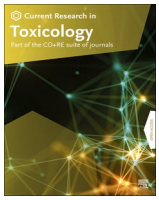
Current Research in Toxicology 7 (2024) 100186

Contents lists available at ScienceDirect

Current Research in Toxicology

journal homepage: www.journals.elsevier.com/current-research-in-toxicology



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

Jente Hoyberghs [a], Axelle Coppens [a], Chloé Bars [a], Chris Van Ginneken [a], Kenn Foubert [b], Steven Van Cruchten [a,*]

[a] Comparative Perinatal Development, Department of Veterinary Sciences, University of Antwerp, Wilrijk, Belgium
[b] Natural Products & Food Research and Analysis-Pharmaceutical Technology (NatuRAPT), Department of Pharmaceutical Sciences, University of Antwerp, Wilrijk, Belgium

ARTICLE INFO

Keywords:
NAM
In vitro
Developmental toxicity
Carbamazepine
Levetiracetam
Zebrafish larvae
Biotransformation
LC-MS

ABSTRACT

Zebrafish embryo-based assays are a promising alternative for animal testing to screen new compounds for developmental toxicity. 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. In the first step, zebrafish embryos were exposed to LTC and CBZ and their non-CYP mediated human metabolites, etiracetam carboxylic acid (ECA) and 9-acridine carboxaldehyde (9ACA), acridine (AI), and acridone (AO), respectively, from 5.25 to 120 hpf and morphologically evaluated. Next, the uptake of all compounds and the formation of the metabolites were assessed using LC-MS methods. 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.

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 (Ball et al., 2014; Braunbeck et al., 2014; Zhao et al., 2015; Augustine-Rauch et al., 2010). Zebrafish embryo-based assays, such as the ZEDTA (Hoyberghs et al., 2020), 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 limb bud micromass test (MM), the rat whole embryo culture (WEC) and the mouse embryonic stem cell test (EST). After all, the EST and MM do not allow assessment in a whole organism, while the WEC only allows assessment during a short period of the organogenesis period (i.e., only for 24–48 h) (Lee et al., 2012; Dimopoulou et al., 2018; Spielmann et al., 2004; Fantel, 1982). Moreover, using zebrafish embryo assays as a

* Corresponding author.
E-mail address: steven.vancruchten@uantwerpen.be (S. Van Cruchten).

https://doi.org/10.1016/j.crtox.2024.100186
Received 6 February 2024; Received in revised form 28 May 2024; Accepted 9 July 2024
Available online 11 July 2024

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 (Kari et al., 2007). 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 h post-fertilization (hpf) until 120 hpf (Westerfield, 2007). In addition, zebrafish embryos have a transparent chorion and embryonic tissue during early development which facilitates detailed observation of morphological changes (Westerfield, 2007; Kimmel et al., 1995). Despite the advantages, the assay suffers from some drawbacks, and further standardization and optimization are needed (Hoyberghs et al., 2020). 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 (Guengerich, 2008). It is already known that CYP-mediated biotransformation is immature during mammalian and human embryofetal development (Verbueken et al., 2017). 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 (Verbueken et al., 2018; Bars et al., 2021; Saad et al., 2016), 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 (Field et al., 2003). 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 (Hoyberghs et al., 2020; Pype, 2018; Giusti et al., 2019; Mattsson et al., 2012; Busquet et al., 2008). 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 (Bars et al., 2021; Furst and Uetrecht, 1995; Donner et al., 2013; Zhu et al., 2015; Han et al., 2019; Isoherranen et al., 2003).

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 (Bars et al., 2021). Interestingly, zebrafish larvae showed malformations after CBZ exposure from 5.25 to 120 hpf in a dose-dependent manner (Bars et al., 2021). 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 (AI), that could be responsible for the developmental toxicity in this species (Bars et al., 2021). This latter suggestion looks pretty plausible as different research groups have already shown that some of CBZ its metabolites, AI, acridone (AO) and 9-acridine carboxaldehyde (9ACA), are more toxic than CBZ itself (Furst and Uetrecht, 1995; Donner et al., 2013; Zhu et al., 2015). 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 (Zhu et al., 2015). It was found that AI had a 96 h half maximal lethal and sublethal effective concentration (EC50) value of 1.0 mg/L, which was much lower than the EC50 value of CBZ, being 60.8 mg/L. The sublethal toxic effects of AI included: edema, malformed spine, rare or no eye and skin pigments, weak heartbeat and weak or no blood circulation. In another study, both AI or 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 (Donner et al., 2013). Moreover, incubation of human lymphocytes with 9ACA, a reactive metabolite of CBZ, resulted in 40 % cell death, while no effect of viability was seen after incubation with CBZ (Furst and Uetrecht, 1995). Interestingly, all three previously mentioned metabolites of CBZ (i.e., 9ACA, AI and AO) 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 Fig. 1) (Furst and Uetrecht, 1995; Furst and Uetrecht, 1993). 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 (Halbach et al., 2020). In addition, the expression and activity of MPO enzymes have already been reported in zebrafish embryos at 18 hpf and 33 hpf, respectively (Lieschke et al., 2001). 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 (Isoherranen et al., 2003; Elgndy et al., 2019), growth retardation (Isoherranen et al., 2003; Elgndy et al., 2019; El Ghareeb et al., 2015), internal organ abnormalities (Elgndy et al., 2019; El Ghareeb et al., 2015) and fetal mortality (Isoherranen et al., 2003) were reported in mammalian studies, no teratogenic effects were found when exposing zebrafish embryos to LTC (Martinez et al., 2018; Lee et al., 2013). 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 (FDA, KEPPRA® (levetiracetam), n.d.) was found to cause growth retardation and several skeletal abnormalities in mice (Isoherranen et al., 2003). 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 (Isoherranen et al., 2003; Patsalos, 2004). 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.

2. Materials and methods

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

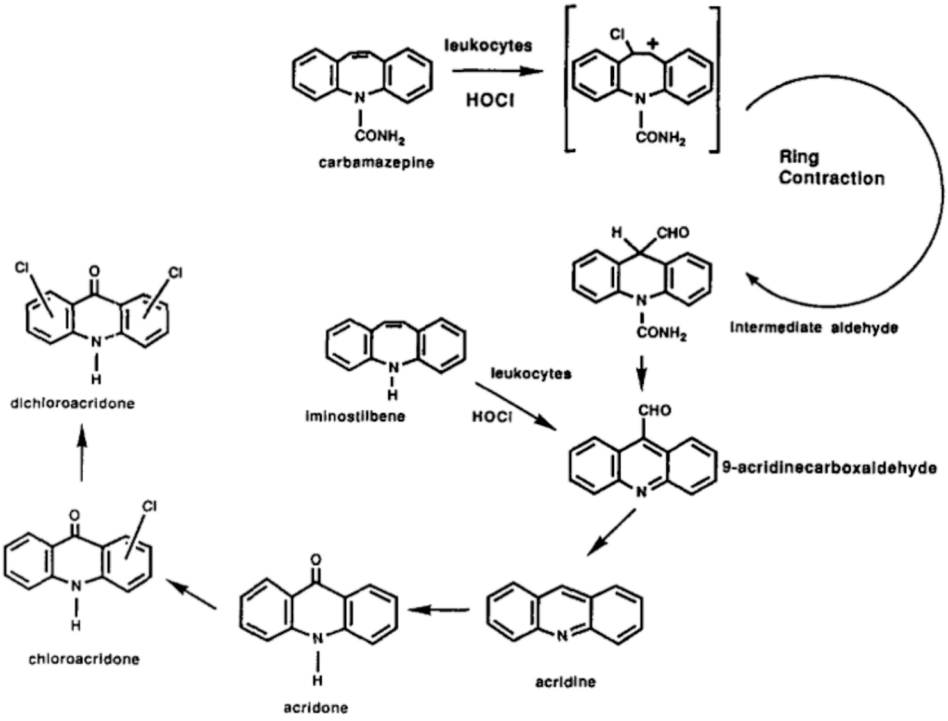


Fig. 1. Proposed pathway of carbamazepine in humans by myeloperoxidases in activated leukocytes (Furst and Uetrecht, 1993).

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; see Supplementary Table 1) in embryo medium. These concentrations were based on concentrations previously used in zebrafish research (Martinez et al., 2018; Lee et al., 2013).
- 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; see Supplementary Table 1) 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; see Supplementary Table 1), 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 (Hoybergh et al., 2021). 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 (Bars et al., 2021; Weigt et al., 2011). 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 (see Supplementary Table 1) (Hoybergh et al., 2021). 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 (Zhu et al., 2015). 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.

2.2. Adult zebrafish housing and egg collection

Experiments were conducted according to our standardized ZEDTA protocol (Hoybergh et al., 2020). 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 h 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. 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. From 3 hpf until exposure (i.e. at the latest at 5.25 hpf), 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 h light/dark cycle, attached on the inside. 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 (Bars et al., 2021). Coagulated eggs and malformed embryos were euthanized with buffered 1 g/L tricaine methane sulfonate (MS-222; pH 7.4).

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 h light/dark cycle). To avoid acidification and oxygen deprivation, the embryo medium or test solution was renewed every 48 h (Pype et al., 2015).

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 (Hoybergh et al., 2020) 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) (Hoybergh et al., 2020). 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 2.5).

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 (Bars et al., 2021) at the zebrafish embryos/larvae exposed to 500 μM CBZ and 10,000 μM LTC (for exposure method see 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 min, 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 min to remove zebrafish tissue from the supernatant. This supernatant was kept at −80 °C until further analysis using UPLC-MS/MS.

2.6. Analytical investigation of the extracted samples

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 Fig. 2) (Mathieu et al., 2011). By adding these, it will be possible to check whether these metabolites also can be found in the extractions of the CBZ samples.

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) (Bars et al., 2021). 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

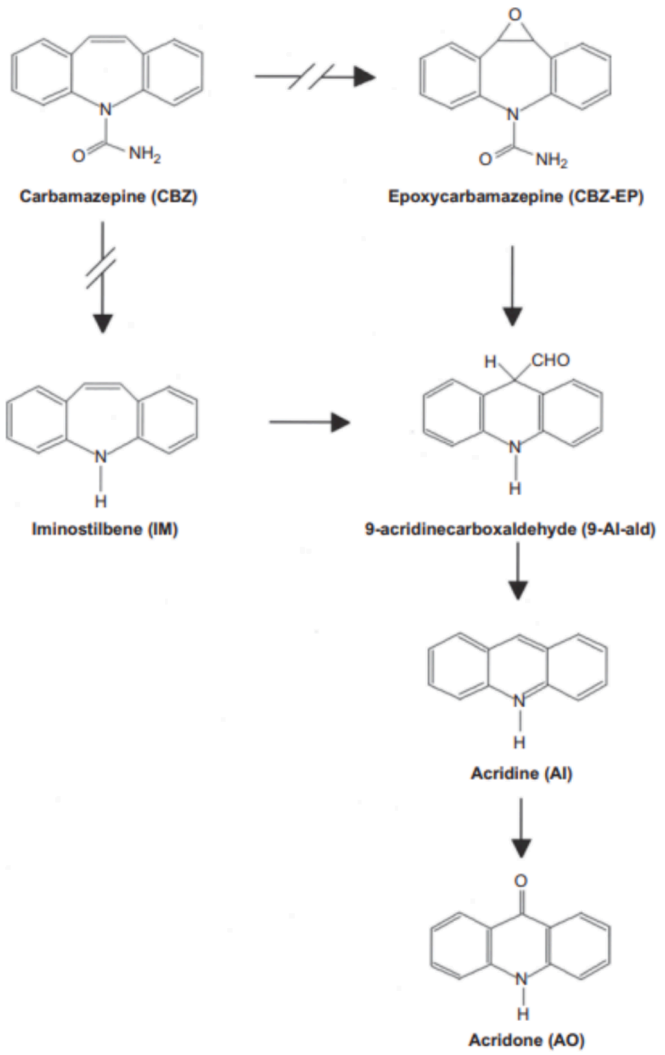


Fig. 2. Proposed pathway of carbamazepine in humans. Metabolism in the liver is indicated with broken arrows. Metabolism in the peripheral blood is indicated with continuous arrows (Mathieu et al., 2011).

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).

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 (0–0.5 min); 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).

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 2), 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).

2.7. Statistics

For the binary data of the morphological investigation of zebrafish

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
	9ACA	$C_{14}H_9NO$	2.89	208.0762	0.000048	0.000014
	AI	$C_{13}H_9N$	1.59	180.0813	0.000066	0.000020
	AO	$C_{13}H_9NO$	2.32	196.0762	0.000023	0.000007
	IM	$C_{14}H_{11}N$	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

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).

3. Results

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 (Hoybergh et al., 2020). 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).

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 (Hoybergh et al., 2020). The pH of all test solutions remained within a physiological range throughout the experiment (i.e., pH 7.23–8.04).

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 3). 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 3) 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 Fig. 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 3). 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 Fig. 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 Fig. 3).

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 alterations 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**

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 4). For the two other concentrations, however, significant malformations were already observed at 24 hpf (see Supplementary Table 4). 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 4). 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 4). 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 4, Table 5 and Fig. 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 4). 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 Fig. 3).

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

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 alterations 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 alterations 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

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 5). At 120 hpf, the number of larvae having this malformation almost doubled ($p = 0.0001$, $p < 0.0001$) (see Table 6 and Fig. 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 5 and Fig. 4).

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 6) 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 6). 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, Fig. 4 and Supplementary Table 6). Moreover, half of the larvae showed a darker pigmentation at 120 hpf in one of the replicates ($p = 0.0004$) (see Table 6, and Fig. 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 6). 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 6), which did not increase further during the following time points (see Supplementary Table 6 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 6). 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 Fig. 4).

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

3.3.1. Levetiracetam and its metabolite

No LTC or ECA > LOD was found in the extracts of the control

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 alterations 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

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). Although LTC uptake was detected but below the LOQ in the first LTC 10,000 μM 24 hpf replicate, the second replicate confirms that the compound can be taken up by zebrafish embryos, as it was biotransformed into its metabolite ECA in this replicate. However, no uptake of ECA could be observed (see Table 7, indicated in orange). Biotransformation of LTC into ECA was observed in the second 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).

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

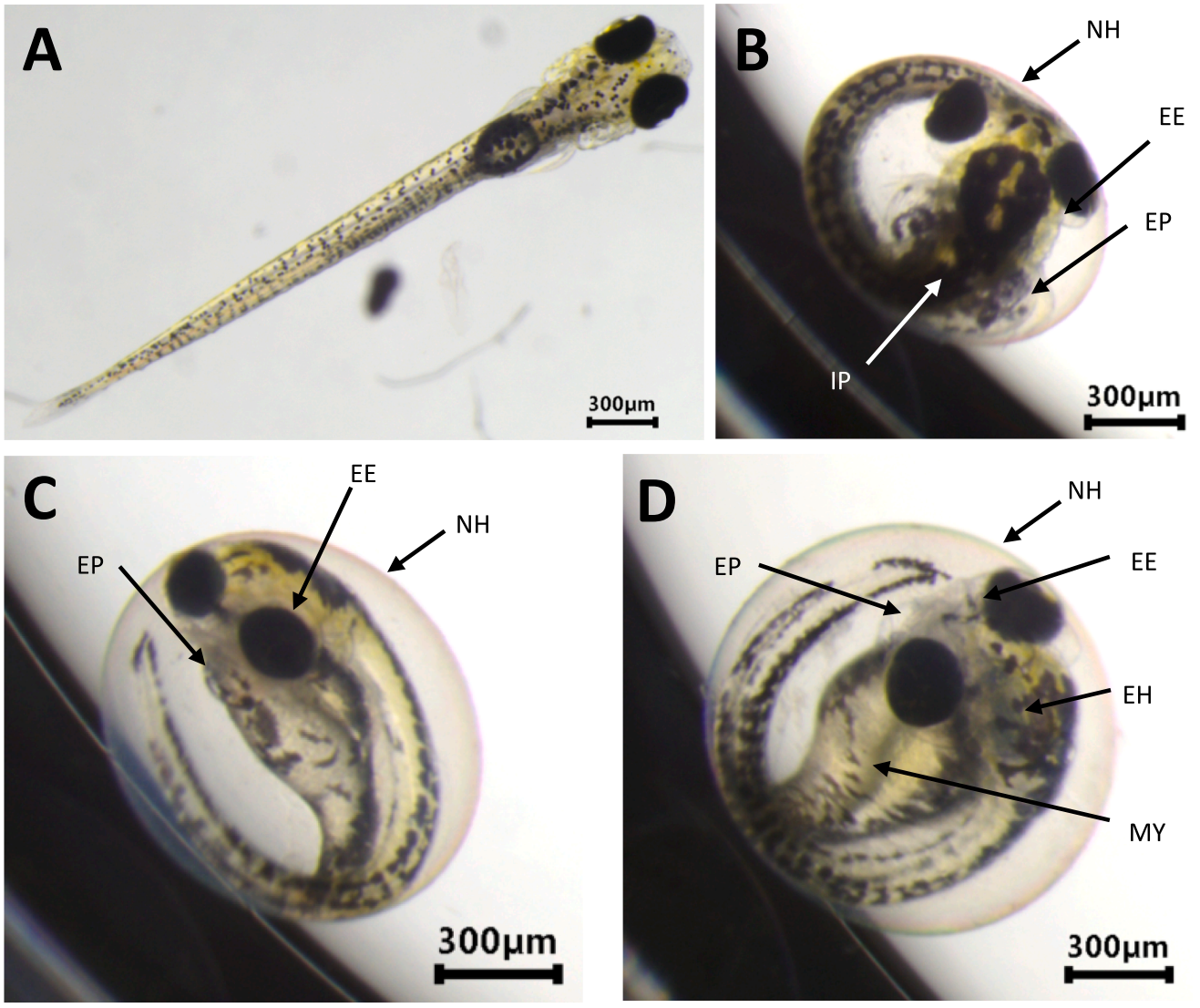


Fig. 3. Larvae exposed to CBZ or AI at 120 hpf. A) Control larva (1 % DMSO) showing no signs of abnormalities. B) Larva treated with 250 µM CBZ. C) Larva treated with 500 µM CBZ. D) Larva treated with 30 µM AI. The larvae depicted in B, C and D show several malformations. Abbreviations: acridine (AI),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

(see Supplementary Table 2). Therefore, additional analytical evaluations were performed to determine the origin of these unexpected peaks (see Supplementary Table 7). 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).

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) (Martinez et al., 2018; Lee et al., 2013). 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 (Isoherranen et al., 2003; Elgndy et al., 2019), growth retardation (Isoherranen et al., 2003; Elgndy et al., 2019; El Ghareeb et al., 2015),

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 alterations 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)

internal organ abnormalities (Elgndy et al., 2019; El Ghareeb et al., 2015) and fetal mortality (Isoherranen et al., 2003) were reported, and for ECA, growth retardation, and several skeletal abnormalities were reported (Isoherranen et al., 2003).

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 the larvae poorly took up LTC. 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 (Isoherranen et al., 2003)). 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 (Guarin et al., 2021). 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 seems not 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

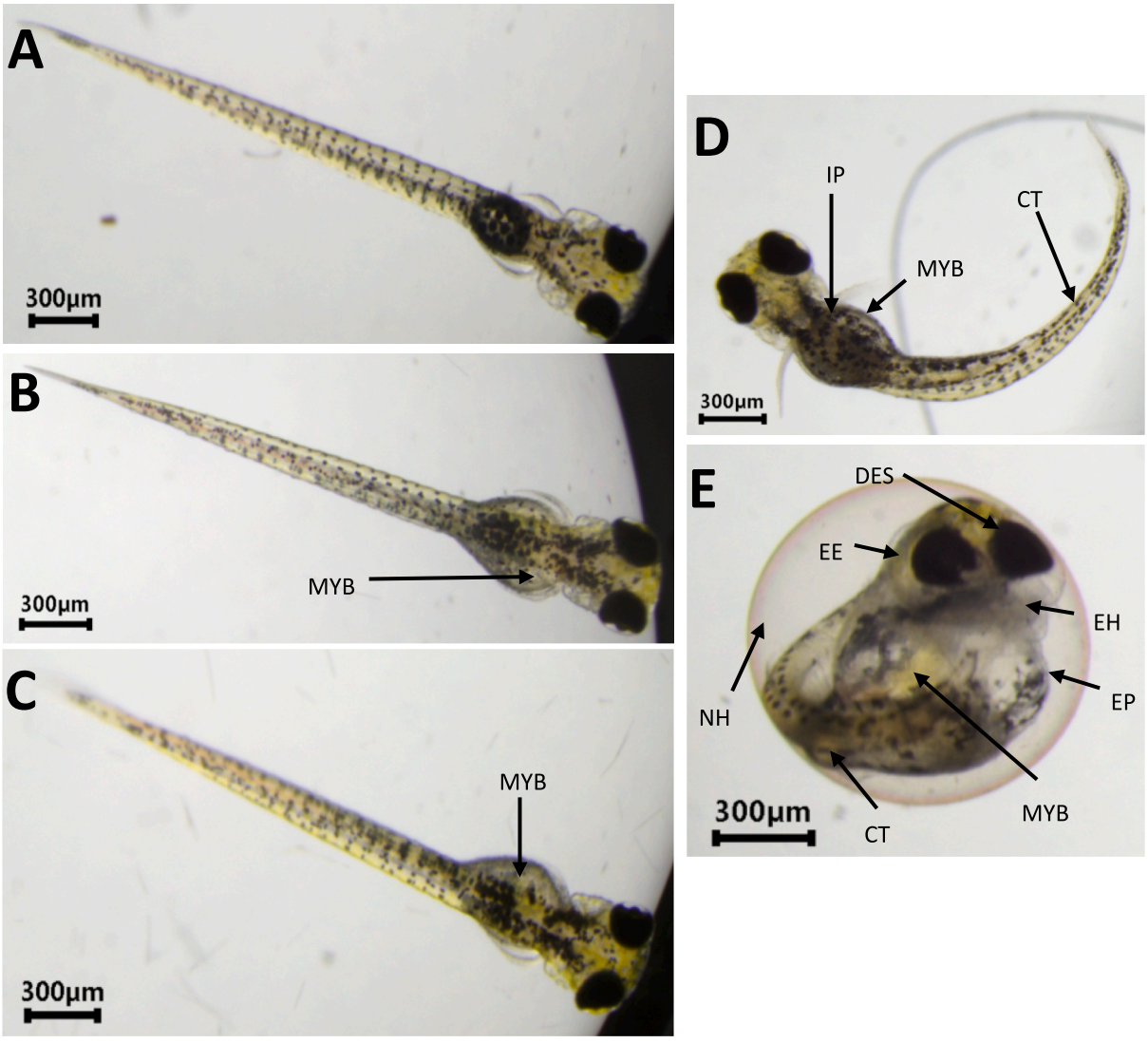


Fig. 4. Larvae exposed to AO or 9ACA at 120 hpf. A) Control larva (1 % DMSO) showing no signs of abnormalities. B) Larva treated with 30 µM AO. C) Larva treated with 60 µM AO. D) Larva treated with 30 µM 9ACA. E) Larva treated with 60 µM 9ACA. The larvae depicted in B, C, D and E show several malformations. Abbreviations: 9-acridine carboxaldehyde (9ACA), acridone (AO), 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).

Table 7
Analytical evaluation results of zebrafish larvae exposed to LTC and ECA. In short: LTC was taken up by zebrafish at 24 and 120 hpf (green), and biotransformed into ECA (light green), while ECA was not taken up (orange). 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.

Table 8
Analytical evaluation results of zebrafish larvae exposed to CBZ, 9ACA, AI and AO. In short: all compounds were taken up by zebrafish at 24 and/or 120 hpf (green), and biotransformed into one or more metabolites from further down the pathway (light green). 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 AI, 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 AO, 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.

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) (Guarin et al., 2021). 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 (DrugBank, n.d. & DrugBank, Levetiracetam, n.d.). 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. This knowledge is also relevant for other drugs that contain ester, amide or thioester bonds (Fukami and Yokoi, 2012), and are metabolized by special or unspecific types of B-esterase, such as several antiarrhythmic and anesthetic drugs (Oesch-Bartlomowicz et al., 2007).

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. However, as the mechanism of action of LTC is still unclear, it should be taken into account that potential differences in mode of action (MOA) in zebrafish could be a possible explanation for the negative results as well. Anyway, 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 (Bars et al., 2021). 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) (Bars et al., 2021; Zhu et al., 2015; van den Brandhof and Montforts, 2010). 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) (Bars et al., 2021). 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) (van den Brandhof and Montforts, 2010). 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) (Zhu et al., 2015). 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 (Zhu et al., 2015). 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 (Zhu et al., 2015). 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) (Zhu et al., 2015). Also, our observed delay in hatching from 72 hpf onwards was in accordance with their findings (Zhu et al., 2015). 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 (Zhu et al., 2015). 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 (Farooq et al., 2015). 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 (Farooq et al., 2015). 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) (Zhu et al., 2015). 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 (Bars et al., 2021; Donner et al., 2013; Zhu et al., 2015; Ghasemian et al., 2017). 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 (Bars et al., 2021), 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 Fig. 1) or CBZ > E-CBZ/IM > 9ACA > AI > AO (see Fig. 2)) was followed (Furst and Uetrecht, 1993; Mathieu et al., 2011). 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 (Mathieu et al., 2011). Anyway, we showed that the MPO homologue, responsible for the metabolization of CBZ into 9ACA, AI and AO is already active at 24 hpf. This finding is also interesting for other drugs that are biotransformed by MPOs, including arylamides such as diclofenac and clozapine (Uetrecht, 1995; Zuurbier et al., 1990).

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 (Isoherranen et al., 2003; Patsalos, 2004) 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 (Furst and Uetrecht, 1993; Mathieu et al., 2011). 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 (Buchan et al., 2019; Elks et al., 2014). 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) (Alexandre-Moreno et al., 2021; Behra et al., 2002).

In conclusion, we found that LTC was biotransformed into ECA, indicating that the special type of B-esterase that is responsible for its biotransformation is already active in 24 hpf zebrafish. In addition, we showed that CBZ was biotransformed into its downstream metabolites (i.e., 9ACA, AI, AO) in 24 hpf zebrafish embryos and 120 hpf larvae, showing that the MPO homologue is already active at 24 hpf. Moreover, exposure to CBZ, 9ACA, AI, and AO caused anomalies in zebrafish embryos and larvae. These three metabolites showed to be more potent than CBZ, with AI being the most potent, followed by 9ACA and AO. Consequently, one or more of these metabolites probably contribute to the teratogenic effects observed in zebrafish larvae after exposure to CBZ. As such, our study gained more insights on developmental toxicity and non-CYP mediated biotransformation of CBZ, LTC and their metabolites, and showed that two non-CYP phase I enzymes, B-esterases and MPOs, that were responsible for the biotransformation of these AEDs are already active in 24 hpf zebrafish embryos.

Funding

This work was supported by a special research fund of the University of Antwerp (grant number 43833).

CRediT authorship contribution statement

Jente Hoyberghs: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Axelle Coppens:** Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Chloé Bars:** Writing – review & editing. **Chris Van Ginneken:** Writing – review & editing. **Kenn Foubert:** Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Steven Van Cruchten:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank the staff of the Zebrafishlab at the University of Antwerp for the supply of the fertilized zebrafish eggs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2024.100186.

References

Alexandre-Moreno, S., Bonet-Fernández, J.-M., Atienzar-Aroca, R., Aroca-Aguilar, J.-D., Escribano, J., 2021. Null cyp1b1 activity in zebrafish leads to variable craniofacial defects associated with altered expression of extracellular matrix and lipid metabolism genes. Int. J. Mol. Sci. 22 https://doi.org/10.3390/ijms22126430.

Augustine-Rauch, K., Zhang, C.X., Panzica-Kelly, J.M., 2010. In vitro developmental toxicology assays: A review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays. Birth Defects Res. Part C - Embryo Today Rev. 90, 87–98. https://doi.org/10.1002/bdrc.20175.

Ball, J.S., Stedman, D.B., Hillegass, J.M., Zhang, C.X., Panzica-kelly, J., Coburn, A., Enright, B.P., Tornesi, B., Amouzadeh, H.R., Hetheridge, M., Gustafson, A.L., Augustine-rauch, K.A., 2014. Fishing for teratogens: A consortium effort for a harmonized zebrafish developmental toxicology assay. Toxicol. Sci. 139, 210–219. https://doi.org/10.1093/toxsci/kfu017.

Bars, C., Hoyberghs, J., Valenzuela, A., Buyssens, L., Ayuso, M., Van Ginneken, C., Labro, A.J., Foubert, K., Van Cruchten, S.J., 2021. Developmental toxicity and biotransformation of two anti-epileptics in zebrafish embryos and early larvae. Int. J. Mol. Sci. 22 https://doi.org/10.3390/ijms222312696.

Behra, M., Cousin, X., Bertrand, C., Vonesch, J.-L., Biellmann, D., Chatonnet, A., Strähle, U., 2002. Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. Nat. Neurosci. 5, 111–118. https://doi.org/10.1038/nn788.

Braunbeck, T., Kais, B., Lammer, E., Otte, J., Schneider, K., Stengel, D., Strecker, R., 2014. The fish embryo test (FET): origin, applications, and future. Environ. Sci. Pollut. Res. 22, 16247–16261. https://doi.org/10.1007/s11356-014-3814-7.

Buchan, K.D., Prajsnar, T.K., Ogryzko, N.V., de Jong, N.W.M., van Gent, M., Kolata, J., Foster, S.J., van Strijp, J.A.G., Renshaw, S.A., 2019. A transgenic zebrafish line for in vivo visualisation of neutrophil myeloperoxidase. PLoS One 14, e0215592.

Busquet, F., Nagel, R., Von Landenberg, F., Mueller, S.O., Huebler, N., Broschard, T.H., 2008. Development of a new screening assay to identify proteratogenic substances using zebrafish Danio rerio embryo combined with an exogenous mammalian metabolic activation system (mDarT). Toxicol. Sci. 104, 177–188. https://doi.org/10.1093/toxsci/kfn065.

Dimopoulou, M., Verhoef, A., Gomes, C.A., van Dongen, C.W., Rietjens, I.M.C.M., Piersma, A.H., van Ravenzwaay, B., 2018. A comparison of the embryonic stem cell test and whole embryo culture assay combined with the BeWo placental passage model for predicting the embryotoxicity of azoles. Toxicol. Lett. 286, 10–21. https://doi.org/10.1016/j.toxlet.2018.01.009.

Donner, E., Kosjek, T., Qualmann, S., Kusk, K.O., Heath, E., Revitt, D.M., Ledin, A., Andersen, H.R., 2013. Ecotoxicity of carbamazepine and its UV photolysis transformation products. Sci. Total Environ. 443, 870–876. https://doi.org/10.1016/j.scitotenv.2012.11.059.

DrugBank, Levetiracetam carboxylic acid metabolite (L057), (n.d.). https://go.drugbank.com/metabolites/DBMET02789 (accessed December 21, 2023).

DrugBank, Levetiracetam, (n.d.). https://go.drugbank.com/drugs/DB01202 (accessed December 21, 2023).

El Ghareeb, A.E.W., Hamdi, H., Eleyan, M., 2015. Teratogenic effects of the anti-epileptic drug (levetiracetam) on albino rat fetuses during pregnancy and lactation. Res. J. Pharm. Biol. Chem. Sci. 6, 1456–1474.

Elgndy, I.S., Hagag, O.G., EL Kholy, S.M.S., Sarg, N.A.S., Farag, A.A.M., 2019. A comparative study of the teratogenic effects of antiepileptic drugs: lamotrigine and levetiracetam on adult albino rats, Egypt. J. Forensic Sci. Appl. Toxicol. 16, 87–111. https://doi.org/10.21608/ejfsat.2016.41640.

Elks, P.M., van der Vaart, M., van Hensbergen, V., Schutz, E., Redd, M.J., Murayama, E., Spaink, H.P., Meijer, A.H., 2014. Mycobacteria counteract a TLR-mediated nitrosative defense mechanism in a zebrafish infection model. PLoS One 9, e100928. https://doi.org/10.1371/journal.pone.0100928.

Fantel, A.G., 1982. Culture of whole rodent embryos in teratogen screening. Teratog. Carcinog. Mutagen. 2, 231–242. https://doi.org/10.1002/1520-6866(1990)2:3/4<231::aid-tcm1770020305>3.0.co;2-1.

Farooq, M., Taha, N.A., Butorac, R.R., Evans, D.A., Elzatahry, A.A., Elsayed, E.A., Wadaan, M.A.M., Al-Deyab, S.S., Cowley, A.H., 2015. Biological screening of newly synthesized BIAN N-heterocyclic gold carbene complexes in zebrafish embryos. Int. J. Mol. Sci. 16, 24718–24731. https://doi.org/10.3390/ijms161024718.

FDA, KEPPRA® (levetiracetam), (n.d.). https://www.accessdata.fda.gov/drugsatfda_docs/label/2009/021035s078s080,021505s021s024lbl.pdf (accessed November 22, 2021).

Field, H.A., Ober, E.A., Roeser, T., Stainier, D.Y.R., 2003. Formation of the digestive system in zebrafish. I. Liver morphogenesis. Dev. Biol. 253, 279–290. https://doi.org/10.1016/S0012-1606(02)00017-9.

Fukami, T., Yokoi, T., 2012. The emerging role of human esterases. Drug Metab. Pharmacokinet. 27, 466–477. https://doi.org/10.2133/dmpk.dmpk-12-rv-042.

Furst, S.M., Uetrecht, J.P., 1993. Carbamazepine metabolism to a reactive intermediate by the myeloperoxidase system of activated neutrophils. Biochem. Pharmacol. 45, 1267–1275. https://doi.org/10.1016/0006-2952(93)90279-6.

Furst, S.M., Uetrecht, J.P., 1995. The effect of carbamazepine and its reactive metabolite, 9-acridine carboxaldehyde, on immune cell function in vitro. Int. J. Immunopharmacol. 17, 445–452. https://doi.org/10.1016/0192-0561(95)00019-X.

Ghasemian, S., Nasuhoglu, D., Omanovic, S., Yargeau, V., 2017. Photoelectrocatalytic degradation of pharmaceutical carbamazepine using Sb-doped Sn80%-W20%-oxide electrodes. Sep. Purif. Technol. 188, 52–59. https://doi.org/10.1016/j.seppur.2017.07.007.

Giusti, A., Nguyen, X.B., Kislyuk, S., Mignot, M., Ranieri, C., Nicolaï, J., Oorts, M., Wu, X., Annaert, P., De Croze, N., Léonard, M., Ny, A., Cabooter, D., de Witte, P., 2019. Safety assessment of compounds after in vitro metabolic conversion using zebrafish eleuthero embryos. Int. J. Mol. Sci. 20 https://doi.org/10.3390/ijms20071712.

Guarin, M., Ny, A., De Croze, N., Maes, J., Léonard, M., Annaert, P., de Witte, P.A.M., 2021. Pharmacokinetics in zebrafish embryos (ZFE) following immersion and intrayolk administration: A fluorescence-based analysis. Pharmaceuticals (Basel) 14. https://doi.org/10.3390/ph14060576.

Guengerich, F.P., 2008. Cytochrome P450 and chemical toxicology. Chem. Res. Toxicol. 21, 70–83. https://doi.org/10.1021/tx700079z.

Halbach, K., Ulrich, N., Goss, K.U., Seiwert, B., Wagner, S., Scholz, S., Luckenbach, T., Bauer, C., Schweiger, N., Reemtsma, T., 2020. Yolk sac of zebrafish embryos as

backpack for chemicals? Environ. Sci. Technol. 54, 10159–10169. https://doi.org/10.1021/acs.est.0c02068.
Han, Y., Ma, M., Oda, Y., Rao, K., Wang, Z., Yang, R., Liu, Y., 2019. Insight into the generation of toxic products during chloramination of carbamazepine: Kinetics, transformation pathway and toxicity. Sci. Total Environ. 679, 221–228. https://doi.org/10.1016/j.scitotenv.2019.04.423.
Hoyberghs, J., Bars, C., Pype, C., Foubert, K., Ayuso Hernando, M., Van Ginneken, C., Ball, J., Van Cruchten, S., 2020. Refinement of the zebrafish embryo developmental toxicity assay. MethodsX 7, 101087. https://doi.org/10.1016/j.mex.2020.101087.
Hoyberghs, J., Bars, C., Ayuso, M., Van Ginneken, C., Foubert, K., Van Cruchten, S., 2021. DMSO concentrations up to 1% are safe to be used in the zebrafish embryo developmental toxicity assay. Front. Toxicol. 3, 804033 https://doi.org/10.3389/ftox.2021.804033.
Isoherranen, N., Spiegelstein, O., Bialer, M., Zhang, J., Merriweather, M., Yagen, B., Roeder, M., Triplett, A.A., Schurig, V., Finnell, R.H., 2003. developmental outcome of levetiracetam, its major metabolite in humans, 2-pyrrolidinone N-butyric acid, and its enantiomer (R)-α -ethyl-oxo-pyrrolidine acetamide in a mouse model of teratogenicity. Epilepsia 44, 1280–1288. https://doi.org/10.1046/j.1528-1157.2003.21503.x.
Kari, G., Rodeck, U., Dicker, A.P., 2007. Zebrafish: An emerging model system for human disease and drug discovery. Clin. Pharmacol. Ther. 82, 70–80. https://doi.org/10.1038/sj.clpt.6100223.
Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310. https://doi.org/10.1002/aja.1002030302.
Lee, H., Inselman, A.L., Kanungo, J., Hansen, D.K., 2012. Alternative models in developmental toxicology. Syst. Biol. Reprod. Med. 58, 10–22. https://doi.org/10.3109/19396368.2011.648302.
Lee, S.H., Kang, J.W., Lin, T., Lee, J.E., Il Jin, D., 2013. Teratogenic potential of antiepileptic drugs in the zebrafish model. Biomed Res. Int. 2013, 726478 https://doi.org/10.1155/2013/726478.
Lieschke, G.J., Oates, A.C., Crowhurst, M.O., Ward, A.C., Layton, J.E., 2001. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. Blood 98, 3087–3096. https://doi.org/10.1182/blood.V98.10.3087.
Martinez, C.S., Feas, D.A., Siri, M., Igartúa, D.E., Chiaramoni, N.S., del, S., Prieto, M.J., 2018. In vivo study of teratogenic and anticonvulsant effects of antiepileptics drugs in zebrafish embryo and larvae. Neurotoxicol. Teratol. 66, 17–24. https://doi.org/10.1016/j.ntt.2018.01.008.
Mathieu, O., Dereure, O., Hillaire-Buys, D., 2011. Presence and ex vivo formation of acridone in blood of patients routinely treated with carbamazepine: Exploration of the 9-acridinecarboxaldehyde pathway. Xenobiotica 41, 91–100. https://doi.org/10.3109/00498254.2010.529955.
Mattsson, A., Ullerås, E., Patring, J., Oskarsson, A., 2012. Albendazole causes stage-dependent developmental toxicity and is deactivated by a mammalian metabolization system in a modified zebrafish embryotoxicity test. Reprod. Toxicol. 34, 31–42. https://doi.org/10.1016/j.reprotox.2012.02.007.
Oesch-Bartlomowicz, B., Oesch, F. (2007) 5.08 - Mechanisms of Toxification and Detoxification which Challenge Drug Candidates and Drugs, in: J.B. Taylor, D.J.B.T.-C.M.C.I.I. Triggle (Eds.), Elsevier, Oxford, 2007: pp. 193–214. https://doi.org/https://doi.org/10.1016/B0-08-045044-X/00124-3.
Patsalos, P.N., 2004. Clinical pharmacokinetics of levetiracetam. Clin. Pharmacokinet. 43, 707–724. https://doi.org/10.2165/00003088-200443110-00002.
Pype, C., Verbueken, E., Saad, M.A., Casteleyn, C.R., Van Ginneken, C.J., Knapen, D., Van Cruchten, S.J., 2015. Incubation at 32.5°C and above causes malformations in the zebrafish embryo. Reprod. Toxicol. 56, 56–63. https://doi.org/10.1016/j.reprotox.2015.05.006.
C. Pype, Optimization of drug metabolism in the metabolic zebrafish developmental toxicity assay (mZEDTA), University of Antwerp, 2018. https://repository.uantwerpen.be/docman/irua/927e44/154692.pdf.
Saad, M., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Maes, L., Cos, P., Van Cruchten, S., 2016. In vitro CYP1A activity in the zebrafish: temporal but low metabolite levels during organogenesis and lack of gender differences in the adult stage. Reprod. Toxicol. 64, 50–56. https://doi.org/10.1016/j.reprotox.2016.03.049.
Spielmann, H., Genschow, E., Brown, N.A., Piersma, A.H., Verhoef, A., Spanjersberg, M.Q.I., Huuskonen, H., Paillard, F., Seiler, A., 2004. Validation of the rat limb bud micromass test in the international ECVAM validation study on three in vitro embryotoxicity tests. Altern. Lab. Anim. 32, 245–274. https://doi.org/10.1177/026119290403200306.
Uetrecht, J.P., 1995. Myeloperoxidase as a generator of drug free radicals. Biochem. Soc. Symp. 61, 163–170. https://doi.org/10.1042/bss0610163.
van den Brandhof, E.-J., Montforts, M., 2010. Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. Ecotoxicol. Environ. Saf. 73, 1862–1866. https://doi.org/10.1016/j.ecoenv.2010.08.031.
Verbueken, E., Alsop, D., Saad, M.A., Pype, C., van Peer, E.M., Casteleyn, C.R., Van Ginneken, C.J., Wilson, J., Van Cruchten, S.J., 2017. In vitro biotransformation of two human CYP3A probe substrates and their inhibition during early zebrafish development. Int. J. Mol. Sci. 18 https://doi.org/10.3390/ijms18010217.
Verbueken, E., Bars, C., Ball, J.S., Periz-Stanacev, J., Marei, W.F.A., Tochwin, A., Gabriëls, I.J., Michiels, E.D.G., Stinckens, E., Vergauwen, L., Knapen, D., Van Ginneken, C.J., Van Cruchten, S.J., 2018. From mRNA expression of drug disposition genes to in vivo assessment of CYP-mediated biotransformation during zebrafish embryonic and larval development. Int. J. Mol. Sci. 19 https://doi.org/10.3390/ijms19123976.
Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H., 2011. Zebrafish (Danio rerio) embryos as a model for testing proteratogens. Toxicology 281, 25–36. https://doi.org/10.1016/j.tox.2011.01.004.
M. Westerfield, The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition, 2007.
Zhao, S., Huang, J., Ye, J., 2015. A fresh look at zebrafish from the perspective of cancer research. J. Exp. Clin. Cancer Res. 34, 1–9. https://doi.org/10.1186/s13046-015-0196-8.
Zhu, L., Santiago-Schübel, B., Xiao, H., Thiele, B., Zhu, Z., Qiu, Y., Hollert, H., Küppers, S., 2015. An efficient laboratory workflow for environmental risk assessment of organic chemicals. Chemosphere 131, 34–40. https://doi.org/10.1016/j.chemosphere.2015.02.031.
Zuurbier, K.W., Bakkenist, A.R., Fokkens, R.H., Nibbering, N.M., Wever, R., Muijsers, A.O., 1990. Interaction of myeloperoxidase with diclofenac. Inhibition of the chlorinating activity of myeloperoxidase by diclofenac and oxidation of diclofenac to dihydroxyazobenzene by myeloperoxidase. Biochem. Pharmacol. 40, 1801–1808. https://doi.org/10.1016/0006-2952(90)90359-s.