium (4°C) on a mesh (Cell strainer 100 µM Nylon, Sterile Falcon®, Durham, NC, USA) to remove test solution present at the outside of the embryos/larvae. Next, 20 embryos or larvae (or the highest possible number if less than 20/concentration survived) were transferred into a cryotube with a cold embryo medium (4°C) to be snap-frozen in liquid nitrogen to ensure death. Then, they were thawed on ice and transferred into a 15 mL tube. The embryo medium was removed as much as possible, and 400 µL of the extraction solvent acetonitrile (ACN) (purity ≥99,9%, Merck) was added. Next, the samples were homogenized by ultrasonication (15 minutes, 30 cycles, high energy (85-90%)) by using an Ultrasonic Processor VCX 130 (Sonics, Newtown, CT, USA), while being kept on ice. The samples were centrifuged twice (Centrifuge 5424 R, Eppendorf, Hamburg, Germany) at 15,000 x g for 15 minutes to remove zebrafish tissue from the supernatant. This supernatant was kept at -80°C until further analysis using UPLC-MS/MS.

6.2.6 Analytical investigation of the extracted samples

6.2.6.1 Calibration curves

To quantify the concentrations that were present in the extracted samples, mixes containing 0.0005 - 1 µg/mL (CBZ experiment) and 0.001 - 1 µg/mL (LTC experiment) of the parents and their metabolite(s) in ACN were used to set up calibration curves. The data were first log-transformed for all compounds to obtain a linear curve. All calibration curves' determination coefficients (R^2) were more than 0.987. Also E-CBZ and IM were added to the CBZ mix as they were proposed as possible metabolites in an alternative metabolization pathway (see Figure 2) [37]. By adding these, it will be possible to check whether these metabolites also can be found in the extractions of the CBZ samples.

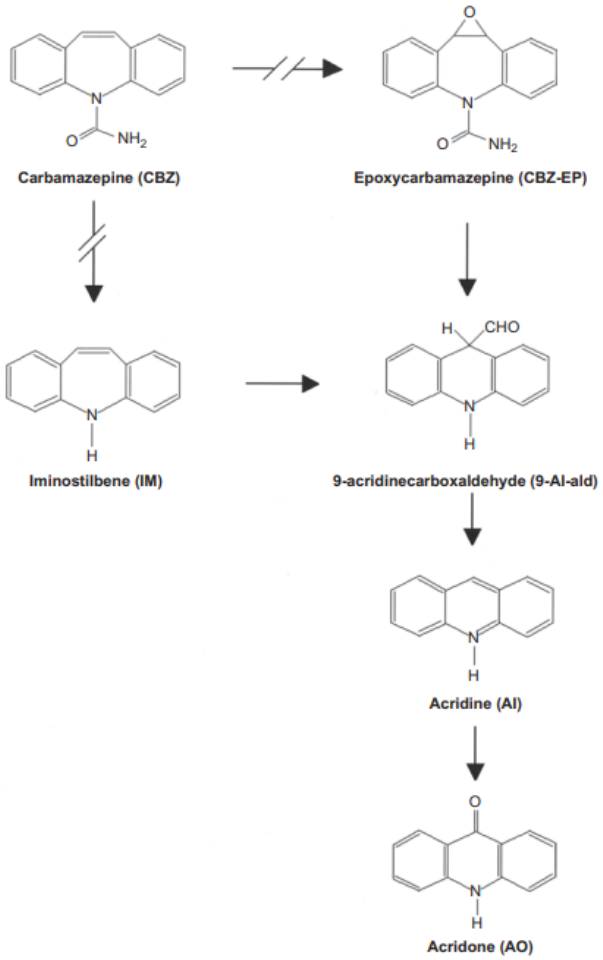

Figure 2. Proposed pathway of carbamazepine in humans. Metabolism in the liver is indicated with broken arrows. Metabolism in the peripheral blood is indicated with continuous arrows [37].

6.2.6.2 Analytical evaluation of carbamazepine and its metabolites

The settings for detecting CBZ and its metabolites were based on Bars et al. (2021) [7]. The chromatographic separation was performed using an Acquity premier UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm) (Waters, Milford, CT, USA). For elution, an HPLC-grade mobile phase consisting of water with 0.1% formic acid (99% ULC/MS grade, CAS 64-18-6, Biosolve, Dieuze, France) (solvent A) and ACN (purity ≥99,9%, Biosolve) containing 0.1% of formic acid (solvent B), was used. A flow rate of 0.5 mL/min was applied, and the solvent gradient program was set as follows: 85% A/15% B (0-0.5 min); 85-0% A/15-100% B (0.5-3.5 min); 0% A/100% B (3.5-4.4 min); 0-85% A/100-15% B (4.4-4.5 min); 85% A/15% B (4.5-6 min). The column was set at 40°C and a full loop injection volume of 10 µL was used. The UPLC instrument was coupled to a Xevo G2-XS QTOF (Waters, Milford, USA) mass spectrometer which was calibrated in positive ionization mode (ESI +) using a sodium formate solution. Data acquisition was done from *m/z* 100 to *m/z* 1500 in the sensitivity mode. Full scan MS data were obtained. Leucine-Enkephalin was used as the lock mass compound. The experimental conditions were as follows: electrospray capillary voltage 1.0 kV, sampling cone voltage 40.0 V, source temperature 120 °C, desolvation temperature 550 °C, cone gas flow 50.0 L/h, and desolvation gas flow 1,000.0 L/h. The limit of quantification (LOQ) and the

limit of detection (LOD) were determined using the signal to noise ratio (see Table 1). All results were analyzed using the MassLynx software (version 4.1) (Waters).

6.2.6.3 Analytical evaluation of levetiracetam and its metabolite

LTC and its metabolite ECA were analyzed using a UPLC-triple quadrupole detector (TQD). The chromatographic separation on the UPLC instrument (Acquity, Waters, Milford, CT, USA) was carried out using an Acquity UPLC premier HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm) (Waters). For elution, an HPLC-grade mobile phase consisting of water with 0.1% formic acid (99% ULC/MS grade, Biosolve) (solvent A) and ACN (purity ≥99,9%, Biosolve) containing 0.1% of formic acid (solvent B) was used. A flow rate of 0.5 mL/min was applied, and the solvent gradient program was set as follows: 95% A/5% B (0-0.5min); 95-60% A/5-40% B (0.5-3.5 min); 60-0% A/40-100% B (3.5-4.4 min); 0-95% A/100-5% B (4.4-4.5 min); 95% A/5% B (4.5-6 min). The column was set at 40°C and an injection volume of 5 µL was used. A solution of LTC with a concentration of 10 µg/mL and a solution of the metabolite with a concentration of 10 µg/mL were used for tuning. The following parameters were used in positive ionization mode (ESI +): capillarity voltage 3.50 kV, cone voltage 18.0 V, extractor voltage 3.00 V, and RF 0.10 V. The source temperature was set at 140°C and the desolvation temperature at 500°C. The desolvation gas flow was fixed at 850 L/h and cone gas flow at 50 L/h. Mass spectrometric analysis was performed in MRM mode using following transitions for LTC: m/z 171 → 69 as a quantifier and 171 → 154 as a qualifier (collision energy, 28 and 5 V respectively). For ECA the transitions were as follows: 172 → 69 and 172 → 126, both with an optimal collision energy of 26 V. The LOQ and LOD were determined by using the signal to noise ratio (see Table 1). All results were analyzed using the MassLynx software (version 4.1) (Waters).

Table 1. Retention time, mass-to-charge ratio, LOQ and LOD of the compounds of interest. Abbreviations: 9-acridine carboxaldehyde (9ACA), acridine (AI), acridone (AO), carbamazepine (CBZ), carbamazepine-10,11-epoxide (E-CBZ), etiracetam carboxylic acid (ECA), iminostilbene (IM), levetiracetam (LTC), limit of detection (LOD), limit of quantification (LOQ), retention time (Rt).

Exp.	Compound	Formula	Rt	*m/z* $[M + H]^+$	LOQ (µM)	LOD (µM)
CBZ	CBZ	$C_{15}H_{12}N_2O$	2.47	237.1082	0.000023	0.000007
	9-acridine carboxaldehyde	$C_{14}H_9NO$	2.89	208.0762	0.000048	0.000014
	Acridine	$C_{13}H_9N$	1.59	180.0813	0.000066	0.000020
	Acridone	$C_{13}H_9NO$	2.32	196.0762	0.000023	0.000007
	Iminostilbene	$C_{14}H_{11}N$	3.39	194.0970	0.000025	0.000007
	E-CBZ	$C_{15}H_{12}N_2O_2$	2.16	253.0977	0.000032	0.000010
LTC	LTC	$C_8H_{14}N_2O_2$	2.05	171.1134	0.000665	0.000200
	ECA	$C_8H_{13}NO_3$	2.50	172.0974	0.000585	0.000176

6.2.6.4 Additional analytical evaluations

As peaks similar to the peaks of interest were detected in the solvent control samples (1% DMSO) of the CBZ experiments (see Supplementary table 1), additional analytical evaluations were performed to determine the origin of these unexpected peaks.

The following additional samples were investigated:

- After removal of the zebrafish larvae, the 1% DMSO medium, in which the larvae resided during the exposure experiments, was stored at -20°C. Three of these 1% DMSO medium samples were subjected to LC-MS analysis.
- Two samples containing extracted zebrafish from the LTC exposure experiment (i.e., no DMSO was used).

6.2.7 Statistics

For the binary data of the morphological investigation of zebrafish embryos, a Fisher Exact test was used. P-values of ≤0.05 were considered to indicate statistically significant differences between the control and test groups. Also, the relative risk (RR) was calculated to indicate the probability of a malformation occurring in the test group versus the probability of that malformation occurring in the control group. All statistical analyses were performed using GraphPad Prism 8.4.0 or newer versions (GraphPad Software, Inc., San Diego, CA, USA).

6.3 Results

6.3.1 Morphological evaluation of zebrafish embryos exposed to levetiracetam and its metabolites

More than or equal to 80% of all eggs were fertilized and the total number of malformed or dead larvae in the control groups (i.e., embryo medium) was ≤10% at the end of the experiments, making all replicates valid [5]. The pH of all LTC test solutions and 1 µM, 10 µM, and 100 µM of ECA remained within a physiological range throughout the experiment (i.e., pH 7.38 - 7.72). The two highest ECA concentrations, 250 µM and 500 µM, had to be adjusted to a physiological pH as they were too acidic to be tolerated by the embryos. After adjustment with 1 M NaOH, these test solutions remained within a physiological pH range of 7.36 - 7.90 throughout the experiment.

For all test concentrations of LTC and ECA, no statistically significant differences were observed between any of the test groups and the embryo medium control group at 120 hpf (see Table 2, Table 3 and Table 4) or at any of the other time points (i.e., 24, 48, 72, and 96 hpf) (data not shown).

Table 2. Overview of lethality and malformations in the medium control group, and the 100 and 1,000 µM LTC test groups at 120 hpf in both replicates. Only parameters showing abnormalities are shown in the tables. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/dead and total malformed (incl. dead), this total number of larvae consisted only of larvae that were alive at 120 hpf. Abbreviation: levetiracetam (LTC).

	Replicate 1			**Replicate 2**		
Parameter	**Control**	**LTC 100 µM**	**LTC 1,000 µM**	**Control**	**LTC 100 µM**	**LTC 1,000 µM**
Coagulation/dead	**2/20**	0/20	**1/20**	0/20	0/20	0/20
Tot. malformed (incl. dead)	**2/20**	0/20	**1/20**	**2/20**	**1/20**	**1/20**
Tot. malformed (excl. dead)	0/18	0/20	0/19	**2/20**	**1/20**	**1/20**
Curved tail	0/18	0/20	0/19	**2/20**	**1/20**	**1/20**

Table 3. Overview of lethality and malformations in the medium control group, and the 1, 10 and 100 µM ECA test groups at 120 hpf in both replicates. Only parameters showing abnormalities are shown in the tables. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/dead and total malformed (incl. dead), this total number of larvae consisted only of larvae that were alive at 120 hpf. Abbreviations: blood accumulation (BA), etiracetam carboxylic acid (ECA).

	Replicate 1				Replicate 2			
Parameter	**Control**	**ECA 1 µM**	**ECA 10 µM**	**ECA 100 µM**	**Control**	**ECA 1 µM**	**ECA 10 µM**	**ECA 100 µM**
Coagulation/dead	**1/20**	0/20	**2/20**	**2/20**	0/20	**1/20**	0/20	0/20
Tot. malformed (incl. dead)	**1/20**	0/20	**4/20**	**3/20**	**1/20**	**1/20**	**1/20**	**2/20**
Tot. malformed (excl. dead)	0/19	0/20	**2/18**	**1/18**	**1/20**	0/19	**1/20**	**2/20**
No hatching	0/19	0/20	**1/18**	0/18	0/20	0/19	0/20	0/20
Curved tail	0/19	0/20	**2/18**	0/18	**1/20**	0/19	**1/20**	**1/20**
Tissue deviation tail	0/19	0/20	0/18	**1/18**	**1/20**	0/19	0/20	**1/20**
Edema head	0/19	0/20	0/18	0/18	0/20	0/19	0/20	**1/20**
Edema pericard	0/19	0/20	0/18	**1/18**	0/20	0/19	0/20	**2/20**
Edema yolk	0/19	0/20	0/18	0/18	0/20	0/19	0/20	**1/20**
BA heart	0/19	0/20	0/18	0/18	0/20	0/19	0/20	**1/20**
Curved fin left	0/19	0/20	0/18	0/18	0/20	0/19	0/20	**1/20**
Curved fin right	0/19	0/20	0/18	0/18	0/20	0/19	0/20	**1/20**

Table 4. Overview of lethality and malformations in the medium control group, the 5,000 and 10,000 µM LTC test groups and the 250 and 500 µM ECA test groups at 120 hpf in A) replicate 1 and B) replicate 2. Only parameters showing abnormalities in at least one of both replicates are shown in the tables. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/dead and total malformed (incl. dead), this total number of larvae consisted only of larvae that were alive at 120 hpf. Abbreviations: etiracetam carboxylic acid (ECA), levetiracetam (LTC).

A)

	Replicate 1				
Parameter	**Control**	**LTC 5,000 µM**	**LTC 10,000 µM**	**ECA 250 µM**	**ECA 500 µM**
Coagulation/dead	0/20	0/20	0/20	0/20	0/20
Tot. malformed (incl. dead)	**1/20**	0/20	**2/20**	**2/20**	**2/20**
Tot. malformed (excl. dead)	**1/20**	0/20	**2/20**	**2/20**	**2/20**
Elbow tail	0/20	0/20	**1/20**	**1/20**	0/20
Curved tail	0/20	0/20	0/20	0/20	**1/20**
Tissue deviation tail	0/20	0/20	**2/20**	**1/20**	**1/20**
Deviation eye	**1/20**	0/20	0/20	0/20	**1/20**

B)

	Replicate 2				
Parameter	**Control**	**LTC 5,000 µM**	**LTC 10,000 µM**	**ECA 250 µM**	**ECA 500 µM**
Coagulation/dead	0/20	**1/20**	0/20	0/20	0/20
Tot. malformed (incl. dead)	0/20	**1/20**	0/20	0/20	**1/20**
Tot. malformed (excl. dead)	0/20	0/19	0/20	0/20	**1/20**
Elbow tail	0/20	0/19	0/20	0/20	0/20
Curved tail	0/20	0/19	0/20	0/20	0/20
Tissue deviation tail	0/20	0/19	0/20	0/20	**1/20**
Deviation eye	0/20	0/19	0/20	0/20	0/20

6.3.2 Morphological evaluation of zebrafish embryos exposed to carbamazepine and its metabolites

More than or equal to 80% of all eggs were fertilized and the total number of malformed or dead larvae in the control groups (i.e., 1% DMSO in embryo medium) was ≤10% at the end of the experiments, making all replicates valid [5]. The pH of all test solutions remained within a physiological range throughout the experiment (i.e., pH 7.23 – 8.04).

6.3.2.1 Carbamazepine

No statistically significant differences with the control groups were observed before 72 hpf in any of the CBZ concentrations for both replicates (data not shown). From 72 hpf onwards, a statistically significant delay in hatching could be observed in both replicates after exposure to 250 and 500 µM CBZ ($p < 0.0001$ for all four) (see Supplementary table 2). This significant hatching delay remained present throughout 96 hpf ($p = 0.0004$ and $p < 0.0001$ for 250 µM, $p < 0.0001$ for both of 500 µM) (see Supplementary table 2) and 120 hpf ($p = 0.0033$ and $p < 0.0001$ for 250 µM, $p < 0.0001$ for both of 500 µM) (see Table 5 and Figure 3). At 96 hpf, larvae treated with the highest concentration of CBZ (i.e., 500 µM) also showed a significantly lower heartrate in both replicates ($p < 0.0001$ for both), and pericardial edema in one of the replicates ($p = 0.0471$) (see Supplementary table 2). The lower heartrate ($p < 0.0001$ for both) and edema of the pericard ($p = 0.0001$, $p = 0.0083$) were also observed at 120 hpf after exposure to 500 µM CBZ and were, at this age, supplemented by a few more malformations: edema around the eyes, characterized by translucid tissues around the eyes (i.e., scored as edema head) ($p < 0.0001$), blood accumulation in the yolk ($p = 0.0471$) and a malformed yolk ($p = 0.0202$) in one of the replicates, and no blood circulation in the tail for almost all larvae in both replicates ($p < 0.0001$ for both) (see Table 5 and Figure 3). Also after exposure to 250 µM CBZ at 120 hpf, some additional malformations were observed: edema around the eyes (i.e., scored as edema head) ($p = 0.0033$ for both), and darker and wider pigmentation spots ($p = 0.0001$, $p = 0.0033$) in both replicates, and edema of the pericard ($p = 0.0033$) in one replicate (see Table 5 and Figure 3).

6.3.2.2 Acridine

No statistically significant differences were observed between 3 µM AI and the control group for all time points in both replicates (see Table 5 and Supplementary table 3). For the two other concentrations, however, significant malformations were already observed at 24 hpf (see Supplementary table 3). All embryos exposed to 300 µM were already coagulated at this time point in both replicates ($p < 0.0001$ for both). Also, in the second replicate of the AI 30 µM test group, a significant increase in coagulations/dead was observed ($p < 0.0001$) as 13 out of the 20 embryos died. Moreover, in both replicates, embryos exposed to 30 µM AI showed to have a smaller yolk, and it looked like substance was leaking out of the yolk ($p = 0.0415$, $p = 0.0120$). At 48 hpf this yolk malformation was also noted ($p = 0.0020$) in the second replicate, and it remained present throughout 72 hpf ($p = 0.0120$) and 96 hpf ($p = 0.0077$) (see Supplementary table 3). Moreover, also the following statistically significant malformations could be noted for 30 µM at 48 hpf: smaller eyes (i.e., scored as deviation eye) in one of the replicates ($p < 0.0001$) and less pigmentation in the

eyes (i.e., scored as deviating pigmentation) in both replicates ($p = 0.0002$, $p < 0.0001$) (see Supplementary table 3). The smaller eye malformation remained for some of the embryos throughout 72 hpf ($p = 0.0020$), 96 hpf ($p = 0.0077$) and 120 hpf ($p = 0.0077$). Starting from 72 hpf, a statistically significant delay in hatching could be observed for both replicates ($p < 0.0001$ for both), which could still be noted at 96 hpf ($p < 0.0001$, $p = 0.0077$) and 120 hpf ($p < 0.0001$, $p = 0.0077$) (see Supplementary table 3, Table 5 and Figure 3). At 96 hpf, one more larva died (i.e., total of 14 out of 20) ($p < 0.0001$) and two additional significant malformations in replicate 2 were noted: edema around the eyes (i.e., scored as edema head) ($p = 0.0077$) and pericard edema ($p = 0.0077$) (see Supplementary table 3). Both remained present at 120 hpf ($p = 0.0077$ and $p < 0.0001$, respectively). At 120 hpf, the following additional statistically significant malformations could be observed in one of the replicates: tissue deviation of the tail ($p = 0.0309$), a lower heartrate and some larvae with a strange vibrating/squeezing movement of their heart (i.e., both scored as deviating heartbeat) ($p = 0.0041$), increased pigmentation across the body ($p = 0.0116$), edema of the yolk ($p = 0.077$), no blood circulation in the tail ($p = 0.0462$) and larvae with either a larger or smaller yolk (i.e., scored as malformation yolk) ($p = 0.0010$) (see Table 5 and Figure 3).

Table 5. Overview of lethality and malformations in the solvent control group (i.e., 1% DMSO), the 250 and 500 µM CBZ test groups and the 3, 30 and 300 µM AI test groups at 120 hpf in A) replicate 1 and B) replicate 2. Only parameters showing abnormalities in at least one of both replicates are shown in the tables. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/dead and total malformed (incl. dead), this total number of larvae consisted only of larvae that were alive at 120 hpf. All test groups were compared with the solvent control group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (Fisher Exact test) and the number in brackets indicates the relative risk. Abbreviations: acridine (AI), blood accumulation (BA), blood circulation (BC), carbamazepine (CBZ).

A)

	Replicate 1					
Parameter	**Control**	**CBZ 250 µM**	**CBZ 500 µM**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**
Coagulation/dead	0/20	0/20	0/20	0/20	**4/20**	**20/20** **** (∞)
Tot. malformed (incl. dead)	0/20	**13/20** **** (2.857)	**20/20** **** (∞)	0/20	**20/20** **** (∞)	**20/20** **** (∞)
Tot. malformed (excl. dead)	0/20	**13/20** **** (2.857)	**20/20** ****(∞)	0/20	**16/16** **** (∞)	0/0
No hatching	0/20	**8/20** ** (1.667)	**20/20** ****(∞)	0/20	**15/16** **** (16.000)	0/0
Elbow tail	0/20	0/20	0/20	0/20	**2/16**	0/0
Curved tail	0/20	0/20	0/20	0/20	**2/16**	0/0
Tissue deviation tail	0/20	0/20	0/20	0/20	**4/16** * (1.333)	0/0
Edema head	0/20	**8/20** ** (1.667)	**12/20** **** (2.500)	0/20	**1/16**	0/0
Edema pericard	0/20	**2/20**	**11/20** *** (2.222)	0/20	**1/16**	0/0
Edema yolk	0/20	**3/20**	0/20	0/20	**1/16**	0/0
BA tail	0/20	0/20	**4/20**	0/20	0/16	0/0
BA heart	0/20	0/20	**3/20**	0/20	0/16	0/0
BA yolk	0/20	0/20	**5/20** * (1.333)	0/20	0/16	0/0
Missing fin left	0/20	0/20	0/20	0/20	**1/16**	0/0
Missing fin right	0/20	0/20	0/20	0/20	**1/16**	0/0
Curved fin left	0/20	0/20	0/20	0/20	0/16	0/0
Curved fin right	0/20	**1/20**	0/20	0/20	0/16	0/0
Malformation yolk	0/20	**4/20**	**6/20** * (1.429)	0/20	**3/16**	0/0
No BC in tail	0/20	0/20	**17/20** **** (6.667)	0/20	**1/16**	0/0
Disturbed BC in tail	0/20	0/20	**1/20**	0/20	0/16	0/0
Deviating Heartbeat	0/20	0/20	**20/20** **** (∞)	0/20	**6/16** ** (1.600)	0/0
Deviating shape of head	0/20	0/20	0/20	0/20	**1/16**	0/0
Deviation eye	0/20	0/20	0/20	0/20	**2/16**	0/0
Deviating pigmentation	0/20	**11/20** *** (2.222)	**4/20**	0/20	**5/16** * (1.455)	0/0

B)

	Replicate 2					
Parameter	**Control**	**CBZ 250 µM**	**CBZ 500 µM**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**
Coagulation/dead	0/20	0/20	0/20	0/20	**14/20** **** (3.333)	**20/20** **** (∞)
Tot. malformed (incl. dead)	0/20	**17/20** **** (6.667)	**20/20** **** (∞)	0/20	**20/20** **** (∞)	**20/20** **** (∞)
Tot. malformed (excl. dead)	0/20	**17/20** **** (6.667)	**20/20** **** (∞)	0/20	**6/6** **** (∞)	0/0
No hatching	0/20	**15/20** **** (4.000)	**19/20** **** (20.000)	0/20	**3/6** ** (2.000)	0/0
Elbow tail	0/20	0/20	0/20	0/20	0/6	0/0
Curved tail	0/20	**1/20**	**1/20**	0/20	0/6	0/0
Tissue deviation tail	0/20	0/20	**1/20**	0/20	**1/6**	0/0
Edema head	0/20	**8/20** ** (1.667)	**4/20**	0/20	**3/6** ** (2.000)	0/0
Edema pericard	0/20	**8/20** ** (1.667)	**7/20** ** (1.538)	0/20	**5/6** **** (6.000)	0/0
Edema yolk	0/20	**2/20**	0/20	0/20	**3/6** ** (2.000)	0/0
BA tail	0/20	**1/20**	**3/20**	0/20	0/6	0/0
BA heart	0/20	0/20	**2/20**	0/20	0/6	0/0
BA yolk	0/20	0/20	**3/20**	0/20	0/6	0/0
Missing fin left	0/20	0/20	0/20	0/20	0/6	0/0
Missing fin right	0/20	0/20	0/20	0/20	0/6	0/0
Curved fin left	0/20	0/20	**1/20**	0/20	0/6	0/0
Curved fin right	0/20	0/20	0/20	0/20	0/6	0/0
Malformation yolk	0/20	**3/20**	**3/20**	0/20	**4/6** ** (3.000)	0/0
No BC in tail	0/20	0/20	**19/20** **** (20.000)	0/20	**2/6** * (1.500)	0/0
Disturbed BC in tail	0/20	0/20	**1/20**	0/20	0/6	0/0
Deviating Heartbeat	0/20	**2/20**	**20/20** **** (∞)	0/20	0/6	0/0
Deviating shape of head	0/20	0/20	0/20	0/20	0/6	0/0
Deviation eye	0/20	0/20	0/20	0/20	**3/6** ** (2.000)	0/0
Deviating pigmentation	0/20	**8/20** ** (1.667)	**4/20**	0/20	0/6	0/0

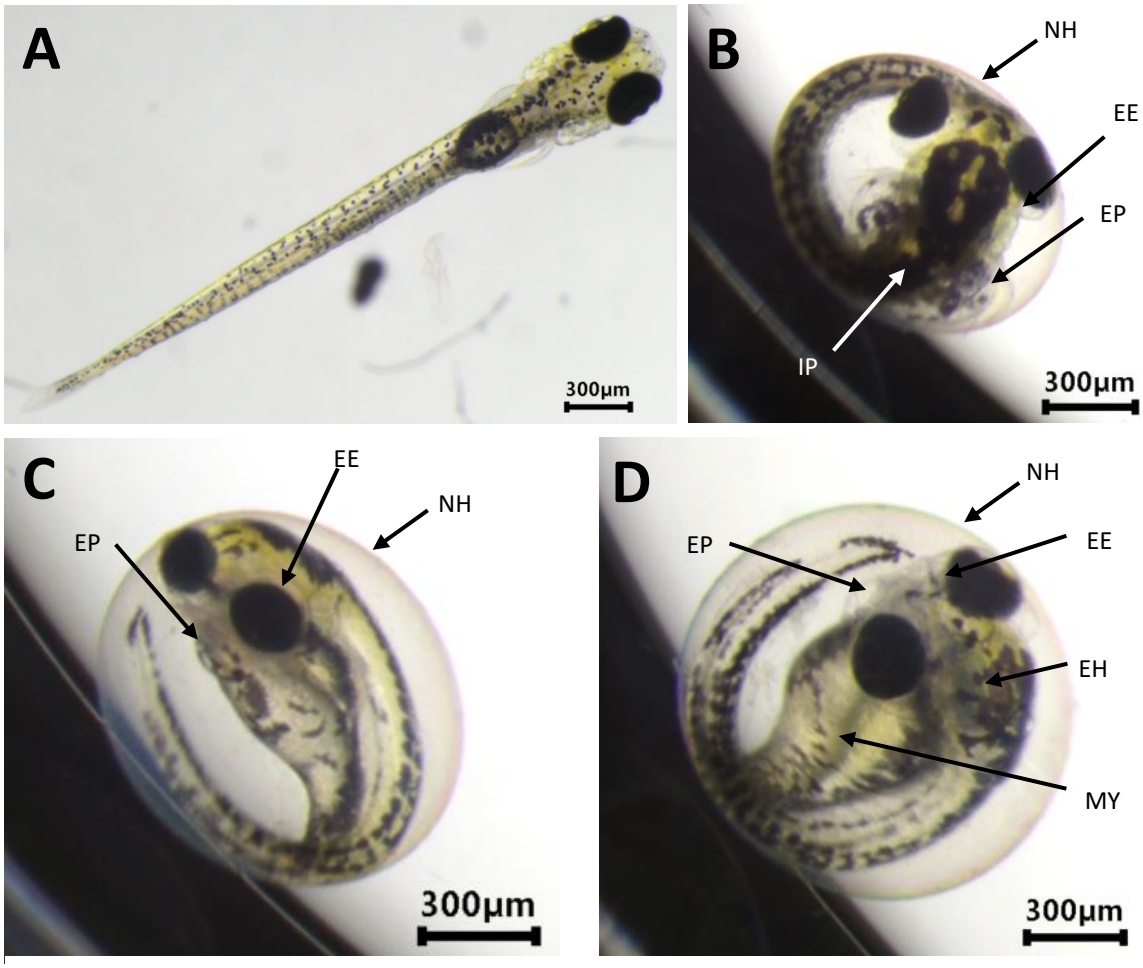

Figure 3. Larvae exposed to carbamazepine or acridine at 120 hpf. A) Control larva (1% DMSO) showing no signs of abnormalities. B) Larva treated with 250 µM CBZ. C) Larva treated with 500 µM CBZ. D) Larva treated with 30 µM acridine. The larvae depicted in B, C and D show several malformations. Abbreviations: carbamazepine (CBZ), edema eyes (EE), edema head (EH), edema pericard (EP), increased pigmentation (IP), malformation yolk (MY), no hatching.

6.3.2.3 Acridone

No statistically significant differences were observed between the lowest concentration of AO and the control group (see Table 6). For 30 µM of AO, no statistically significant differences were observed before 72 hpf (data not shown). From this time point onwards, a significant delay in hatching ($p < 0.0001$) was seen in replicate 2 and remained present until 96 hpf ($p = 0.0463$). At 96 hpf, larvae exposed to 30 µM AO also showed a significantly larger yolk in both replicates ($p = 0.0202$, $p = 0.0197$) (see Supplementary table 4). At 120 hpf, the number of larvae having this malformation almost doubled ($p = 0.0001$, $p < 0.0001$) (see Table 6 and Figure 4). Also, for larvae exposed to 60 µM, a larger yolk was observed at 96 hpf in replicate 2 ($p = 0.0202$) and at 120 hpf in both replicates ($p = 0.0001$, $p = 0.0083$) (see Table 6, Supplementary table 4 and Figure 4).

6.3.2.4 9-acridine carboxaldehyde

Just like for AO, no statistically significant differences could be observed between 3 µM of 9ACA and the control group at any of the time points (see Table 6 and Supplementary table 5) and between 30 µM of 9ACA and the control group before 72 hpf. At 72 hpf, a significant delay in

hatching could be observed in one of the replicates at 30 µM ($p = 0.0083$) (see Supplementary table 5). In this replicate, a statistically significant number of curved tails could be observed at 96 hpf ($p = 0.0471$) and 120 hpf ($p = 0.0083$) (see Table 6, Figure 4 and Supplementary table 5). Moreover, half of the larvae showed a darker pigmentation at 120 hpf in one of the replicates ($p = 0.0004$) (see Table 6, and Figure 4). For the highest concentration of 9ACA (i.e., 60 µM), the total number of embryos with at least one malformation (i.e., total malformed incl. and excl. dead) was already significant at 24 hpf in one replicate ($p = 0.0033$ and $p = 0.0142$, respectively) (see Supplementary table 5). However, when looking at each of the parameters separately at this point, no significant increase in abnormalities could be noted compared to the control group (data not shown). At 48 hpf, the eyes' pigmentation was less intense ($p = 0.0014$, $p = 0.0012$). At 72 hpf, the following malformations were observed: tissue deviation of the tail ($p = 0.0093$) and a larger yolk ($p = 0.0261$) in one of the replicates, and a delay in hatching in both replicates ($p < 0.0001$ for both). Moreover, there was a significant increase in coagulations/dead larvae ($p = 0.0471$) at this time point in one replicate (see Supplementary table 5), which did not increase further during the following time points (see Supplementary table 5 and Table 6). In addition, most of the previously mentioned malformations remained present throughout the following time points, and some new malformations were observed. To be more specific, at 96 hpf, these statistically significant malformations included a delay in hatching ($p < 0.0001$ for both) and a larger yolk ($p = 0.0010$, $p = 0.0012$) in both replicates, and a curved tail ($p = 0.0261$), no blood circulation in the tail ($p = 0.0261$) and edema of the pericard ($p = 0.0031$) in one replicate (see Supplementary table 5). At 120 hpf, a delay in hatching ($p = 0.0003$, $p = 0.0011$), a curved tail ($p = 0.0031$, $p = 0.0463$), a larger yolk ($p < 0.0001$ for both) and darker pigmentation of the body ($p = 0.0261$, $p < 0.0001$) was observed in both replicates and tissue deviation of the tail ($p = 0.0274$), edema of the eyes (i.e., scored as edema head) ($p = 0.0031$), pericard edema ($p = 0.0031$), yolk edema ($p = 0.0093$), no blood circulation in the tail ($p = 0.0010$), smaller eyes ($p = 0.0261$) and a deviation of the mouth ($p = 0.0093$) was found in one of the replicates (see Table 6 and Figure 4).

Table 6. Overview of lethality and malformations in the solvent control group (i.e., 1% DMSO) and the 3, 30 and 60 µM AO and 9ACA test groups at 120 hpf in A) replicate 1 and B) replicate 2. Only parameters showing abnormalities in at least one of both replicates are shown in the tables. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/dead and total malformed (incl. dead), this total number of larvae consisted only of larvae that were alive at 120 hpf. All test groups were compared with the solvent control group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (Fisher Exact test) and the number in brackets indicates the relative risk. Abbreviations: 9-acridine carboxaldehyde (9ACA), acridone (AO), blood accumulation (BA), blood circulation (BC).

A)

	Replicate 1						
Parameter	**Control**	**AO 3 µM**	**AO 30 µM**	**AO 60 µM**	**9ACA 3 µM**	**9ACA 30 µM**	**9ACA 60 µM**
Coagulation/dead	0/20	0/20	0/20	0/20	0/20	0/20	**5/20** *(1.333)
Tot. malformed (incl. dead)	**1/20**	**1/20**	**12/20** *** (2.375)	**12/20** *** (2.375)	**6/20**	**8/20** * (1.583)	**20/20** **** (∞)
Tot. malformed (excl. dead)	**1/20**	**1/20**	**12/20** *** (2.375)	**12/20** *** (2.375)	**6/20**	**8/20** * (1.583)	**15/15** **** (∞)
No hatching	0/20	0/20	0/20	0/20	0/20	0/20	**8/15** *** (2.143)
Elbow tail	0/20	**1/20**	0/20	**1/20**	**1/20**	**3/20**	**3/15**
Curved tail	0/20	**1/20**	**4/20**	0/20	**1/20**	**7/20** ** (1.538)	**6/15** ** (1.667)
Tissue deviation tail	**1/20**	**1/20**	0/20	0/20	**3/20**	**2/20**	**6/15** * (1.583)
Edema head	0/20	0/20	0/20	0/20	**1/20**	**1/20**	**6/15** ** (1.667)
Edema pericard	0/20	0/20	**1/20**	0/20	**1/20**	0/20	**6/15** ** (1.667)
Edema yolk	0/20	0/20	0/20	0/20	0/20	0/20	**5/15** ** (1.500)
BA tail	0/20	**1/20**	0/20	**4/20**	**1/20**	0/20	0/15
BA head	0/20	0/20	0/20	0/20	0/20	0/20	0/15
BA yolk	0/20	0/20	0/20	0/20	0/20	0/20	**2/15**
Curved fin left	0/20	0/20	0/20	0/20	**1/20**	0/20	0/15
Curved fin right	0/20	0/20	0/20	0/20	**1/20**	0/20	**1/15**
Malformation yolk	0/20	**1/20**	**11/20** *** (2.222)	**11/20** *** (2.222)	**3/20**	**1/20**	**13/15** **** (7.500)
No BC in tail	0/20	0/20	0/20	0/20	0/20	**1/20**	**7/15** *** (1.875)
Deviating heartbeat	0/20	0/20	0/20	0/20	0/20	0/20	**2/15**
Deviating shape of head	0/20	0/20	0/20	0/20	0/20	0/20	**3/15**
Deviation mouth	0/20	0/20	**1/20**	0/20	**1/20**	0/20	**5/15** ** (1.500)
Deviation eye	0/20	0/20	0/20	0/20	**1/20**	**1/20**	**4/15** * (1.364)
Deviating pigmentation	0/20	0/20	**1/20**	**1/20**	**1/20**	**3/20**	**4/15** * (1.364)

B)

	Replicate 2						
Parameter	**Control**	**AO 3 µM**	**AO 30 µM**	**AO 60 µM**	**9ACA 3 µM**	**9ACA 30 µM**	**9ACA 60 µM**
Coagulation/dead	**1/20**	0/20	**1/20**	0/20	0/20	0/20	**1/20**
Tot. malformed (incl. dead)	**1/20**	0/20	**14/20** **** (3.167)	**9/20** ** (1.727)	**1/20**	**14/20** **** (3.167)	**20/20** **** (∞)
Tot. malformed (excl. dead)	0/19	0/20	**13/19** **** (3.167)	**9/20** ** (1.818)	**1/20**	**14/20** **** (3.333)	**19/19** **** (∞)
No hatching	0/19	0/20	**1/19**	0/20	0/20	0/20	**9/19** ** (1.900)
Elbow tail	0/19	0/20	**1/19**	0/20	0/20	0/20	0/19
Curved tail	0/19	0/20	**1/19**	**1/20**	0/20	**1/20**	**5/19** * (1.357)
Tissue deviation tail	0/19	0/20	**1/19**	**1/20**	**1/20**	**1/20**	**3/19**
Edema head	0/19	0/20	0/19	0/20	0/20	0/20	**2/19**
Edema pericard	0/19	0/20	**3/19**	0/20	0/20	0/20	**2/19**
Edema yolk	0/19	0/20	0/19	0/20	0/20	0/20	**2/19**
BA tail	0/19	0/20	**1/19**	0/20	0/20	0/20	0/19
BA head	0/19	0/20	0/19	0/20	0/20	0/20	**1/19**
BA yolk	0/19	0/20	**1/19**	0/20	0/20	0/20	0/19
Curved fin left	0/19	0/20	0/19	0/20	0/20	0/20	0/19
Curved fin right	0/19	0/20	0/19	0/20	0/20	0/20	0/19
Malformation yolk	0/19	0/20	**13/19** **** (3.167)	**7/20** ** (1.538)	0/20	**3/20**	**16/19** **** (6.333)
No BC in tail	0/19	0/20	0/19	0/20	0/20	0/20	**1/19**
Deviating heartbeat	0/19	0/20	0/19	0/20	0/20	0/20	0/19
Deviating shape of head	0/19	0/20	0/19	0/20	0/20	0/20	**1/19**
Deviation mouth	0/19	0/20	0/19	0/20	0/20	0/20	**2/19**
Deviation eye	0/19	0/20	0/19	0/20	0/20	0/20	0/19
Deviating pigmentation	0/19	0/20	**4/19**	**1/20**	0/20	**10/20** *** (2.000)	**12/19** **** (2.714)

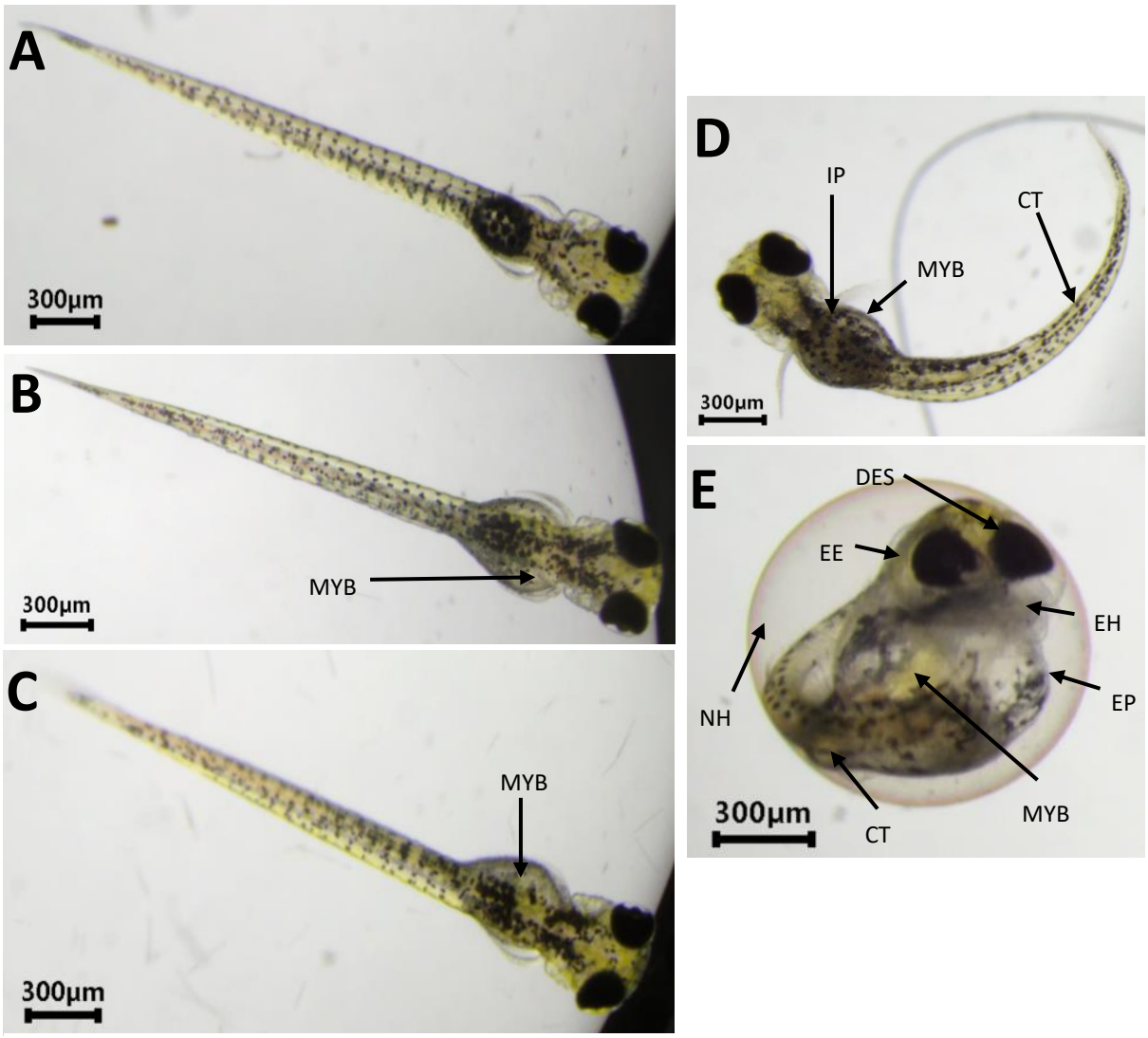

Figure 4. Larvae exposed to acridone or 9-acridine carboxaldehyde at 120 hpf. A) Control larva (1% DMSO) showing no signs of abnormalities. B) Larva treated with 30 µM acridone. C) Larva treated with 60 µM acridone. D) Larva treated with 30 µM 9ACA. E) Larva treated with 60 µM 9ACA. The larvae depicted in B, C, D and E show several malformations. Abbreviations: curved tail (CT), deviation eyes (shape) (DES), edema eyes (EE), edema head (EH), edema pericard (EP), increased pigmentation (IP), malformation yolk (bigger) (MYB), no hatching (NH).

6.3.3 Compound uptake and metabolization products in whole zebrafish embryos/larvae extracts

6.3.3.1 Levetiracetam and its metabolite

No LTC or ECA >LOD was found in the extracts of the control embryos and larvae that were exposed to embryo medium (data not shown). Uptake of LTC was detected in 24 and 120 hpf zebrafish embryos and larvae exposed to 10,000 µM LTC (see Table 7, indicated in green). In one of the 24 hpf replicates, the uptake was lower than the LOQ, while in the other replicate, the uptake was above the upper limit of quantification. No uptake of ECA could be observed (see Table 7, indicated in orange). Biotransformation of LTC into ECA was observed in one replicate of 24 hpf embryos and in both replicates of 120 hpf zebrafish larvae exposed to 10,000 µM LTC (see Table 7, indicated in light green). Moreover, no LTC >LOD was observed in the zebrafish larvae exposed to 500 µM ECA (see Table 7, indicated in gray).

Table 7. Analytical evaluation results of zebrafish larvae exposed to LTC and ECA. Abbreviations: etiracetam carboxylic acid (ECA), levetiracetam (LTC), hours post-fertilization (hpf), limit of detection (LOD), limit of quantification (LOQ), replicate 1 (R1), replicate 2 (R2).

Sample (zebrafish extract)	Concentration of detected compounds (µM)	
	LTC	ECA
LTC 10,000 µM R1 24 hpf	<LOQ	<LOD
LTC 10,000 µM R2 24 hpf	>5.8751*	0.4125
LTC 10,000 µM R1 120 hpf	0.0442	0.0341
LTC 10,000 µM R2 120 hpf	0.0170	0.0098
ECA 500 µM R1 120 hpf	<LOD	<LOD
ECA 500 µM R2 120 hpf	<LOD	<LOD

The shown concentrations are indicative and not exact as no internal standard was used.
*Value was far above the upper limit of quantification (indicated as > upper limit of quantification). The AUC corresponding to the upper limit of quantification was 54,664 and the AUC of the sample was 223,749.

6.3.3.2 Carbamazepine and its metabolites

After investigation of the LC-MS chromatograms, compounds with characteristics similar to the peaks of interest were detected in the extracts of the control embryos and larvae that were exposed to 1% DMSO (see Supplementary table 1). Therefore, additional analytical evaluations were performed to determine the origin of these unexpected peaks (see Supplementary table 6). Since no peaks of interest were found in the 1% DMSO medium samples of the additional analytical evaluations, the presence of contamination could be excluded (see discussion for more details). As such, a cut-off value could be set for each compound of interest. This cut-off value was based on the highest compound concentration detected in the control samples.

Compound uptake was observed in 120 hpf zebrafish larvae exposed to 500 µM CBZ, 60 µM 9ACA, 30 µM AI, and 60 µM AO. Moreover, uptake of CBZ was also determined and detected in 24 hpf zebrafish embryos (see Table 8, indicated in green). Metabolization of CBZ into 9ACA was observed in one replicate in 24 hpf embryos and for both replicates in 120 hpf larvae. Moreover, CBZ was also metabolized into AO (one replicate), IM (two replicates), and E-CBZ (two replicates) in 24 hpf zebrafish embryos. In the 120 hpf larvae, CBZ was also metabolized into E-CBZ (two replicates). 9ACA was metabolized into AI and, subsequently, into AO (both replicates). In the larvae exposed

to AI, metabolization to AO was observed (both replicates) (see Table 8, indicated in light green). No E-CBZ, IM, and CBZ were detected in the extracts of zebrafish larvae exposed to 9ACA, AI, or AO. Also, no 9ACA and AI were detected in the extracts of zebrafish larvae that were exposed to, respectively, AI and AO, and AO (see Table 8, indicated in gray).

Table 8. Analytical evaluation results of zebrafish larvae exposed to CBZ, 9ACA, AI, and AO. Abbreviations: 9-acridine carboxaldehyde (9ACA), acridine (AI), acridone (AO), carbamazepine (CBZ), carbamazepine-10,11-epoxide (E-CBZ), hours post-fertilization (hpf), iminostilbene (IM), limit of detection (LOD), limit of quantification (LOQ), replicate 1 (R1), replicate 2 (R2).

Sample (zebrafish extract)	Concentration of detected compounds (µM)					
	CBZ	9ACA	AI	AO	IM	E-CBZ
CBZ 500 µM R1 24 hpf	6.3739	<cut-off	<cut-off	<cut-off	0.0051	0.0164
CBZ 500 µM R2 24 hpf	8.9238	0.0485	<cut-off	0.0154	0.0032	0.0346
CBZ 500 µM R1 120 hpf	0.7965	0.0154	<cut-off	<cut-off	<cut-off	0.1071
CBZ 500 µM R2 120 hpf	2.4100	0.0040	<cut-off	<cut-off	<cut-off	0.0520
9ACA 60 µM R1 120 hpf	<cut-off	0.0497	>5.5797*	0.4292	<LOD	<LOD
9ACA 60 µM R2 120 hpf	<LOD	0.0184	>5.5797*	0.2141	<LOD	<LOD
AI 30 µM R1 120 hpf	<LOD	<LOD	>5.5797*	0.0662	<LOD	<LOD
AI 30 µM R2 120 hpf	<LOD	<LOD	>5.5797*	0.0394	<LOD	<LOD
AO 60 µM R1 120 hpf	<LOQ	<LOD	<LOD	>5.1224*	<LOD	<LOD
AO 60 µM R2 120 hpf	<LOD	<LOD	<cut-off	>5.1224*	<LOD	<LOD
Cut-off (based on controls)	0.0061	0.0008	0.1440	0.0120	0.0027	0.0077

The shown concentrations are indicative and not exact as a cut-off, and no internal standard was used.
*Value was far above the upper limit of quantification (indicated as > upper limit of quantification). For the detection of acridine, the AUC corresponding to the upper limit of quantification was 599,929, and the AUC of the samples was (from top to bottom): 899,563; 741,683; 1,985,534 and 1,297,891. For the detection of acridone, the AUC corresponding to the upper limit of quantification was 430,229, and the AUC of the samples was (from top to bottom) 1,025,875 and 710,852.

6.4 Discussion

This study aimed to investigate whether LTC and CBZ are biotransformed into non-CYP-mediated metabolites in zebrafish embryos/larvae and whether one or more of these metabolites cause developmental toxicity in this species.

The morphological evaluation of zebrafish embryos exposed to 1-10,000 µM LTC and 1-500 µM ECA revealed that, up until 120 hpf, no significant malformations were observed for any of the concentrations compared to the control group. This is in agreement with what was previously found for LTC by Martinez et al. (2018) and Lee et al. (2013) [32,33]. As no studies examined ECA yet in zebrafish embryos, the results of our study cannot be compared to other studies. As mentioned in the introduction, the negative results in zebrafish embryo assays contrast with what was found in *in vivo* studies in mammals. For LTC, malformations such as skeletal abnormalities [26,30], growth retardation [26,30,31], internal organ abnormalities [30,31] and fetal mortality [26] were reported, and for ECA, growth retardation, and several skeletal abnormalities were reported [26].

To rule out a false negative result in zebrafish embryo assays due to lack of uptake, the uptake of both the parent and the metabolite at the highest concentrations was determined at 120 hpf. The assessment revealed that LTC was poorly taken up by the larvae. In zebrafish embryos/larvae, the

concentration was between 0.0000029 mg/mL (i.e., 0.0170 µM) and 0.0010000 mg/mL (i.e., 5.8751 µM; the upper limit of quantification), which is ~1,000 times lower than the plasma concentration for which skeletal effects were seen in mice (i.e., 1.20 ± 0.35 mg/mL [26]). For ECA, no concentrations above the LOD could be detected. This means that the metabolite might not have been taken up by the embryos/larvae or might have been present in concentrations too low to detect. This limited uptake of LTC and lack of uptake of ECA is likely due to their relatively hydrophilic properties, as it has been shown that the membrane of the skin of embryos can form an obstacle for the uptake of hydrophilic compounds [38]. Consequently, potential teratogenic properties of LTC and ECA might have been missed. This emphasizes the need to always determine uptake, especially for compounds that are negative in zebrafish embryo assays. Otherwise, this can lead to misinterpretation of the results as the compound appears to be non-teratogenic while it was not taken up. Exposing zebrafish embryos to LTC and ECA using intra-yolk microinjection techniques might increase the uptake, as Guarin et al. (2021) compared intrabody exposure and distribution in non-yolk body parts after immersion and micro-injection and recommended the use of microinjection for less lipophilic compounds (i.e., logD between -1.96 and 1.07) [38]. Assuming that the logD value is comparable to the logP value at a physiological pH, LTC and ECA fall within this range as LTC has a logP of -0.6 and ECA has a predicted logP of around -0.1 or 0.21 [39,40]. Interestingly, ECA could be detected in the extracts of 120 hpf zebrafish larvae exposed to 10,000 µM LTC (both replicates) and in extracts of 24 hpf zebrafish embryos exposed to 10,000 µM LTC (second replicate). In the first replicate of 24 hpf zebrafish embryos exposed to 10,000 µM LTC, no ECA >LOD is present. In this replicate, the uptake of LTC was also much lower (i.e., <LOQ in the first replicate vs. >5.8751 in the second replicate), resulting in less LTC inside the embryo to metabolize. Nevertheless, the presence of ECA in one replicate of 24 hpf embryos and two replicates of 120 hpf larvae shows that 24 hpf zebrafish embryos and 120 hpf larvae can metabolize LTC into its metabolite ECA. As such, this indicates that the special type of B-esterase is already active at 24 hpf. Concentrations up to 10,000 µM LTC and 500 µM ECA showed to be non-teratogenic in zebrafish larvae, however, this is likely due to, respectively, a limited uptake or lack of uptake. Repeating the exposure of LTC and ECA to zebrafish embryos using micro-injection techniques instead of immersion, may provide a definitive answer whether the metabolite is teratogenic to zebrafish embryos or not.

Zebrafish larvae exposed to CBZ, 9ACA, AI and AO showed several malformations. For CBZ, a clear dose-response could be noted, as the number of affected embryos/larvae increased with the concentration. Also in the study by Bars et al. (2021), a dose-response was noted [7]. Moreover, most of the malformations that were found in zebrafish larvae exposed to 250 µM CBZ are in accordance with the studies by Bars et al. (2021), Zhu et al. (2015), and van den Brandhof et al. (2010) [7,24,41]. Like our study, Bars et al. (2021) observed a hatching delay from 72 hpf onwards, and edemas around the eye and pericardial edemas at 120 hpf. Increased pigmentation was also observed in that study, but already at 96 hpf, which is earlier than in our study (i.e., 120 hpf) [7]. Van den Brandhof et al. (2010) reported the presence of heart abnormalities and delayed hatching. Also, in their study, no hatching was observed in larvae exposed to 122 mg/L CBZ (i.e., 516.4 µM) and higher at 72 hpf. In contrast to our findings, they observed pericardial edema and delayed heartbeat after exposure to much higher concentrations of CBZ, i.e., 244.5 mg/L or 1034.8 µM.

However, it must be noted that larvae were only exposed to the compound until 72 hpf, and these effects were visible from 96 hpf onwards in our study. Moreover, they observed tail deformation after exposure to 61.2 mg/L (i.e., 259 µM), which was not observed in our study at 72 hpf (or later) [41]. Delayed hatching was also reported by Zhu et al. (2015), as well as the presence of weak heartbeats and blood circulation at concentrations of 50 mg/L and 100 mg/L (i.e., 211.6 µM and 423.2 µM) [24]. Blood accumulation in the yolk and yolk malformations, observed in our study at 120 hpf in the 500 µM CBZ group, were not observed in any of these other studies.

From the three metabolites, only AI had already been tested in a zebrafish embryo assay before [24]. Hence, for the other two metabolites, our results cannot be compared with the literature. For 9ACA, we observed no anomalies at 3 µM, some malformations at 30 µM, and many malformations and increased lethality at 60 µM. For AI, we observed no anomalies at 3 µM, many malformations and increased lethality at 30 µM, and 100% lethality at 300 µM. These results are in line with what has been reported before in the literature [24]. At 48 hpf, 30 µM AI showed no or weak pigmented eyes in our study, whereas this effect was no longer present during the following time points, which was in line with the data by Zhu et al. (2015) [24]. Also, our observed delay in hatching from 72 hpf onwards was in accordance with their findings [24]. In the study by Zhu et al. (2015), edemas, a weak heartbeat and weak blood circulation were noted at 72 hpf. In our study, these sub-lethal effects were observed later (i.e., edema of the eyes and pericard from 96 hpf onwards and a weak heartbeat and blood circulation at 120 hpf). However, as concentrations of 0.625 – 10 mg/L (i.e., 3.4 – 55.8 µM) were used in their study, and it was not specified at which concentration these effects were seen, it is possible that these malformations were observed at a higher concentration than what was used in our study. Also, the malformed spines reported at 96 hpf, which we did not observe, might be due to exposure to a higher concentration. On the other hand, some significant malformations were only seen in our investigation at 120 hpf: tissue deviation of the tail, malformation of the yolk, and darker and enlarged body pigmentation dots. However, as we have a longer exposure window, it cannot be ruled out that these malformations would have been present if they also prolonged their exposure window [24]. At 24 hpf, we observed embryos with smaller yolks and what seemed to be a leakage of the yolk content after exposure to 30 µM AI, which was also not reported by Zhu et al. (2015). This yolk malformation remained for some embryos until 96 hpf. By just looking at the larvae, even without measuring the larval length, it was also noticed that some of these embryos appeared shorter. We hypothesized that a lack of sufficient nutrients due to the leakage of the yolk may have caused impeded growth. A study with potential chemotherapeutic agents also observed the combination of leaking yolks and a shorter larval length and suggested the same theory [42]. They tested this hypothesis by puncturing the yolk of untreated zebrafish embryos at 24 hpf. The larvae that survived this intervention showed no significant difference in body length compared to the controls. This implies that a potential lack of nutrients was not the exclusive reason but that the compound itself may have influenced the overall cell proliferation and embryonic growth [42]. In our study, already at 24 hpf, a 100% lethality was observed at 300 µM of AI. This is not surprising, as Zhu et al. (2015) observed ~90-100% lethality at much lower concentrations (i.e., at 10 mg/L or 55.8 µM) [24]. For both 9ACA and AI, a clear dose-response could be observed in our study, as the number of malformations increased with concentration. For AO, we observed no anomalies at 3 µM and some malformations at 30 µM and

60 µM. For this compound, however, no dose-response could be observed as the number of malformed larvae is higher at 30 µM than at 60 µM AO. Overall, we noticed that the older the larvae became, the more malformations became visible.

Based on our gross morphology investigation for AI and on literature data, AI appears to be more potent than CBZ [7,23,24,43]. When we compare our observations for 30 µM AI with the 31.25 µM of CBZ that was already tested in our research group before (see [7]), we see several malformations at 30 µM AI, while no malformations were observed at 31.25 µM of CBZ, except for swim bladder inflation. When comparing the three metabolites, AO appeared to be the least teratogenic metabolite as it clearly showed less malformations compared with AI and 9ACA at similar or lower concentrations. For these other two metabolites, AI can be referred to as the most potent, as all larvae in both replicates showed to have at least one malformation or died after exposure to 30 µM AI, while this was only 8/20 (replicate 1) and 14/20 (replicate 2) larvae for 9ACA.

Interestingly, for some of the compounds similar anomalies could be observed. These include: increased pigmentation for 250 µM CBZ, 30 and 60 µM 9ACA and 30 µM AI; malformed (bigger) yolks for 500 µM CBZ, 60 µM 9ACA and 30 and 60 µM AI; deviating (slower) heartbeat for 500 µM CBZ and 30 µM AI; delay in hatching for 250 and 500 µM CBZ, 30 µM AI and 60 µM 9ACA; no BC in the tail for 500 µM CBZ, 60 µM 9ACA, 30 µM AI; and edema of the pericard for 250 µM and 500 µM CBZ and 30 µM AI. Some similar anomalies were even very particular, being the weak pigmented eyes at 48 hpf for 60 µM 9ACA and 30 µM AI, and the edema around the eyes at 120 hpf for 250 µM CBZ and 30 µM AI. Based on these observations, we hypothesize that some of the malformations might be caused by one or more formed metabolite(s) from further down the biotransformation pathway.

An LC-MS analysis was performed to assess uptake and to check whether CBZ was metabolized into the metabolites. CBZ was taken up by the 120 hpf zebrafish larvae. However, when checking for biotransformation, compounds with characteristics similar to the peaks of interest were detected in the extracts of the control embryos and larvae that were exposed to 1% DMSO. Therefore, additional analytical evaluations were performed on the test medium where the larvae resided to ensure the test medium was not contaminated. Since absence of the compounds of interest in the test medium in which the larvae were immersed was observed, we hypothesized that the origin of the peaks was from zebrafish tissue. However, additional analytical evaluations on samples containing extracted zebrafish from the LTC exposure experiment revealed that no peaks of interest were present in these samples. As such, we speculate that the peaks are due to a potential effect of DMSO on zebrafish (tissues). Further research is needed to determine the exact origin of these peaks, as this might lead to an overinterpretation, or even misinterpretation, of analytical evaluations. Still, as we were interested in identifying whether the metabolites could be formed, rather than performing an absolute quantification, we applied a cut-off value based on the highest concentration of compound detected in the control samples for each compound of interest rather than performing an absolute quantification. Our results showed that 120 hpf zebrafish larvae can metabolize CBZ into 9ACA and E-CBZ; 9ACA into AI and AO; and AI into AO. Moreover, also at 24 hpf, metabolization of CBZ into 9ACA, AO, IM, and E-CBZ was observed. However, at this timepoint,

less E-CBZ and 9ACA are formed than at 120 hpf. AI was the only metabolite that was not detected in 24 hpf extracts, however, based on the known human pathways, AO is formed via 9ACA and AI. Therefore, we suspect that AI was already further transformed into AO or that AI was present, but below the cut-off value. As IM and E-CBZ are detected in embryos that were exposed to CBZ, it is not clear which pathway (i.e., CBZ > intermediate > 9ACA > AI > AO (see Figure 1) or CBZ > E-CBZ/IM > 9ACA > AI > AO (see Figure 2)) was followed [27,37]. Therefore, further analytical evaluation of zebrafish embryos exposed to E-CBZ and IM could help further understand the pathway. If 9ACA, AI, and AO are formed out of E-CBZ and/or IM, the second pathway CBZ > E-CBZ/IM > 9ACA > AI > AO (potentially in combination with the first pathway) is followed [37]. As 9ACA is detected in larvae exposed to CBZ, and AI and AO are detected in larvae exposed to 9ACA, all three metabolites could be responsible for the teratogenic effects observed after exposure to CBZ. This is strengthened by the fact that our morphological evaluation indicated similar anomalies by the parent and (some of) the metabolites. An obvious example is the edema around the eyes that was detected in larvae exposed to 250 µM CBZ and in larvae exposed to 30 µM AI. As such, the teratogenic effects of CBZ are likely to be caused by one or more of the formed metabolites or by a combination of the parent and the metabolites, and not only by the parent compound.

Based on the pathways that are known in man, our data also suggests that B-esterases (see introduction) are responsible for the biotransformation of LTC into ECA [26,35] in zebrafish embryos and larvae, and myeloperoxidases are responsible for the biotransformation of CBZ (or, as explained before, E-CBZ and/or IM) into 9ACA, AI and AO [27,37]. To confirm this, the experiment could be repeated in zebrafish embryos where B-esterases and MPOs are knocked out or knocked down. For CBZ, investigating the *mpx−/−NL144* "spotless" mutant line might be interesting as they express a non-functional zebrafish myeloperoxidase [44,45]. However, caution should be taken as normal embryonic development may be impaired in knock-out and knock-down models, which has been demonstrated by, for example, the *cyp1b1* knock-out zebrafish model in the study by Alexandre-Moreno et al. (2021) and the *AChEs*^{b55} knock-down zebrafish embryos in the study by Behra et al. (2002) [46,47].

In conclusion, as LTC and ECA were, respectively, poorly, or not taken up by zebrafish larvae during the exposure experiments, we could not determine whether LTC and ECA are teratogenic. Repeating the exposure of LTC and ECA to zebrafish using micro-injection techniques instead of immersion may reveal whether these compounds are teratogenic. However, as LTC was biotransformed into ECA, the special type of B-esterase is active at 24 and 120 hpf. CBZ, 9ACA, AI, and AO were taken up by zebrafish larvae. Moreover, the MPO homologue is already active at 24 hpf, as CBZ was biotransformed into 9ACA and AO in 24 hpf zebrafish embryos and into 9ACA in 120 hpf larvae. 9ACA was also found to be biotransformed into AI and AO, and AI into AO. Exposure to CBZ, 9ACA, AI, and AO caused anomalies in zebrafish embryos and larvae. These three metabolites are more potent than CBZ, with AI being the most potent, followed by 9ACA and AO. As such, one or more of these metabolites probably contribute to the teratogenic effects observed in zebrafish larvae after exposure to CBZ.

6.5 Supplementary tables

Supplementary table 1. Analytical evaluation of the control samples (zebrafish extracts). Abbreviations: 9-acridine carboxaldehyde (9ACA), acridine (AI), acridone (AO), carbamazepine (CBZ), carbamazepine-10,11-epoxide (E-CBZ), carbamazepine-acridine experiment (E1), 9ACA-acridone experiment (E2), hours post-fertilization (hpf), iminostilbene (IM), limit of detection (LOD), limit of quantification (LOQ), replicate 1 (R1), replicate 2 (R2).

Sample (zebrafish extract)	Concentration of detected compounds (µM)					
	CBZ	9ACA	AI	AO	IM	E-CBZ
1% DMSO R1 24 hpf (E1)	<LOQ	<LOQ	<LOQ	0.0002	0.0003	<LOQ
1% DMSO R1 120 hpf (E1)	<LOQ	<LOQ	<LOQ	0.0009	0.0027*	0.0077*
1% DMSO R1 120 hpf (E2)	<LOQ	0.0008*	0.0737	0.0017	<LOQ	<LOQ
1% DMSO R2 24 hpf (E1)	0.0061*	<LOD	0.1440*	0.0120*	<LOD	<LOQ
1% DMSO R2 120 hpf (E1)	<LOQ	<LOD	<LOQ	0.0004	<LOD	<LOQ
1% DMSO R2 120 hpf (E2)	<LOQ	<LOQ	<LOQ	0.0031	<LOQ	<LOQ

*Used as a cut-off value

Supplementary table 2. Overview of lethality and malformations in the solvent control group (i.e., 1% DMSO) and the 250 and 500 µM CBZ test groups at A) 72 hpf and B) 96 hpf in both replicates. Only parameters showing significant abnormalities in at least one of both replicates are shown in the tables. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/dead and total malformed (incl. dead), this total number of larvae consisted only of larvae that were alive. All test groups were compared with the solvent control group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (Fisher Exact test) and the number in brackets indicates the relative risk. Abbreviations: carbamazepine (CBZ), hours post-fertilization (hpf).

A) 72 hpf

	Replicate 1			Replicate 2		
Parameter	Control	CBZ 250 µM	CBZ 500 µM	Control	CBZ 250 µM	CBZ 500 µM
Coagulation/dead	0/20	0/20	0/20	0/20	0/20	0/20
Tot. malformed (incl. dead)	0/20	**12/20** **** (2.500)	**20/20** ****(∞)	0/20	**19/20** **** (20.000)	**20/20** **** (∞)
Tot. malformed (excl. dead)	0/20	**12/20** **** (2.500)	**20/20** ****(∞)	0/20	**19/20** **** (20.000)	**20/20** **** (∞)
No hatching	0/20	**12/20** **** (2.500)	**20/20** ****(∞)	0/20	**18/20** **** (10.00)	**20/20** **** (∞)

B) 96 hpf

	Replicate 1			Replicate 2		
Parameter	Control	CBZ 250 µM	CBZ 500 µM	Control	CBZ 250 µM	CBZ 500 µM
Coagulation/dead	0/20	0/20	0/20	0/20	0/20	0/20
Tot. malformed (incl. dead)	0/20	**11/20** *** (2.222)	**20/20** **** (∞)	0/20	**16/20** **** (5.000)	**20/20** **** (∞)
Tot. malformed (excl. dead)	0/20	**11/20** *** (2.222)	**20/20** **** (∞)	0/20	**16/20** **** (5.000)	**20/20** **** (∞)
No hatching	0/20	**10/20** *** (2.000)	**20/20** **** (∞)	0/20	**15/20** **** (4.000)	**20/20** **** (∞)
Edema pericard	0/20	0/20	**4/20**	0/20	**2/20**	**5/20** * (1.333)
Deviating heartbeat	0/20	0/20	**18/20** **** (10.00)	0/20	0/20	**16/20** **** (5.000)

Supplementary table 3. Overview of lethality and malformations in the solvent control group (i.e., 1% DMSO) and the 3, 30 and 300 µM acridine test groups at A) 24 hpf and B) 48 hpf, C) 72 hpf and D) 96 hpf in both replicates. Only parameters showing significant abnormalities in at least one of both replicates are shown in the tables. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/dead and total malformed (incl. dead), this total number of larvae consisted only of larvae that were alive. All test groups were compared with the solvent control group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (Fisher Exact test) and the number in brackets indicates the relative risk. Abbreviations: acridine (AI), hours post-fertilization (hpf).

A) 24 hpf

	Replicate 1				Replicate 2			
Parameter	**Control**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**	**Control**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**
Coagulation/dead	0/20	0/20	**2/20**	**20/20** **** (∞)	0/20	0/20	**13/20** **** (2.857)	**20/20** **** (∞)
Tot. malformed (incl. dead)	0/20	0/20	**6/20** * (1.429)	**20/20** **** (∞)	0/20	0/20	**18/20** **** (10.00)	**20/20** **** (∞)
Tot. malformed (excl. dead)	0/20	0/20	**4/18** * (1.286)	0/0	0/20	0/20	**5/7** *** (3.500)	0/0
Malformation yolk	0/20	0/20	**4/18** * (1.286)	0/0	0/20	0/20	**3/7** * (1.750)	0/0

B) 48 hpf

	Replicate 1				Replicate 2			
Parameter	**Control**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**	**Control**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**
Coagulation/dead	0/20	0/20	**3/20**	**20/20** **** (∞)	0/20	0/20	**13/20** **** (2.857)	**20/20** **** (∞)
Tot. malformed (incl. dead)	0/20	0/20	**14/20** **** (3.333)	**20/20** **** (∞)	0/20	0/20	**20/20** **** (∞)	**20/20** **** (∞)
Tot. malformed (excl. dead)	0/20	0/20	**11/17** **** (2.833)	0/0	0/20	0/20	**7/7** **** (∞)	0/0
Malformation yolk	0/20	0/20	**3/17**	0/0	0/20	0/20	**4/7** ** (2.333)	0/0
Deviation eye	0/20	0/20	0/17	0/0	0/20	0/20	**7/7** **** (∞)	0/0
Deviating pigmentation	0/20	0/20	**9/17** *** (2.125)	0/0	0/20	0/20	**7/7** **** (∞)	0/0

C) 72 hpf

	Replicate 1				Replicate 2			
Parameter	**Control**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**	**Control**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**
Coagulation/dead	0/20	0/20	**3/20**	**20/20** **** (∞)	0/20	0/20	**13/20** **** (2.857)	**20/20** **** (∞)
Tot. malformed (incl. dead)	0/20	0/20	**20/20** **** (∞)	**20/20** **** (∞)	0/20	**1/20**	**20/20** **** (∞)	**20/20** **** (∞)
Tot. malformed (excl. dead)	0/20	0/20	**17/17** **** (∞)	0/0	0/20	**1/20**	**7/7** **** (∞)	0/0
No hatching	0/20	0/20	**17/17** **** (∞)	0/0	0/20	0/20	**6/7** **** (7.000)	0/0
Malformation yolk	0/20	0/20	**3/17**	0/0	0/20	0/20	**3/7** * (1.750)	0/0
Deviation eye	0/20	0/20	**2/17**	0/0	0/20	0/20	**4/7** ** (2.333)	0/0

D) 96 hpf

	Replicate 1				Replicate 2			
Parameter	**Control**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**	**Control**	**AI 3 µM**	**AI 30 µM**	**AI 300 µM**
Coagulation/dead	0/20	0/20	**3/20**	**20/20** **** (∞)	0/20	0/20	**14/20** **** (3.333)	**20/20** **** (∞)
Tot. malformed (incl. dead)	0/20	0/20	**20/20** **** (∞)	**20/20** **** (∞)	0/20	**1/20**	**20/20** **** (∞)	**20/20** **** (∞)
Tot. malformed (excl. dead)	0/20	0/20	**17/17** **** (∞)	0/0	0/20	**1/20**	**6/6** **** (∞)	0/0
No hatching	0/20	0/20	**16/17** **** (17.00)	0/0	0/20	0/20	**3/6** ** (2.000)	0/0
Edema head	0/20	0/20	0/17	0/0	0/20	0/20	**3/6** ** (2.000)	0/0
Edema pericard	0/20	0/20	**1/17**	0/0	0/20	0/20	**3/6** ** (2.000)	0/0
Malformation yolk	0/20	0/20	**3/17**	0/0	0/20	0/20	**3/6** ** (2.000)	0/0
Deviation eye	0/20	0/20	**2/17**	0/0	0/20	0/20	**3/6** ** (2.000)	0/0

Supplementary table 4. Overview of lethality and malformations in the solvent control group (i.e., 1% DMSO) and the 3, 30 and 60 µM acridone test groups at A) 72 hpf and B) 96 hpf in both replicates. Only parameters showing significant abnormalities in at least one of both replicates are shown in the tables. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/dead and total malformed (incl. dead), this total number of larvae consisted only of larvae that were alive. All test groups were compared with the solvent control group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (Fisher Exact test) and the number in brackets indicates the relative risk. Abbreviations: acridone (AO), hours post-fertilization (hpf).

A) 72 hpf

	Replicate 1				Replicate 2			
Parameter	**Control**	**AO 3 µM**	**AO 30 µM**	**AO 60 µM**	**Control**	**AO 3 µM**	**AO 30 µM**	**AO 60 µM**
Coagulation/dead	0/20	0/20	0/20	0/20	0/20	0/20	**1/20**	0/20
Tot. malformed (incl. dead)	0/20	**1/20**	**5/20** * (1.333)	**1/20**	**1/20**	0/20	**13/20** *** (2.714)	**5/20**
Tot. malformed (excl. dead)	0/20	**1/20**	**5/20** * (1.333)	**1/20**	**1/20**	0/20	**12/19** *** (2.579)	**5/20**
No hatching	0/20	0/20	**4/20**	**1/20**	0/20	0/20	**12/19** **** (2.714)	**3/20**

B) 96 hpf

	Replicate 1				Replicate 2			
Parameter	**Control**	**AO 3 µM**	**AO 30 µM**	**AO 60 µM**	**Control**	**AO 3 µM**	**AO 30 µM**	**AO 60 µM**
Coagulation/dead	0/20	0/20	0/20	0/20	**1/20**	0/20	**1/20**	0/20
Tot. malformed (incl. dead)	**1/20**	**1/20**	**10/20** ** (1.900)	**4/20**	**1/20**	0/20	**13/20** *** (2.714)	**8/20** * (1.583)
Tot. malformed (excl. dead)	**1/20**	**1/20**	**10/20** ** (1.900)	**4/20**	0/19	0/20	**12/19** **** (2.714)	**8/20** ** (1.667)
No hatching	0/20	0/20	**1/20**	0/20	0/19	0/20	**5/19** * (1.357)	0/20
Malformation yolk	0/20	**1/20**	**6/20** * (1.429)	**4/20**	0/19	0/20	**6/19** * (1.462)	**6/20** * (1.429)

Supplementary table 5. Overview of lethality and malformations in the solvent control group (i.e., 1% DMSO) and the 3, 30 and 60 µM 9-acridine carboxaldehyde test groups at A) 24 hpf and B) 48 hpf, C) 72 hpf and D) 96 hpf in both replicates. Only parameters showing significant abnormalities in at least one of both replicates are shown in the tables. The ratio of affected larvae/total number of larvae is shown for each parameter and each group. For all parameters, except for coagulation/dead and total malformed (incl. dead), this total number of larvae consisted only of larvae that were alive. All test groups were compared with the solvent control group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (Fisher Exact test) and the number in brackets indicates the relative risk. Abbreviations: 9-acridine carboxaldehyde (9ACA), hours post-fertilization (hpf).

A) 24 hpf

	Replicate 1				Replicate 2			
Parameter	**Control**	**9ACA 3 µM**	**9ACA 30 µM**	**9ACA 60 µM**	**Control**	**9ACA 3 µM**	**9ACA 30 µM**	**9ACA 60 µM**
Coagulation/dead	0/20	0/20	0/20	**3/20**	0/20	0/20	0/20	0/20
Tot. malformed (incl. dead)	0/20	0/20	0/20	**8/20** ** (1.667)	0/20	0/20	0/20	0/20
Tot. malformed (excl. dead)	0/20	0/20	0/20	**5/17** * (1.417)	0/20	0/20	0/20	0/20

B) 48 hpf

	Replicate 1				Replicate 2			
Parameter	**Control**	**9ACA 3 µM**	**9ACA 30 µM**	**9ACA 60 µM**	**Control**	**9ACA 3 µM**	**9ACA 30 µM**	**9ACA 60 µM**
Coagulation/dead	0/20	0/20	0/20	**4/20**	0/20	0/20	0/20	0/20
Tot. malformed (incl. dead)	0/20	0/20	0/20	**13/20** **** (2.857)	**1/20**	0/20	**1/20**	**10/20** ** (1.900)
Tot. malformed (excl. dead)	0/20	0/20	0/20	**9/16** *** (2.286)	**1/20**	0/20	**1/20**	**10/20** ** (1.900)
Deviating pigmentation	0/20	0/20	0/20	**7/16** ** (1.778)	0/20	0/20	**1/20**	**9/20** ** (1.818)

C) 72 hpf

	Replicate 1				Replicate 2			
Parameter	**Control**	**9ACA 3 µM**	**9ACA 30 µM**	**9ACA 60 µM**	**Control**	**9ACA 3 µM**	**9ACA 30 µM**	**9ACA 60 µM**
Coagulation/dead	0/20	0/20	0/20	**5/20** * (1.333)	0/20	0/20	0/20	0/20
Tot. malformed (incl. dead)	0/20	**3/20**	**12/20** **** (2.500)	**19/20** **** (20.00)	**1/20**	0/20	**1/20**	**17/20** **** (6.333)
Tot. malformed (excl. dead)	0/20	**3/20**	**12/20** **** (2.500)	**14/15** **** (15.00)	**1/20**	0/20	**1/20**	**17/20** **** (6.333)
No hatching	0/20	0/20	**7/20** ** (1.538)	**12/15** **** (5.00)	0/20	0/20	0/20	**16/20** **** (5.000)
Tissue deviation tail	0/20	**3/20**	**1/20**	**5/15** ** (1.500)	0/20	0/20	**1/20**	**2/20**
Malformation yolk	0/20	0/20	0/20	**4/15** * (1.364)	0/20	0/20	0/20	**1/20**

D) 96 hpf

Parameter	Replicate 1				Replicate 2			
	Control	9ACA 3 µM	9ACA 30 µM	9ACA 60 µM	Control	9ACA 3 µM	9ACA 30 µM	9ACA 60 µM
Coagulation/dead	0/20	0/20	0/20	**5/20** * (1.333)	**1/20**	0/20	0/20	0/20
Tot. malformed (incl. dead)	**1/20**	**5/20**	**8/20** * (1.583)	**19/20** **** (19.00)	**1/20**	**1/20**	**5/20**	**19/20** **** (19.00)
Tot. malformed (excl. dead)	**1/20**	**5/20**	**8/20** * (1.583)	**14/15** **** (14.25)	0/19	**1/20**	**5/20** * (1.333)	**19/20** **** (20.00)
No hatching	0/20	0/20	**2/20**	**10/15** **** (3.000)	0/19	0/20	0/20	**12/20** **** (2.500)
Curved tail	0/20	**1/20**	**5/20** * (1.333)	**4/15** * (1.364)	0/19	0/20	**1/20**	**2/20**
Edema pericard	0/20	0/20	0/20	**6/15** ** (1.667)	0/19	0/20	0/20	**2/20**
Malformation yolk	0/20	**1/20**	**1/20**	**7/15** *** (1.875)	0/19	0/20	**3/20**	**9/20** ** (1.818)
No BC in tail	0/20	0/20	0/20	**4/15** * (1.364)	0/19	0/20	0/20	**1/20**

Supplementary table 6. Results of the additional analytical evaluations. Abbreviations: 9-acridine carboxaldehyde (9ACA), acridine (AI), acridone (AO), carbamazepine (CBZ), carbamazepine-10,11-epoxide (E-CBZ), carbamazepine-acridine experiment (E1), 9ACA-acridone experiment (E2), etiracetam carboxylic acid (ECA), hours post-fertilization (hpf), iminostilbene (IM), limit of detection (LOD), replicate 1 (R1), replicate 2 (R2).

Sample (zebrafish extract or medium)	Concentration of detected compounds (µM)					
	CBZ	9ACA	AI	AO	IM	E-CBZ
Medium 1% DMSO R1 24 hpf (E1)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Medium 1% DMSO R2 120 hpf (E1)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Medium 1% DMSO R2 24 hpf (E2)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Extract 250 µM ECA R1 120 hpf	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Extract 250 µM ECA R2 120 hpf	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

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CHAPTER 7:
General discussion and future perspectives

The main goal of this thesis was to standardize and optimize the ZEDTA towards a sensitive screening assay for drug-induced birth defects. In this chapter, an overview of the main results of this thesis will be given (i.e., presented as chapters 3-6). Subsequently, these results are placed in a broader perspective and further steps are proposed to increase the sensitivity of the ZEDTA.

7.1 Overview of the results

Chapter 3 – Development of a standardized ZEDTA protocol that can be used as a modular system depending on the compound of interest.

- A standardized and optimized ZEDTA protocol was developed.
- A pre-incubation metabolic activation system using human liver microsomes was developed (i.e., mZEDTA):
    - The 1:10 diluted ultracentrifuged pre-incubation protocol was tested with a blank control and proved to be non-embryotoxic.
    - Validation with a tool compound, carbamazepine, proved the formation of the metabolite.
    - Further research should investigate whether the concentration of the formed toxic metabolite(s) in the diluted protocol is sufficiently high to cause malformations in zebrafish embryos and larvae.
- A skeletal staining protocol that can be added to the ZEDTA was developed (i.e., sZEDTA):
    - The live alizarin red skeletal staining protocol was tested with a tool compound, rosiglitazone. Exposure to this compound caused significant differences in bone intensities compared to the solvent control.
    - This skeletal staining protocol was further qualified with proprietary and non-proprietary compounds (see chapter 5). However, this showed that biological variability in bone development hampers the use of a skeletal staining.
- This standardized ZEDTA can be used as a modular system, depending on the compound of interest. The mZEDTA can be added for compounds that need metabolic activation. Although it initially seemed that the sZEDTA could be added to detect skeletal teratogens (see chapter 3), the use of a skeletal staining was not possible due to biological variability in bone development (see chapter 5).

Chapter 4 – Determination of the maximal concentration of DMSO that can be safely used as a solvent in the ZEDTA.

- DMSO concentrations up to 1% are safe to be used in the ZEDTA:
    - No increase in lethality or malformations were observed when using DMSO concentrations up to 1%.
    - Combining ascorbic acid or hydrochlorothiazide with 1% DMSO did not cause any developmental toxicity in zebrafish embryos/larvae.
- Extending the exposure period in the ZEDTA to 120 hpf instead of 96 hpf makes the assay more sensitive, as exposure to 2% DMSO significantly increased the total number of

malformed/dead larvae at the end of the exposure period (120 hpf), and not at 96 hpf, when compared to the start of the exposure (5.25 hpf).

Chapter 5 – Does implementation of a skeletal staining increase the sensitivity of zebrafish embryo developmental toxicity assays?

- Biological variability in (onset of) bone development hampers the use of skeletal staining methods in zebrafish embryo developmental toxicity assays:
    - There was a high variability in staining in larvae exposed to mammalian skeletal teratogens, as well as between control larvae originating from the same clutch of zebrafish.
- Assessment of larval length can be included as a standard endpoint in future zebrafish embryo developmental toxicity assays as determination of larval length revealed signs of growth retardation in larvae exposed to compound 5 and 9.

Chapter 6 – Assessing developmental toxicity and non-CYP mediated biotransformation of two anti-epileptics and their human metabolites in zebrafish embryos and larvae.

- Due to the hydrophilic properties of levetiracetam (LTC) and etiracetam carboxylic acid (ECA), the compounds were, respectively, poorly or not taken up by zebrafish embryos/larvae during the exposure experiments.
    - The special type of B-esterase was found to be active at 24 and 120 hpf, as LTC was biotransformed into ECA.
    - No malformations were observed in zebrafish embryos and larvae exposed to LTC or ECA, but no conclusions on the teratogenicity of these compounds could be made due to the fact this might be due to the limited uptake.
    - This emphasizes the need to always determine uptake in the ZEDTA, especially for negative compounds. Otherwise, this can lead to false negative results.
    - Research by others revealed that exposure via micro-injection showed a higher compound uptake for compounds with a logD within the range of -1.96 and 1.07. Assuming that the logD value is comparable to the logP value at a physiological pH, LTC and ECA fall within this range as LTC has a logP of -0.6 and ECA has a predicted logP of around -0.1 or 0.21. As such, repeating the experiments using micro-injection techniques may reveal whether the parent and/or metabolite is teratogenic or not.
- The MPO homologue is already active at 24 hpf, as 9-acridine carboxaldehyde (9ACA), acridine (AI) and acridone (AO) are formed. Moreover, they are found to be more potent than the parent, carbamazepine (CBZ), and therefore, one or more of these metabolites probably contributes to the teratogenic effects that are observed in zebrafish larvae after exposure to carbamazepine.
    - CBZ was biotransformed into 9ACA, AO, IM and E-CBZ in 24 hpf zebrafish embryos, and into 9ACA and E-CBZ in 120 hpf larvae. Moreover, 9ACA was also found to be biotransformed into AI and AO, and AI into AO.
    - Exposure to CBZ, 9ACA, AI and AO caused anomalies in zebrafish embryos and larvae.

- All three metabolites are found to be more potent than CBZ, with AI being the most potent, followed by 9ACA and AO.

7.2 Standardization of the ZEDTA

A literature search by our group (unpublished data) revealed that, due to the use of different zebrafish embryo assay protocols by different labs, discordant results in teratogenicity classification for the same compounds are obtained [1–9]. The use of limited exposure windows, the use of different endpoints and several other differences in study design are believed to be potential causes for discordant classification of compounds. Moreover, a recent study that characterized the sources of variability in zebrafish embryo assay protocols reported that the use of dechorionated or chorion-intact embryos and static or semi-static approaches for (renewal of) exposure media could also potentially influence study outcomes [10]. This shows the clear need for standardization of zebrafish embryo assay protocols in order to reduce discordances in teratogenicity classification. However, as the ZEDTA might be considered for regulatory use in the future (see 7.4), internal validation, thus also internal standardization, of the ZEDTA protocol is required. After all, the ICH S5 guideline does not require a cross-laboratory validation for potential regulatory use, but qualification (i.e., internal validation) is required. To this end, we took different sources of variability into account when developing our modular ZEDTA protocol (chapter 3). The most important choices, adjustments and considerations we made for developing our standardized modular protocol, as well as future recommendations for further standardization are discussed below. However, we would first like to point out that a different protocol was used in chapter 5 of this thesis, as the skeletal staining experiments were carried out at the University of Exeter when we started standardizing our ZEDTA at the UAntwerp. As such, the experiments in chapter 5 were conducted according to the standard protocol of the University of Exeter (described in section 5.2).

Zebrafish strain

Currently, several wild type strains, such as AB, TU, TL and WIK, are used in zebrafish embryo assays [1,3,11]. Interestingly, several strain-specific differences in response to stimuli have been reported in behavioral studies [12–14]. Although data about strain-specific differences in response to toxicants is limited (reviewed by [10]), one study reported that two strains, AB and TU, show differences in mortality and inflammatory responses after exposure to 2,4,6-trinitrobenzenesulfonate [11]. As such, to avoid potential inter-strain differences in sensitivity to compounds, we included only the wild type AB strain in our standardized assay. However, it is not important which strain is used, as long as, within a lab/protocol, always the same strain is used and validated.

Temperature

The optimal temperature for zebrafish development is known to be between 26 and 28.5°C [15]. As the developmental rate of zebrafish changes when using different temperatures, it is important to incubate all embryos at the same temperature [16]. In addition, using one temperature will avoid potential additional variation within a test due to differences in temperature. Kimmel et al. (1995) described all stages and timing of embryonic development at 28.5°C [16]. To allow easy comparison

of our observations with the normal embryonic development described in this study, we chose 28.5°C as incubation temperature in our standardized modular ZEDTA protocol. Moreover, this temperature can also be used in the mZEDTA, as previous research showed that human liver microsomes have the same CYP activity at 28.5°C than at 37°C (i.e., the temperature at which they are considered to function optimally [17]) [18]. Hence, incubation at 28.5°C was the best choice for our protocol.

Embryo medium

Previous research by our group showed that a TRIS-buffered medium allowed both normal development of control embryos and sufficiently high CYP activity [19]. This is in contrast to other media, which either caused embryotoxicity or insufficient CYP activity [19]. Therefore, the use of TRIS-buffered medium provided opportunities for including the mZEDTA in our standardized ZEDTA protocol. As the same media can be used in the mZEDTA and ZEDTA, a compound dissolved in TRIS-buffered medium can be activated with a MAS using microsomes (mZEDTA; see chapter 3 and 7.3), and then, the TRIS-buffered medium containing the parent and metabolites of interest can be exposed to zebrafish embryos (ZEDTA). As such, we included a TRIS-buffered medium to our protocol, as the use of this medium is required if a compound requires the use of a MAS.

Solvent

Due to the frequent occurring need for DMSO concentrations higher than 0.01% to dissolve compounds in an aqueous medium, we investigated the highest concentration of DMSO that can be safely used in our standardized ZEDTA. Our results (chapter 4) indicated that 1% of DMSO is the maximum tolerated concentration. Therefore, if possible, we recommend to always use ≤1% DMSO in our standardized assay. As such, compounds or concentrations that are not soluble in ≤1% DMSO are less suited to be tested in the ZEDTA. In chapter 6, for example, the maximal concentration of 9-acridine carboxaldehyde and acridone that could be tested was 60 µM, as higher concentrations could not be reached due to precipitation of the compounds. If solvents other than ≤1% DMSO need to be used, a solvent control experiment should be performed first as the solvent and/or concentration may be toxic for zebrafish embryos.

When using zebrafish embryos for other types of toxicity or when other endpoints than gross morphology are examined, the DMSO concentrations presumably need to be further reduced, as reported in other studies [20–22]. For example, Hallare et al. (2004, 2006) found that DMSO induces a concentration-dependent increase in heat shock protein 70 production, and therefore recommend the use of DMSO concentrations below 0.01% for stress protein analysis [20,21]. Chen et al. (2011) reported that concentrations as low as 0.01% DMSO already can alter locomotor activity [22].

pH

Exposure to extreme pH levels and drastic changes in pH level are known to have a negative impact on zebrafish development [23,24]. During the exposure experiments of chapter 6, we noticed that the two highest concentrations of etiracetam carboxylic acid were too acidic to be tolerated by the embryos (i.e., pH <5). Other studies have also mentioned that the pH of the incubation medium

may change after addition of a test compound, and that this can even result in alterations in bioavailability of the test compound [25,26] or lethality [10]. Although not (yet) included in our standardized protocol in chapter 3, we added pH measurement (and if needed pH adjustment) to our protocol in the next chapters to make sure a physiological pH is maintained throughout the experiments. As described in the zFET [27]; if the pH of a test solution is not in a range of pH 6.5-8.5, the pH should be adjusted prior exposure using HCl or NaOH, while changing the concentration of the test solution as minimal as possible.

Exposure length

The main organogenesis period of zebrafish, raised at 28.5°C, is from 5 ¼ hpf until 96-120 hpf [16]. If zebrafish embryos are not exposed to the compound during this period, the critical window for teratogenic action can be missed or exposure duration can be insufficient to exert teratogenic effects. In the study by Pruvot et al. (2012), for example, only a limited exposure window from 48-144 hpf was used. As a consequence, the teratogenic potential of carbamazepine, lithium chloride, pentobarbital and caffeine was missed, while these compounds showed to be teratogenic in other studies where exposure already started at 5 ¼ hpf [4,6,7,9]. For our standardized ZEDTA, we chose an exposure period from 5 ¼ hpf until 120 hpf. In many of the zebrafish embryo assays that are described in literature, as well as in the zFET, the exposure period already ends at 96 hpf. Extending the exposure period to 120 hpf, however, already proved to make the assay more sensitive, as exposure to 2% DMSO significantly increased the total number of malformed/dead larvae at the end of the exposure period (120 hpf), and not at 96 hpf, when compared to the start of the exposure (5.25 hpf) (see chapter 4). Therefore, we recommend exposure until 120 hpf in zebrafish embryo assays.

Renewal of test solution

There are three approaches in which zebrafish embryos can be exposed to a test compound via their incubation medium. In the static approach, the test solution remains the same during the entire experiment, without replacing it. In the semi-static approach, the test solution is replaced with fresh test solution at certain timepoints. In the flow-through approach, the test solution is constantly circulating, and the concentration is monitored and adjusted [10]. This last approach, however, requires the need for a special set-up and requires a significant amount of test compound. In our standardized ZEDTA, we mention the use of the static approach, unless the test solution is degraded at 120 h with more than 20% of the nominal concentration and a semi-static exposure approach should be used. However, to avoid acidification and oxygen deprivation [15], the test solution was renewed every 48 h in the experiments in chapter 4 and 6[4]. Hence, a semi-static approach, instead of a static approach, was used. The renewal of test medium, however, might increase lethality and malformations as the embryos are manipulated more. On the other hand, when uptake is limited, false negative results might be more likely in a static approach [10]. As such, the use of different approaches in labs may result in discordant results. A future study that

[4] Chapter 5 is not mentioned here, as these experiments were performed by researchers of the university of Exeter.

investigates the teratogenicity classification of identical compounds exposed to zebrafish embryos via a static and semi-static approach would be very useful to identify the discordances in outcome between both approaches. Moreover, based on this information a more informed decision could be made on which approach should be included in the ZEDTA, and as such this would allow further standardization.

Chorion-intact or dechorionated embryos

The chorion is an acellular envelope that can function as a barrier for compounds that have a molecular weight of more than 3 kDa. Besides the molecular weight, also the physicochemical and electrostatic properties, ionic charge and used DMSO concentration can affect the ability of a compound to pass through the chorion (reviewed by [10]). Therefore, some research groups prefer to use dechorionated embryos to make sure the embryos are exposed to the compound of interest. However, the removal of the chorion has disadvantages as well. The process of removing the chorion is laborious and special equipment and training is needed to avoid damaging the embryo. Moreover, using dechorionated embryos prevents to use hatching as an endpoint [10] and increases the number of malformed and death embryos [28,29]. As, in our opinion, the advantage of a potential better exposure to small molecule drugs does not outweigh the disadvantages of chorion removal, we chose to use chorion-intact embryos in our protocol.

Compound administration

In our standardized ZEDTA, zebrafish embryos are exposed to a test compound by adding it to the incubation medium (i.e., via immersion). A test compound can also be delivered directly to the embryo by using a micro-injection technique. Micro-injection has the advantage that the test compound does not have to pass through the chorion and the skin of the zebrafish. However, difficulties with delivering consistent volumes of chemical to the embryos were reported due to variations in inner diameters of the needles. Moreover, the injected test compound might get trapped in the yolk or perivitelline space instead of entering the embryo [10]. Therefore, we decided to expose embryos via the incubation medium and, subsequently, determine if the compound was taken up. However, for hydrophilic compounds that are poorly or not taken up via immersion, the use of intra-yolk micro-injection techniques might increase the uptake. This was demonstrated by Guarin et al. (2021), who compared the absorption and distribution of less lipophilic (i.e., logD[5] range between -1.96 and 1.07) fluorescent compounds after immersion and micro-injection. The relative distribution data showed that, at ≤72 hpf[6], the compounds were poorly taken up by the embryos, while about 20-30% of compound entered non-yolk parts after intra-yolk micro-injection. They hypothesized that the fast consumption of the yolk influences the biodistribution of intra-yolk administered compounds. Also, the relative exposure data of the non-yolk body parts showed that the compound exposure was higher when using micro-injection than when using immersion. Therefore, they recommended the use of micro-injection for less lipophilic compounds [30]. As

[5] LogD is the distribution constant, and describes the lipophilicity of a compound. A negative logD means that a compound is less lipophilic (and more hydrophilic) [74].

[6] At later time points is was difficult to distinguish the yolk from the rest of the body.

such, it might be interesting to explore the use of intra-yolk micro-injection techniques in the ZEDTA for compounds that have logD values between -1.96 and 1.07 (e.g. LTC and ECA, see chapter 6). However, caution should be taken as another study showed that micro-injection and immersion can result in differences in ADME, resulting in differences in physiological responses [31]. As such, micro-injection should be used as a complementary exposure method, rather than as a substitute for immersion [31].

Test concentrations

As we want to predict the teratogenicity in humans and reduce the amount of laboratory animals used for developmental toxicity testing, the test concentrations we use in the ZEDTA should be based on the concentrations to which a mammalian fetus would be exposed. To allow the use of relevant concentrations in alternative methods, Daston et al. (2014) made a list of reference compounds in which maximal maternal plasma concentrations (i.e., C_{max}) and associated developmental toxicities in rats are reported [32]. For most of these reference compounds, teratogenic effects were observed in the µM range. As we use compounds that were not reported in this reference list, we decided to chose our concentrations based on what was used in zebrafish embryo assays before [4,33–35]. These nominal concentrations were in the µM range, or even higher as we wanted to detect the formed metabolites in chapter 6. However, when comparing the internal concentration we found in zebrafish embryos with the C_{max} reported in *in vivo* studies, we noted that the internal concentrations in zebrafish embryos were more than 10 times (i.e., for CBZ) to more than 1000 times (i.e., for LTC) lower (see Table 1). If teratogenic effects can already be observed at these lower concentrations (e.g. as with CBZ), the chosen test concentrations can be used. However, if no teratogenic effects are observed at these lower concentrations (e.g. as with LTC), and exposure to higher nominal concentrations does not increase the uptake, caution should be taken as the compound might be less suited to be evaluated in the ZEDTA due to, for example, uptake issues. As such, we recommend to chose test concentrations based on what is reported in literature, but to always confirm the chosen concentrations by comparing the internal concentrations with the C_{max} reported in *in vivo* studies.

Table 1. Comparison of C_{max} reported in *in vivo* studies and the maximal internal concentration found in zebrafish embryos. Abbreviations: carbamazepine (CBZ), levetiracetam (LTC), lowest observed adverse effect level (LOAEL).

Compound	C_{max} *in vivo* (mg/mL)	Effect	Max. internal conc. ZEDTA (mg/mL)	Effect
CBZ	0.065 (rat LOAEL) [36] 0.029 (rabbit LOAEL) [36]	↑ resorptions and kinked tails ↓ # fetuses ↑ resorptions	0.0021075 mg/ml (i.e., 8.92 µM)*	Several malformations (see chapter 6)
LTC	1.20 ± 0.35 (mice) [37]	Skeletal malformations and more	0.0010000 mg/ml (i.e., 5.8751 µM)**	None

*The nominal concentration was 500 µM
**The nominal concentration was 10,000 µM

Morphological endpoints

The list of morphological endpoints that is used by the Zebrafishlab at the UAntwerp is very extensive, therefore, we used the same endpoints in our ZEDTA protocol (see chapter 3). While assessing these endpoints in the experiments of chapter 4 and 6[7], however, we observed a great variability in timing of swim bladder inflation in control larvae. Therefore, we decided to no longer include swim bladder inflation as a morphological endpoint in our protocol. By our knowledge, no research about the variability in swim bladder inflation timing has been done yet. This information could be very useful to decide whether or not this endpoint should be included in future standardized zebrafish embryo assays. However, until further research is conducted, we recommend to not include this parameter as an endpoint. On the other hand, including larval length as one of the standard endpoints in future zebrafish embryo developmental toxicity assays should be further explored. In chapter 5, we found that exposure to certain compounds decreased the length of zebrafish larvae, which is a sign of growth retardation.

Morphological assessment

Morphological assessment is subjective and could lead to discordant results between labs and to misclassification of compounds [38]. Therefore, a few years ago, a software program, FishInspector, was developed to analyze the images that were taken by an automated zebrafish handling and imaging system [38]. Automatization cannot only minimize observer bias on the one hand, but can also increase throughput of the assay on the other hand as manual morphological assessment is quite tedious and time-consuming. However, as the number of endpoints is still limited, further programming is needed to make the system more comprehensive. Next, the use of this automated morphological assessment in our ZEDTA protocol can be explored.

To summarize, several sources of variability, including the exposure length, choice of embryo medium, incubation temperature, and many more, were taken into account when developing our modular ZEDTA protocol. In addition, during this PhD, some adjustments to the standardized protocol (of chapter 3) have already been made to allow further standardization. These adjustments include: using ≤1% DMSO as a solvent if a compound is not soluble in embryo medium, inclusion of pH measurement, exclusion of swim bladder inflation as an endpoint, and inclusion of larval length as an endpoint. However, exclusion of swim bladder as an endpoint is just a temporary recommendation based on our observations until further research about the variability in timing is known. Also, future research is needed to make a more informed decision about the method of test solution renewal, and whether or not using micro-injection for compounds with a logD between - 1.96 and 1.07 is improving compound uptake. Moreover, further programming of morphological endpoints into the FishInspector software should be done to allow inclusion of an automated morphological assessment. In addition to the ones discussed above, there are more sources of variability in zebrafish embryo assay protocols that potentially can mean a difference in outcome. These include breeding, feeding, disease monitoring, and many more [10]. As such, also these

[7] Chapter 5 is not mentioned here, as these experiments were performed by researchers of the university of Exeter.

parameters should be internally standardized to reduce variability in classification. As a consequence, in the future, our standardized protocol needs to be further adjusted based on new discoveries.

7.3 Increasing the sensitivity of the ZEDTA

Besides the presence of inter- and intra-laboratory discordances, other causes for false negative results in the ZEDTA can be: the lack of compound uptake by zebrafish embryos [1], inter-species differences in mode of action [9], the limited number of skeletal endpoints in zebrafish embryo assays [1,2,4,39], and the immature intrinsic CYP-mediated biotransformation capacity of zebrafish embryos and young larvae [17].

For the first one, the lack of compound uptake, we included an uptake analysis in our standardized ZEDTA protocol. If no or only a small amount of compound is detected, a false negative result due to compound uptake is likely. The limited number of skeletal endpoints and the immature biotransformation capacity will be discussed below.

7.3.1 A skeletal evaluation method for increasing the number of endpoints

Skeletal staining methods

In this thesis, we investigated whether the sensitivity of the ZEDTA could be increased by including a skeletal staining method. In a preliminary experiment using only one replicate, the AR live staining method was tested with a positive control, rosiglitazone (chapter 3). As these results looked promising, the use of several staining methods for skeletal evaluation was further investigated in chapter 6. However, the use of more than one replicate showed that there was a lot of variability between replicates. Additional experiments on control larvae showed that biological variability in (onset of) bone development is clearly present in zebrafish larvae and hampers the use of skeletal staining methods in zebrafish embryo developmental toxicity assays, including our standardized ZEDTA.

Transgenic zebrafish lines

An alternative strategy that can be explored for allowing skeletal evaluation of zebrafish larvae is the use of transgenic fluorescent zebrafish lines. In contrast to skeletal staining methods, the use of transgenic lines allows visualization of cartilages and bones in real time at any desired timepoint without the need for staining procedures [40]. In this way, the (abnormal) development of skeletal structures can easily be followed during all embryonic and larval stages. As skeletal development can be followed more precisely at different ages, it might be possible to get a better understanding of the extend of the biological variability and/or pick-up the effects that are treatment-related. Promising cartilage and bone transgenic lines for skeletal assessment include the *Tg(Hsa.RUNX2:EGFP)* bone line and the *Tg(col2a1a:EGFP)* cartilage line [40]. The *Tg(Hsa.RUNX2:EGFP)* line is a transgenic bone line that expresses EGFP in newly forming bones in the appendicular (i.e., the fins and limb gridles) and the craniofacial skeleton, and as such allows

investigating bone development. The *Tg(col2a1a:EGFP)* line visualizes craniofacial chondrocyte formation and distribution, and as such allows following cartilage development (reviewed by [40]). However, it is important to mention that biological variability in onset of bone development may also hamper the use of transgenic zebrafish lines for skeletal assessment.

Nevertheless, also without the use of skeletal visualization techniques, some treatment-related bone effects can be detected in zebrafish embryo assays. If a compound causes severe skeletal malformations, this can already be seen during the assessment of gross morphology (e.g. in chapter 5, exposure to 1,000 µM of compound 5 caused morphological abnormalities). However, subtle treatment-related bone effects that are not picked-up by gross morphology will be missed if no skeletal assessment can be included. As such, if further research shows that also the use of a transgenic line also does not allow skeletal assessment, the inability to pick up subtle treatment-related bone effects is a limitation that should be taken into account when using zebrafish embryo assays.

7.3.2 Methods to encompass the low biotransformation capacity

Metabolic activation systems

Most *in vitro* developmental and reproductive toxicology models, including the EST, WEC and FETAX, lack biotransformation capacity. Also the ZEDTA is known to have a limited CYP-mediated biotransformation capacity [19,41–48]. To circumvent the immature intrinsic CYP-mediated biotransformation capacity of young zebrafish, our research group, as well as other research groups, have been exploring the use of an exogenous metabolic activation system (see chapter 3) [15,19,47,49]. The principle is as follows: adding a MAS to the test medium will allow metabolic activation of drugs and chemicals. In this way, embryos/larvae that have an immature intrinsic biotransformation capacity can be exposed to the metabolites of a compound of interest [19,50]. The advantage of using a MAS instead of exposing zebrafish embryos to the commercially bought metabolites is that a MAS can also be used when the metabolites are still unknown (e.g. for new drugs and chemicals).

In our standardized ZEDTA assay, we described the use of a pre-incubation system in which the compounds of interest were first metabolically activated and then exposed to the zebrafish embryos. The use of a pre-incubation system, instead of a co-incubation system, was based on previous research [19]. Pype et al. (2018) and Mattsson et al. (2012) found that co-incubation of zebrafish embryos with a MAS was impossible as these MAS protocols are toxic for zebrafish embryos [19,51]. Even after optimizing the factors that contributed to the toxicity (i.e., used temperature, medium composition, and the presence of reactive oxygen species in the medium), the MAS remained embryotoxic [19]. As such, it was found that the microsomes itself were toxic for zebrafish embryos, and a pre-incubation system was proposed [19,51]. In chapter 3, the use of a 1:10 diluted ultracentrifuged pre-incubation system was further explored, and this resulted in a non-embryotoxic metabolic activation protocol that is generating the toxic metabolite(s). However, further research should be done in order to reveal whether the concentration of the formed active metabolite(s) is sufficiently high to cause malformations in zebrafish embryos and larvae. If the

concentrations of the formed metabolite(s) are too low to cause an effect, the samples need to be more concentrated or less diluted. Higher concentrations of the metabolite can be obtained by up concentration procedures [47], but these are more labor-intensive and costly. As the use of less diluted samples would cause embryotoxicity [19], additional steps for detoxification need to be performed. Possible strategies include:

- Adding cooled (4°C) **acetonitrile or methanol** prior to ultracentrifugation of the samples to stop CYP reactions (i.e., by denaturing the microsomal proteins [52]).
- An **ultrasonication** step [47], as this is known to interfere with the secondary structure of cytochromes and proteins in general [53].
- Using **bovine serum albumin** as it may have a potential benefit since it is well-recognized for its antioxidant capacity [54] and our group already showed that radicals are present in the medium [49].

Besides microsomes, there are also other metabolic activation systems, such as hepatocytes and the S9 fraction, that can, and have been used in alternative assays.

- Incubation strategies using **hepatocytes** have the advantage that phase I and phase II enzymes, as well as all cofactors are present. As such, all metabolic pathways of interest can be covered. However, the use of hepatocytes is labor-intensive and quite expensive [46]. Mouse, rat, rabbit, and even human hepatocytes have been used before in the EST and WEC [41–43]. Moreover, hepatocytes can also be used in organ-on-chips and multi-organ-chips (see further).
- The **S9 fraction** contains phase I and phase II enzymes, and have been used in the MM and WEC before [44–46]. The S9 fraction is present in the supernatant after differential centrifugation of liver tissue at 9,000x g. In contrast to hepatocytes is the use of the S9 fraction inexpensive and easy to use [46]. However, it requires the addition of co-factors as they are not present. Caution should be taken when adding co-factors, as earlier research by our group has shown that co-factors can be embryotoxic [49].
- Liver **microsomes** are subcellular fractions of the endoplasmic reticulum [46], that mainly contain uridine 5'-diphosphoglucuronosyltransferase (UGT), flavin-containing monooxygenase (FMO) and CYP enzymes, and lack cytosolic enzymes and co-factors. They are obtained by differential centrifugation at 100,000x g, and can be prepared from different species, from which rat and human microsomes are mostly used in alternative assays. The use of microsomes has the advantage of being inexpensive and allowing high-throughput automatization [46], and they have been used in the WEC, FETAX and zebrafish embryo assays before [19,42,47,48].

Consequently, we opted for a MAS using microsomes to circumvent the immature intrinsic CYP-mediated biotransformation capacity. In addition, as we want to mimic the human situation, and the use of microsomes of another species might render other metabolites, we opted to use human microsomes. If further research shows that also other enzymes (i.e., besides the CYPs) are immature (see next paragraph), it might be that another human metabolic activation system would be more

well-suited. For cytosolic enzymes, for example, the S9 fraction should be used instead of microsomes. Therefore, it is important to tailor the choice of a MAS to the enzyme of interest.

As mentioned in the introduction, knowledge about the activity of other non-CYP phase I enzymes involved in the biotransformation of xenobiotics in zebrafish embryos is rather limited or not available at all. This information is, however, crucial as a limited biotransformation capacity may result in false negative results. Therefore, we investigated the biotransformation capacity of zebrafish embryos/larvae for, presumably, MPOs and a special type of B-esterase enzymes and the teratogenic potential of the formed metabolites (see chapter 6). Although we cannot be sure that the involved enzymes were indeed B-esterases and MPOs until the experiments are repeated with knock-out/knock-down models, our results suggest that both enzymes are already active at 24 hpf. After all, at this age, metabolites were found that are formed in man using these enzymes. As such, similar to what was known about other B-esterases (see introduction) [55,56], the special type of B-esterase or its homologue is also already active in 24 hpf zebrafish embryos. The MPO homologue appeared to be active earlier than what was reported before (see introduction), i.e., at 24 hpf instead of at 33 hpf [57]. However, as mentioned before, it is important to note that this is all based on the assumption that the same pathways are followed as in the human situation.

Although the non-CYP phase I enzymes we investigated were already active at 24 hpf, other enzymes might not be active (or only become active later on), and consequently, could be responsible for false negative results in zebrafish embryo assays. For this, a MAS can be implemented. However, to do this, more research is needed to further elucidate the (non-CYP-mediated) biotransformation capacity of xenobiotics in zebrafish embryos and young larvae.

Combining multi-organ chips and zebrafish embryo assays

An organ-on-chip (OOC) is a three-dimensional microfluidic system that contains miniature tissues. They are designed to maintain tissue-specific functions and to control cell microenvironments and are therefore relevant for mimicking human physiology [58]. While single-organ chips enable investigating a specific organ of interest, multi-organ chips (MOCs) allow investigating the interaction of multiple organs. OOCs and especially MOCs can be used to study various types of metabolism, including drug biotransformation and drug toxicity [59]. The tissues/organs that are included in the chip for these purposes are the liver, as it is the most important drug-metabolizing organ, and one or more target and/or suspected off-target tissues/organs. As such, MOCs allow to monitor the generation of metabolites and the effect and/or accumulation in the tissues/organs of interest [59]. To this end, MOCs can be useful to screen for drug-related toxicity (see Figure 1) [60,61]. However, there might be opportunities to combine OOCs/MOCs with zebrafish embryo assays. This would work as follows: the OOC/MOC contains a miniature liver that generates the metabolites of interest. These metabolites are then transferred to the zebrafish embryo, and as such a combination of the parent compound and the generated human metabolites of interest are exposed to the zebrafish embryos. In case of a MOC, it might be that, at the same time, also toxicity on another in the chip included organ/tissue of interest can be assessed. However, caution should be taken as previous research has showed that incubation media and co-factors can cause

embryotoxicity [49]. As such, the fluid of the OOC/MOC should be tested and optimized for exposure to zebrafish embryos.

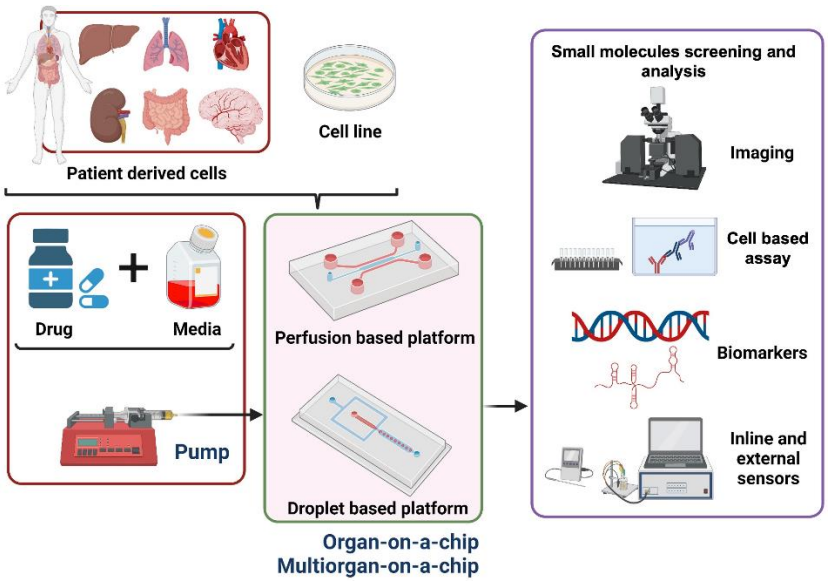

Figure 1. The use of OOCs and MOCs for small molecule screening [61].

In summary, to circumvent the limited CYP-mediated biotransformation capacity of zebrafish embryos in our standardized ZEDTA, we included a MAS with human microsomes. We opted to use microsomes as they contain the enzymes of interest and are the most concentrated (i.e., fewer other enzymes are present than in, for example, the S9 fraction). However, if future research shows that also other enzymes are immature, the choice of MAS should be tailored on the enzyme of interest (e.g. S9 for cytosolic enzymes). Although not much research has been done yet about the combination of OOCs/MOCs and zebrafish embryo assays, this approach might be promising as well. We hypothesize that, with the right combination of tissues/organs, MOCs might even enable exposure of zebrafish embryos to metabolites generated by non-hepatic enzymes. Nevertheless, as mainly CYP-mediated metabolism is responsible for proteratogenicity, the use of a MAS with microsomes will already be very valuable in preventing false negatives due to limited biotransformation activity of zebrafish embryos. Therefore, as a next step, it should be investigated whether the concentration of the formed toxic metabolite(s) in the diluted pre-incubation protocol is sufficiently high to cause malformations in zebrafish embryos and larvae.

7.4 From screening to regulatory use

7.4.1 The ZEDTA versus other alternatives

Several alternatives for developmental toxicity testing in mammals have been developed and have been, and still are, used for screening purposes in early development of drugs and chemicals [36].

The use of zebrafish as an alternative model has, as mentioned earlier in the introduction, many advantages [50,62]. The most important advantage, when compared to the three validated alternative assays (see introduction), is that the ZEDTA allows compound exposure in a whole vertebrate organism during the organogenesis period [33]. Moreover, the accuracy of the ZEDTA is estimated to be around 80-85% for pharmaceuticals [63,64], which is similar or higher than the accuracy of the EST (78%), WEC (80%) and MM (70%) [65]. Only very recently, another alternative, the ReproTracker, was developed. This is a human stem cell-based biomarker *in vitro* assay that can be used for early-phase developmental toxicity screening of drugs and chemicals [66,67]. Although it is not validated (yet), the assay appears to be very promising as it uses human cells, and not animal cells/tissue, and the accuracy is estimated to be 85% (i.e., specificity 84% and sensitivity 85%) [67]. Nevertheless, also here the complexity of a whole organism is lacking as the assay is stem cell-based [68]. The same applies to the OOCs and MOCs. As such, the ZEDTA remains the only alternative that provides increased physiological relevance due to the assessment of a whole vertebrate animal during the organogenesis period with [10,33], unlike the FETAX [48], a (relatively) high accuracy [63,64].

7.4.2 Using the ZEDTA in a regulatory context

Up until now, the use of alternative assays in developmental and reproductive toxicology testing of pharmaceuticals in a regulatory context is very limited. However, it is stated in the approved third revision of the ICH S5 guideline on detection of toxicity to reproduction for human pharmaceuticals that alternative assays have the potential to defer or replace *in vivo* studies in mammals in certain circumstances [36]. The guideline does not list any specific alternatives, but it mentions that a use of multiple alternatives together in a tiered or battery approach is expected. To use alternatives in a regulatory context, they should be properly qualified (i.e., internally validated) and should at least be as safe for humans as the conventional *in vivo* mammalian studies [36]. This qualification includes, among others, mentioning potential limitations of the assay(s) and its/their differences with man and the *in vivo* mammalian studies.

At this moment, several stakeholders from the pharmaceutical, chemical and cosmetic industries are already using (i.e., Bristol-Myers Squibb, Pfizer, AstraZeneca) or exploring the use of (i.e., Johnson and Johnson) the ZEDTA for screening purposes [1–3,7]. However, to further convince them to include the ZEDTA (whether or not combined with other NAMs) in their regulatory submissions for developmental toxicity testing, the ZEDTA (and potential other NAMs) should be qualified with several reference compounds. In the ICH S5 guideline, a list of reference compounds that can be used for qualifying NAMs is included [36]. This list consists of 29 compounds that proved to be teratogenic in mammals, and three non-teratogens, and they represent a variety of mode of actions and physicochemical properties (see Table 2).

Very recently, Weiner et al. (2023) have validated their zebrafish embryo assay with these 32 compounds. They correctly classified 3/3 non-teratogenic compounds and 23/29 teratogenic compounds (i.e., a sensitivity of 79.31%). Hence, 6 compounds proved to be false negative: aspirin, cytarabine, phenytoin, pomalidomide, thalidomide, and vismodegib [69]. Based on their results,

aspirin proved to be toxic instead of teratogenic. However, this toxic effect could be due to the fact that the pH of the test solution was lower than 7 [69]. In our assay, the outcome of aspirin might be different as the pH of the test compounds is adjusted to fall within the physiological range, and because MAS is included. After all, we suspect that aspirin needs biotransformation to exert its teratogenic potential, since it is known to have an active mammalian metabolite, salicylic salt [70]. This assumption is strengthened by the fact that Teixido et al. (2013) reported that this metabolite is indeed teratogenic in zebrafish larvae [7]. Vismodegib was also not classified as a teratogen by Weiner et al. (2023). Although >98% of vismodegib is not metabolized in men, biotransformation by mainly CYP2C9 and CYP3A4 results in several human metabolites (i.e., M1 metabolite, M4 metabolite, M3 metabolite, M5 metabolite and M14 metabolite) from which it is not known whether they are active [71]. In contrast to Weiner et al. (2023), other studies correctly classified phenytoin as a teratogen [33,72]. Song et al. (2021) reported tachycardia, which was not considered as an endpoint by Weiner et al. (2023) and explains the difference in classification [69,72]. Bars et al. (2021), however, observed a deviating pigmentation from 72 hpf onwards, and curvature of the tail in 5 dpf larvae, both at concentrations within the 1-5,000 µM range that was tested by Weiner et al. (2023) [33,69]. Moreover, Bars et al. (2021) found that the active human metabolite of phenytoin, 5-(4-hydroxyphenyl)-5-phenylhydantoin, is not teratogenic in zebrafish larvae [33]. As such, it might be that phenytoin is a teratogen, but that it was missed by Weiner et al. (2023). Cytarabine and thalidomide were found to have a low uptake, and as such, this can be the reason for the missed teratogenic effect [69]. However, it has been suggested that for thalidomide, and pomalidomide as well, species-specific differences in metabolic pathways could also be a potential reason for false negative results [73]. An example of this is the lack of SALL4, a zinc finger transcription factor, in zebrafish, which is present in several other species, including man and rabbit [73].

Table 2. ICH S5 reference compounds list for qualifying NAMs.

Acitretin[a]	Fluconazole[a]	Pomalidomide[a]
Aspirin[a]	5-Fluorouracil[a]	Ribavirin[c]
Bosentan[c]	Hydroxyurea[a]	Tacrolimus[c]
Busulfan[a]	Ibrutinib[c]	Thalidomide[a]
Carbamazepine[a]	Ibuprofen[a]	Topiramate[a]
Cisplatin[c]	Imatinib[c]	Tretinoin (all-trans-retinoic acid)[a]
Cyclophosphamide[a]	Isotretinoin (13-cis-retinoic acid)[a]	Trimethadione[a]
Cytarabine[a]	Methotrexate[a]	Valproic acid[a]
Dabrafenib[c]	Pazopanib[c]	Vismodegib[b]
Dasatinib[c]	Phenytoin (Diphenylhydantoin)[a]	Saxagliptin[d]
Cetirizine[d]	Vildagliptin[d]	

[a]human teratogen, [b]presumed human teratogen, [c]rat and/or rabbit teratogen, [d]non-teratogen

In summary, Weiner et al. (2023) internally validated their zebrafish developmental defects assay with the ICH S5 reference list and obtained an accuracy of ~81-90% [69]. In our ZEDTA, pH adjustment and the use of a MAS are included, and therefore we assume that, once our ZEDTA is further standardized and optimized, it might be very promising for teratogenic classification of pharmaceuticals.

As such, when our ZEDTA is further standardized and optimized (see 7.2 and 7.3), it should be internally validated using the ICH S5 reference compounds. This will allow us to detect and mechanistically explain its potential differences in outcome with man/*in vivo* studies. One of the strategies that can be used for qualification of the ZEDTA using the 32 ICH S5 reference compounds is the following (see also Figure 2):

1. Depending on the compound of interest, first metabolically activate the compound of interest using the **mZEDTA** protocol.
2. Evaluation of all ICH S5 compounds using the standardized **ZEDTA** protocol.
3. **Uptake** assessment of compounds that are false negative in the ZEDTA.
4. Check literature whether there are species differences (zebrafish vs human/rat/rabbit) in **metabolic pathways** for compounds that show uptake but are negative in ZEDTA.
5. If commercially available, expose zebrafish embryos to the **active human metabolite** of compounds that have metabolic species differences.

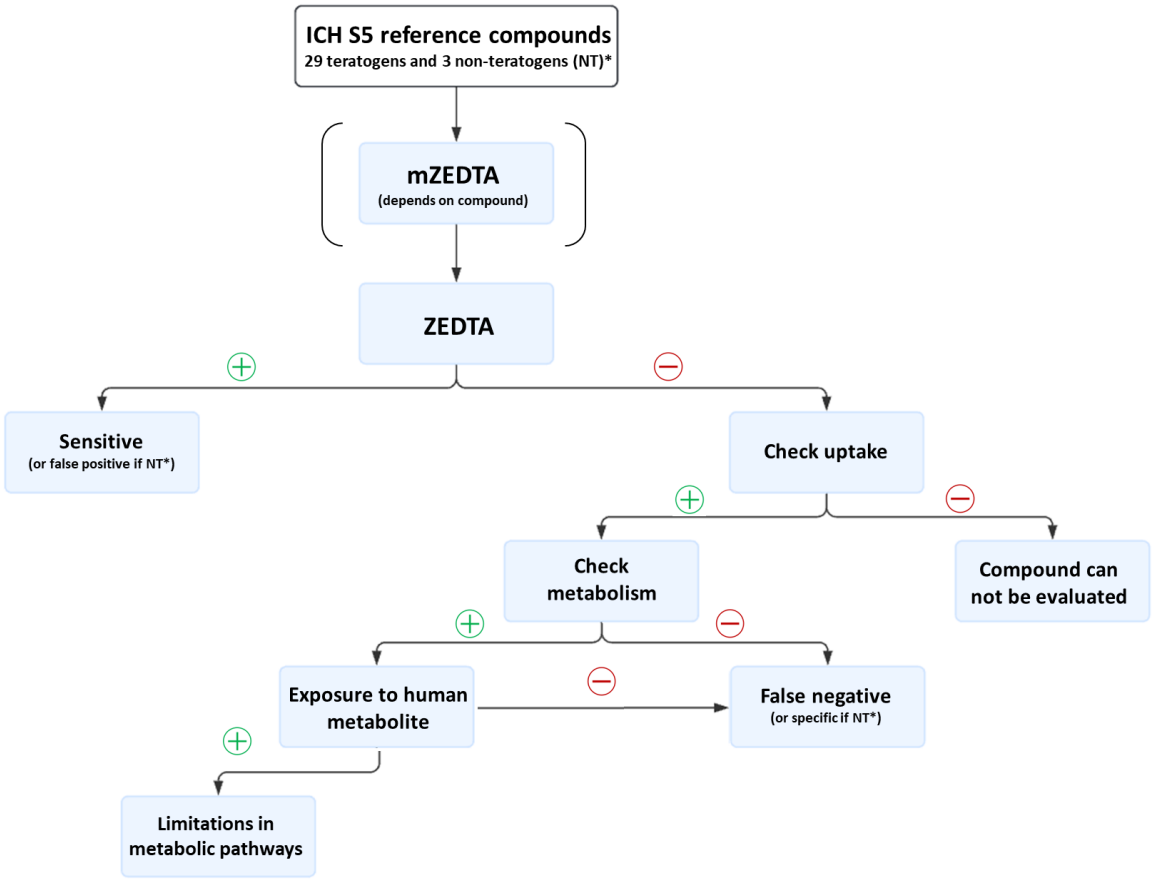

Figure 2. A flowchart for qualifying the ZEDTA using the 32 ICH S5 reference compounds.

In my opinion, in the long-term, the ZEDTA may have the potential to be used together with other alternatives for regulatory purposes. At this moment, the ZEDTA seems promising to test compounds that are properly taken up by the embryos and larvae and are soluble in water or a

combination of ≤1% DMSO and water. For compounds that are not taken up or have a poor solubility, zebrafish embryo assays are less suited. However, the use of both intra-yolk micro-injection techniques and immersion might be considered for compounds with a logD between -1.96 and 1.07, as Guarin et al. (2021) found that, for these compounds, exposure is better than by using immersion [30]. Also, for compounds that are suspected to cause skeletal malformations or for compounds for which species-specific differences are suspected, zebrafish embryo assays are less suited. On the other hand, for compounds that might need bioactivation to exert their teratogenic potential, the use of the optimized ZEDTA might be a good choice as most alternative models lack biotransformation capacity. However, for these compounds the use of a MOC might be promising too. Nevertheless, I believe that for compounds that, based on their physicochemical properties, have the potential to be tested in the ZEDTA, the ZEDTA should be the number one choice as it is the only alternative that provides increased physiological relevance due to the assessment of a whole vertebrate animal during the main organogenesis period. However, there is still a long way to go before it can be considered for regulatory use. Therefore, I think the first point of focus should be on improving the assay for its use as a screening tool. In this way, it can already decrease the number of animals that are used for developmental toxicity testing. For this, efforts should be made to further standardize and optimize the ZEDTA in order to reduce false positive and false negative results as much as possible (see 7.2 and 7.3). In summary, the following strategies can be explored:

- Determine which method of test solution renewal is the best
- Investigate whether or not to also use micro-injection for compounds with a logD between - 1.96 and 1.07
- Further programming of morphological endpoints of interest into the FishInspector software and use this software for an automated morphological assessment
- Investigate whether swim bladder inflation should be included as a morphological endpoint
- Determine whether transgenic bone and cartilage zebrafish lines can be used to allow a skeletal evaluation of zebrafish larvae
- Check whether the concentration of the active metabolite(s) that are formed by using our pre-incubation MAS is sufficiently high to cause malformations in zebrafish embryos and larvae. If not, the use of concentrated or less diluted (with additional steps for detoxification) samples should be explored
- Investigate the possibilities of combining OOCs/MOCs with zebrafish embryo assays

Subsequently, if the ZEDTA is further standardized and optimized, the ‘final’ ZEDTA can then be qualified in a next step and considered for regulatory submission.

7.5 General conclusion

To conclude, a standardized ZEDTA protocol that can be extended with a metabolic activation system was developed. Although it initially seemed that the sensitivity of the ZEDTA could be increased by including skeletal assessment, we found that biological variability in bone development hampers the use of a skeletal staining method. In addition, we also obtained more information about the non-CYP-mediated biotransformation capacity of zebrafish embryos and young larvae and the teratogenic potential of the formed metabolites. As such, this doctoral thesis was a first step in further standardizing and optimizing the ZEDTA to increase its sensitivity. However, in the future, efforts should be made to further optimize the assay in order to reduce false positive and false negative results.

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Summary

Within Europe, new approach methodologies for toxicity assessment of xenobiotics become very important. Several pharmaceutical, (agro)chemical and cosmetic companies are currently using zebrafish embryo assays as an alternative for animal testing to screen new compounds for developmental toxicity. The use of a zebrafish embryo assay is considered to be very promising, as it is the only alternative assay that allows assessment of a whole vertebrate animal during the main organogenesis period with a relatively high accuracy. However, it still suffers from some limitations. Inter- and intra-laboratory discordances in teratogenicity classification of identical compounds, as well as false negative and false positive results are reported for known mammalian teratogens and non-teratogens, respectively. For safety reasons, false negative results are more critical than false positive results, as teratogens may be missed. Causes for these false negative results include: inter-species differences in mode of action, issues with compound uptake, the limited biotransformation capacity and the limited number of morphological endpoints in zebrafish embryo assays. Therefore, the aim of this doctoral thesis was to further standardize and optimize the Zebrafish Embryo Developmental Toxicity Assay (ZEDTA) in order to increase the sensitivity of this screening assay, and as such better predict birth defects caused by drugs during the 1st trimester of pregnancy.

To increase the number of morphological endpoints in zebrafish embryo assays, and consequently also increase its sensitivity, we investigated whether a skeletal assessment could be included. Unfortunately, we found that biological variability in bone development hampers the use of skeletal staining.

To reduce the amount of discordances in teratogenicity classification due to methodological differences, we developed a standardized and optimized ZEDTA protocol. Moreover, this protocol can be used as a modular system depending on the compound of interest, by adding a pre-incubation metabolic activation system using human liver microsomes (mZEDTA). As such, our standardized and optimized ZEDTA includes a method to encompass the problem of a limited cytochrome P450 (CYP)-mediated biotransformation capacity in zebrafish embryos. However, it should be investigated whether the concentration of the formed toxic metabolite(s) in the diluted protocol is sufficiently high to cause malformations in zebrafish embryos and larvae.

In addition, we also investigated the non-CYP-mediated biotransformation capacity and the teratogenic potential of the formed metabolites. The non-CYP enzymes we investigated were active from 24 hpf onwards. The three metabolites of carbamazepine that were formed via non-CYP enzymes, were more potent than the parent compound, with acridine being the most potent, followed by 9-acridine carboxaldehyde and acridone.

As such, this doctoral thesis was a first step in further standardizing and optimizing the ZEDTA in order to increase its sensitivity for screening of teratogenic drugs. In the future, efforts should be made to further optimize the assay to reduce false positive and false negative results even more.

Samenvatting

Binnen Europa worden alternatieve methoden voor de toxiciteitsbeoordeling van xenobiotica steeds belangrijker. Verschillende farmaceutische, (agro)chemische en cosmetische bedrijven gebruiken het zebravisembryo reeds als alternatief voor dierproeven om nieuwe compounds te screenen op teratogeniciteit. Het gebruik van een zebravisembryotest is veelbelovend, omdat het de enige alternatieve test is die het mogelijk maakt om een geheel gewerveld dier tijdens de organogenese met een relatief hoge nauwkeurigheid te beoordelen. Toch kampt de test ook met enkele beperkingen. Zo zijn er inter- en intralaboratoriumverschillen gerapporteerd in de teratogeniteitsclassificatie van identieke compounds, en worden er zowel vals-negatieve als vals-positieve resultaten gerapporteerd voor, respectievelijk, reeds gekende teratogene en niet-teratogene stoffen in zoogdieren. Vanuit veiligheidsoogpunt zijn de vals-negatieve resultaten belangrijker dan de vals-positieve, omdat teratogene stoffen gemist kunnen worden. Oorzaken voor deze vals-negatieve resultaten zijn onder andere: species-verschillen, problemen met de opname van compounds, de beperkte biotransformatiecapaciteit en het beperkte aantal morfologische eindpunten in zebravisembryotesten. Daarom was het doel van dit proefschrift om de zebravisembryo ontwikkelingstoxiciteitstest (ZEDTA) verder te standaardiseren en optimaliseren om de gevoeligheid van de screeningstest te vergroten en zo geboorteafwijkingen veroorzaakt door geneesmiddelen beter te voorspellen.

Om het aantal morfologische eindpunten in de zebravisembryotest te vergroten, en daarmee ook zijn gevoeligheid te vergroten, hebben we onderzocht of er een skeletale beoordelingsmethode aan toegevoegd kan worden. Helaas hebben we ontdekt dat het gebruik van een skeletale kleuring niet mogelijk is door biologische variabiliteit in botontwikkeling bij zebravislarven.

Om het aantal discordanties in de teratogeniciteitsclassificatie als gevolg van methodologische verschillen te verminderen, hebben we een gestandaardiseerd en geoptimaliseerd ZEDTA-protocol ontwikkeld. Dit protocol kan bovendien ook als een modulair systeem gebruikt worden door er een pre-incubatie metabolisch activeringssysteem met humane levermicrosomen (mZEDTA) aan toe te voegen. Bij gevolg omvat onze gestandaardiseerde en geoptimaliseerde ZEDTA dus ook een methode om het probleem van een beperkte cytochroom P450 (CYP)-gemedieerde biotransformatiecapaciteit in zebravisembryo's te omzeilen. In de toekomst moet er echter nog wel onderzocht worden of de concentratie van de gevormde toxische metaboliet(en) in het verdunde protocol voldoende hoog is om malformaties bij zebravisembryo's en -larven te kunnen veroorzaken.

Daarnaast hebben we onderzoek gedaan naar het niet-CYP-gemedieerde biotransformatie-vermogen en de potentieel teratogene eigenschappen van de gevormde metabolieten. De door ons onderzochte niet-CYP-enzymen waren actief vanaf 24 uren na de fertilisatie. De drie metabolieten van carbamazepine die via niet-CYP-enzymen werden gevormd, waren meer potent dan de parent,

waarbij acridine de meest potente was, gevolgd door 9-acridinecarboxaldehyde en vervolgens acridone.

Dit proefschrift was een eerste stap in het verder standaardiseren en optimaliseren van de ZEDTA om de gevoeligheid van de screeningstest te vergroten en zo geboorteafwijkingen veroorzaakt door geneesmiddelen beter te voorspellen. In de toekomst moet er verder onderzoek worden gedaan om de test nog verder te optimaliseren om zo vals-positieve en vals-negatieve resultaten te verminderen.

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Academic Curriculum Vitae

Personalia

Name: Jente Hoyberghs
Date of birth: 4 January 1996
Place of birth: Turnhout
Nationality: Belgian

Scientific work experience

Associate Scientist Discovery Pharmaceutics (ad interim) **April 2024 – present**
Johnson & Johnson (via Randstad)

PhD researcher **November 2019 – February 2024**
Laboratory of Comparative Perinatal Development, University of Antwerp
PhD thesis: 'Advancing the Zebrafish Embryo Developmental Toxicity Assay (ZEDTA) towards a sensitive screening assay'

Student job **August 2018**
Laboratory of Comparative Perinatal Development, University of Antwerp

Education

Master in Molecular and Cellular Biomedical Sciences, minor in Research **2017 – 2019**
Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp
Master thesis: 'Transmission dynamics of extended-spectrum beta-lactamase-producing Enterobacteriaceae in clinical settings' (Medical Microbiology, University of Antwerp)
Graduated with great distinction

Bachelor in Biomedical Sciences **2014 – 2017**
Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp
Bachelor thesis: 'Optimization of a cartilage and bone staining in zebrafish larvae for developmental toxicity testing' (Laboratory of Comparative Perinatal Development, University of Antwerp)
Graduated with great distinction

Honours College **2015 – 2017**
Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp

Secondary school **2008 – 2014**
Koninklijk Atheneum Malle
Sciences and mathematics

Teaching

- Seminar 'The zebrafish as an animal model for embryological development'
  Bachelor of Veterinary Sciences

- Practical 'Laboratory Animal Sciences: zebrafish'
  Master of Biomedical Sciences

Student supervision

- Supervisor master thesis Axelle Coppens (Biomedical Sciences, University of Antwerp)
  Title: Bioactivation of carbamazepine and levetiracetam by other phase I enzymes than CYPs in zebrafish embryos
  Academic year: 2021 – 2022

- Supervisor bachelor thesis Houda Boulayoune (Farmaceutische en Biologische Laboratoriumtechnologie, AP)
  Title: Bioactivatie van carbamazepine en levetiracetam door fase I enzymen verschillend van CYP-enzymen
  Academic year: 2021 – 2022

- Supervisor GIP Zoë Hagenaers (Biotechnische Wetenschappen, VITO Hoogstraten)
  Title: Het testen van geneesmiddelen op zebravisembryo's
  Academic year: 2020 – 2021

- Supervisor bachelor thesis Benjamin Pellis (Biomedical Sciences, University of Antwerp)
  Title: Safe drugs during pregnancy: what can we learn from alternative models?
  Academic year: 2019 – 2020

Scientific trainings, courses & certificates

Certificates

- FELASA C certificate (University of Antwerp, November 2018)
- Linguapolis' Summer Course 'Academic English: Reading, Writing and Speaking' (University of Antwerp, September 2017)
- Honours College (University of Antwerp, 2015-2017)

Trainings & courses

- Designing research projects according to the 3Rs
- Excel: intermediate tips and tricks & database management and pivot tables
- Word: long documents
- Powerpoint
- Creative problem solving
- Communication skills
- Connecting communication
- Communicating science to nonscientists
- Under pressure
- Personal effectiveness
- Creating a scientific poster
- Speed reading

Presentations at conferences

BelTox meeting **2022**
Antwerp, Belgium
Poster presentation: DMSO concentrations up to 1% are safe to be used in the Zebrafish Embryo Developmental Toxicity Assay (ZEDTA)

European Teratology Society Conference **2022**
Antwerp, Belgium
Oral & poster presentation: Assessing developmental toxicity and non-CYP mediated biotransformation of two anti-epileptics and their human metabolites in zebrafish embryos and larvae

BelTox meeting **2019**
Brussels, Belgium
Poster presentation: Refinement of the zebrafish embryo assay for developmental toxicity testing

Student Research Conference **2017**
Middelburg, The Netherlands
Oral presentation: Optimalisatie van een kraakbeen- en botkleuring in de zebravislarve

Publications

Hoyberghs J, Bars C, Ayuso M, Van Ginneken C, Foubert K, Van Cruchten S. DMSO Concentrations up to 1% are Safe to be Used in the Zebrafish Embryo Developmental Toxicity Assay. Front Toxicol. 2021 Dec 21;3:804033. doi: 10.3389/ftox.2021.804033. PMID: 35295145; PMCID: PMC8915880.

Bars C, **Hoyberghs J**, Valenzuela A, Buyssens L, Ayuso M, Van Ginneken C, Labro AJ, Foubert K, Van Cruchten SJ. Developmental Toxicity and Biotransformation of Two Anti-Epileptics in Zebrafish Embryos and Early Larvae. Int J Mol Sci. 2021 Nov 24;22(23):12696. doi: 10.3390/ijms222312696. PMID: 34884510; PMCID: PMC8657848.

Hoyberghs J, Bars C, Pype C, Foubert K, Ayuso Hernando M, Van Ginneken C, Ball J, Van Cruchten S. Refinement of the zebrafish embryo developmental toxicity assay. MethodsX. 2020 Oct 7;7:101087. doi: 10.1016/j.mex.2020.101087. PMID: 33134094; PMCID: PMC7588703.

In progress (as first author)

- Assessing developmental toxicity and non-CYP mediated biotransformation of two anti-epileptics and their human metabolites in zebrafish embryos and larvae

- Biological variability hampers the use of skeletal staining methods in zebrafish embryo developmental toxicity assays