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A quantitative *in vivo* assay for craniofacial developmental toxicity of histone deacetylases

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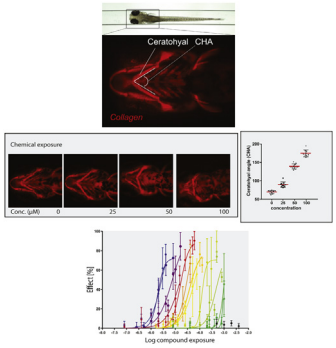
HIGHLIGHTS

- Histone deacetylase inhibitors can cause craniofacial developmental toxicity.
- The ceratohyal angle is a quantitative measure of this developmental toxicity.
- The ceratohyal angle assay is a simple *in vivo* tool for chemical risk assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

Many bony features of the face develop from endochondral ossification of preexisting collagen-rich cartilage structures. The proper development of these cartilage structures is essential to the morphological formation of the face. The developmental programs governing the formation of the pre-bone facial cartilages are sensitive to chemical compounds that disturb histone acetylation patterns and chromatin structure. We have taken advantage of this fact to develop a quantitative morphological assay of craniofacial developmental toxicity based on the distortion and deterioration of facial cartilage structures in zebrafish larvae upon exposure to increasing concentrations of several well-described histone deacetylase inhibitors. In this assay, we measure the angle formed by the developing ceratohyal bone as a precise, sensitive and quantitative proxy for the overall developmental status of facial cartilages. Using the well-established developmental toxicant and histone deacetylase-inhibiting compound valproic acid along with 12 structurally related compounds, we demonstrate the applicability of the ceratohyal angle assay to investigate structure-activity relationships.

1. Introduction

One of the most difficult research areas to develop alternatives to animal testing is chemical risk assessment for developmental toxicity. As developmental processes are very often characterized by complex mechanisms of cell migration and differentiation, simple cellular systems often fail to adequately capture central aspects, and often provide limited insights. One example is the development of the embryonic craniofacial features, which depend on precisely controlled and highly complex programs of neural crest cell migration and cellular differentiation. Classically, craniofacial developmental toxicity has been assessed in rodent models by administering test compounds to the pregnant female

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and examining pups at defined gestational stages or at birth (Binkerd et al., 1988; Vorhees, 1987). In addition to ethical concerns about this approach, such experiments are laborious and expensive. The zebrafish larva is a well-established model of early craniofacial development (Kimmel et al., 2001), and several studies have utilized zebrafish larvae and the preceding embryonic stages with various morphological endpoints as an approach to assess developmental toxicity of various chemicals at a non-protected stage of vertebrate development (Hermsen et al., 2011; Jomaa et al., 2014; Zoupa and Machera, 2017). Such approaches are flexible and have undeniable merit by demonstrating the ability of chemical compounds to affect gross morphological distortions and acute

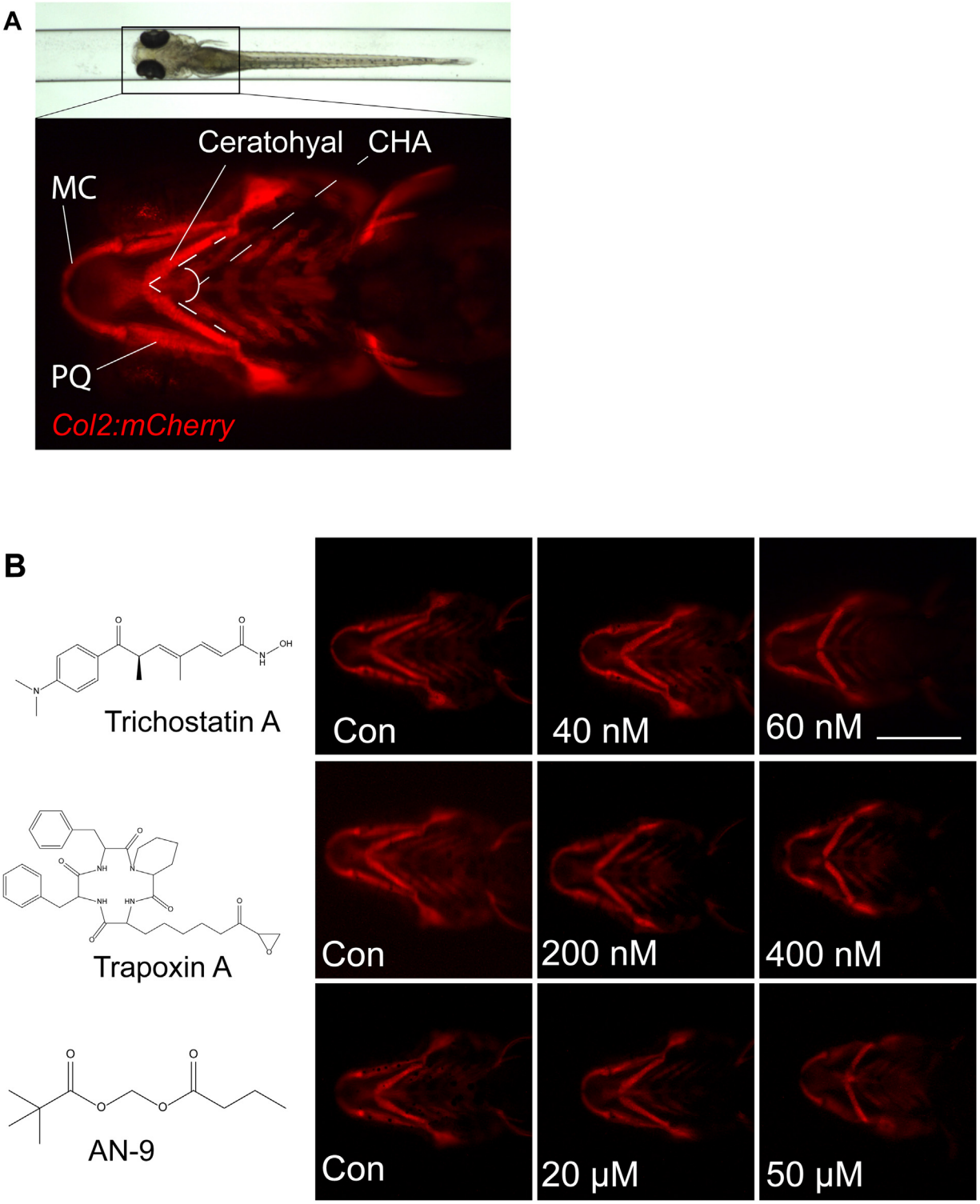


Fig. 1. A) Top: brightfield image of a 5 DPF zebrafish in ventral view. Bottom: representative image of normal cartilage development at 5 DPF, fluorescent ventral view image of the *col2:mCherry* collagen reporter line. Specific cartilage structures are identified: MC – Meckel's Cartilage; PQ – Palatoquadrate. The ceratohyal angle (CHA) is indicated in dashed lines. **B)** Histone deacetylase inhibitors with different chemical structures have similar deteriorating effects on facial cartilaginous features, manifested by the expansion of the CHA at 5 DPF. Scalebar represents 500 μm.

toxicity in a developing organism. However, the semiquantitative data provided by such approaches are ill suited for rigorous statistical analysis.

Utilizing genetically encoded fluorescent reporter lines may be a way to improve sensitivity and bring the zebrafish embryo approaches towards more molecularly informative endpoints. Zebrafish embryos are widely employed in the research of craniofacial development and a wide variety of fluorescent markers have been developed to follow the morphological development of craniofacial features (Brinkley et al., 2016; Eames et al., 2013; Hammond and Schulte-Merker, 2009). Most bony features of the face are formed by endochondral ossification of pre-existing collagen-rich cartilage structures, which are visible in fluorescent microscopy images of embryos and early larvae of the zebrafish type II collagen reporter line (*col2:mCherry*) (Hammond and Schulte-Merker, 2009). Several histone deacetylases (HDACs) have recognized, important functions in the regulatory pathways governing embryonic craniofacial development (reviewed in Westendorf et al., 2015). Both genetic and chemical inhibition of HDAC activity have been shown to result in distortions of craniofacial morphology in zebrafish embryos (DeLaurier et al., 2012; Ignatius et al., 2013). One chemical HDAC inhibitor which has received much attention in this regard is the anti-epileptic drug valproic acid (VPA), which is known to cause facial dysmorphism in mammals if administered during pregnancy (Mutlu-Albayrak et al., 2017).

In this study, we have taken advantage of the well-described craniofacial developmental toxicity effects of HDAC inhibitors to design a translational and quantitative morphological assay to measure craniofacial developmental toxicity based on a sensitive fluorescent reporter zebrafish line. Applying the exposure regime of the OECD fish embryo acute toxicity (FET) test guideline 236 (OECD, 2013) to the *col2:mCherry* zebrafish reporter line, the assay assesses the status of facial cartilages at 5 days post fertilization (DPF). At this stage, the formation of several prominent cartilaginous features has proceeded to a stage that can be clearly identified (Fig. 1A). One such facial cartilage feature is the morphologically distinct V-shaped ceratohyal cartilage. The morphology of this structure is highly dynamic, and the small angle of its V-shape decreases as the cartilaginous structures of the face develop (see supplemental Fig. 1). Together with the palatoquadrate and Meckel's cartilage, the ceratohyal forms the most prominent cartilage features in the ventral part of the developing face (Fig. 1A), and these structures will eventually form the major bony structures of the lower jaw (Eames et al., 2013). Several different methods have been devised to use the morphology of these structures to assess craniofacial developmental toxicity (Staal et al., 2018). Here we use a variation on the method reported by Staal and coworkers (Staal et al., 2018) to employ a morphology-based assessment of craniofacial developmental toxicity based on the ceratohyal angle. As an assay for craniofacial developmental toxicity, the ceratohyal angle (CHA) assay assesses the morphological appearance of the ceratohyal cartilage as a quantitative surrogate of overall facial cartilage development. Utilizing automated larval positioning, the ceratohyal assay balances throughput, simplicity and sensitivity, in a non-protected vertebrate model that captures the complexity of developmental processes. We utilize the assay to assess structurally diverse HDAC inhibitors, as well as structurally similar VPA analogs, demonstrating the CHA assay to be a flexible and highly relevant complement to existing craniofacial developmental toxicity testing batteries.

2. Materials and methods

2.1. Animal handling

Transgenic zebrafish of the fluorescent collagen 2 (*col2a1a*) reporter *Tg(Col2a1aBAC:mcherry*^{hu5910Tg}) (Hammond and Schulte-Merker, 2009) were handled in compliance with local animal welfare regulations and maintained according to standard protocols (zfin.org). All studies in this work were performed on embryos before the free feeding stage (up to 120 h post-fertilization (HPF)), and the stages of zebrafish embryonic development utilized were not protected under animal experimentation law according to the EU Animal Protection Directive 2010/63/EU. Adult zebrafish were kept at 28 °C in an aquarium system with light day/night cycle of 14/10 h. Embryos were harvested at 28 °C in egg water (60 μg/mL sea salt, Seramarin, Heinsberg, Germany).

2.2. Exposure protocol

Embryos were exposed to test compounds following the instructions outlined in the OECD test guideline TG236, with only minor modifications as detailed in supplementary materials. Briefly, after harvest, eggs were transferred to Danieau's solution (30x), 2011 (pH 7,6) (Cold Spring Harb. Protoc., 2011), and, as soon as fertilization and cell division could be confirmed, embryos were transferred to individual wells in 24-well plates in 2 mL of exposure medium. Plastic ware was pre-incubated with the test compounds at the appropriate concentrations for 24 h before the addition of test embryos. In all cases, the exposure medium as well as negative controls, contained 0,1% DMSO and was exchanged once daily, starting immediately after embryos were placed in 24 well plates. All embryos and subsequent larval stages were assessed daily by light microscopy for manifestations of toxicity. All images were recorded at 120 HPF. An initial range-finding experiment in five-fold dilution steps was performed to define the relevant test range, before definitive testing was performed in biological triplicate with 8–10 embryos per exposure concentration in each biological replication. Full materials and methods including detailed descriptions of exposure regime and full descriptions of exposure compounds are provided in supplementary materials.

2.3. Image acquisition and analysis

Prior to imaging, test groups were anesthetized in 0,02 % buffered 3-aminobenzoic acid ethyl ester (Tricaine; Sigma-Aldrich, Zwijndrecht, Netherlands), and then fixed with 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) at 4 °C for at least 3 h prior to microscopic analysis. Automated embryo positioning and rotation was achieved using VAST Bioimager platform (Union Biometrica, Hollister, United States), and ventral view fluorescent images were acquired manually at 10x magnification using a Leica TCS SL microscope equipped with a Leica DFC450C camera (Leica Microsystems, Wetzlar, Germany). The ceratohyal angle was measured manually using the Fiji distribution of ImageJ (Schindelin et al., 2012). To quantify the responses, CHA measurements derived from embryos exposed to test compounds were transformed into a relative response by normalizing against the mean measurements in age-matched sibling control larvae. Data normalization is achieved by converting CHA measurements into relative values spanning from 0% response, defined as the mean measurement in age-matched sibling controls, to 100 %, defined as 180° (the ceratohyal cartilage forming a straight line; Fig. 2). Normalization allowed for the pooling of data across several biological replications without results being impacted by natural batch-variation in the control groups. Normalized CHA measurements were plotted against log-transformed exposure concentrations and dose-response curves were drawn by non-linear regression using Graphpad Prism statistical software (Graphpad Prism v. 8.1.1).

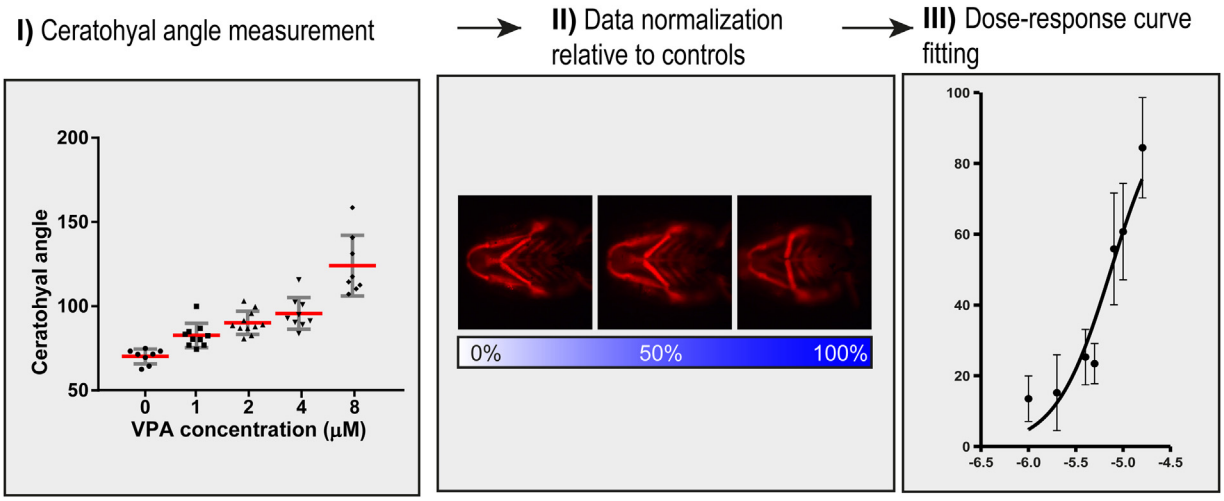


Fig. 2. Data acquisition and transformation steps involved in the CHA assay provided with valproic acid as an example: **I)** Raw data is acquired by measuring CHA in ventral view images of 5 DPF embryos, after exposure to test compounds. **II)** Data points are normalized relative to sibling control, a CHA value of 180° is defined as 100 % response **III)** Plotting CHA responses relative to log-transformed exposure values dose-response curves can be drawn for the extraction of effective concentrations.

3. Results and discussion

3.1. The CHA assay provides a quantitative measure of craniofacial developmental toxicity

As the facial cartilages form and grow in size, the CHA becomes smaller until, at 5 DPF, it resembles a < with a small angle of approximately 65° in ventral views of the head (Fig. 1A). Testing three well-described histone deacetylase inhibitors, Trichostatin A, Trapoxin A and AN-9, we noticed that the dose-dependent deterioration of the facial cartilages including Meckel's cartilage and the palatoquadrate was inversely proportional to the CHA (Fig. 1B). As the overall cartilage development increasingly deteriorates along with the elevation of histone deacetylase inhibitor dosages in the exposure medium, the CHA widens towards an approximate maximum of 180° (Fig. 2). The definition of maximal effect was based on empirical observations, since CHA measurements of 180° are often associated with concentrations close to lethal and accompanied by very severe retardation of other cartilage structures, sometimes to the point when they cannot be readily identified. Compared with other facial cartilage structures which has been assessed as markers of developmental toxicity *e.g.* Meckel's cartilage and the palatoquadrate (Staal et al., 2018), the ceratohyal remains very easy to identify even at high-effect dosages. While the widening of the ceratohyal angle is very sensitive to histone deacetylase inhibitors the ceratohyal remains readily identifiable, whereas Meckel's cartilage and the palatoquadrate becomes drastically diminished and distorted. We plotted normalized effect values against log-transformed exposure concentrations and fitted dose-response curves to the datapoints by non-linear regression in order to extract effective dose estimates such as EC_{10} values, thus enabling comparative evaluation of potencies of different developmental toxicants (Fig. 2).

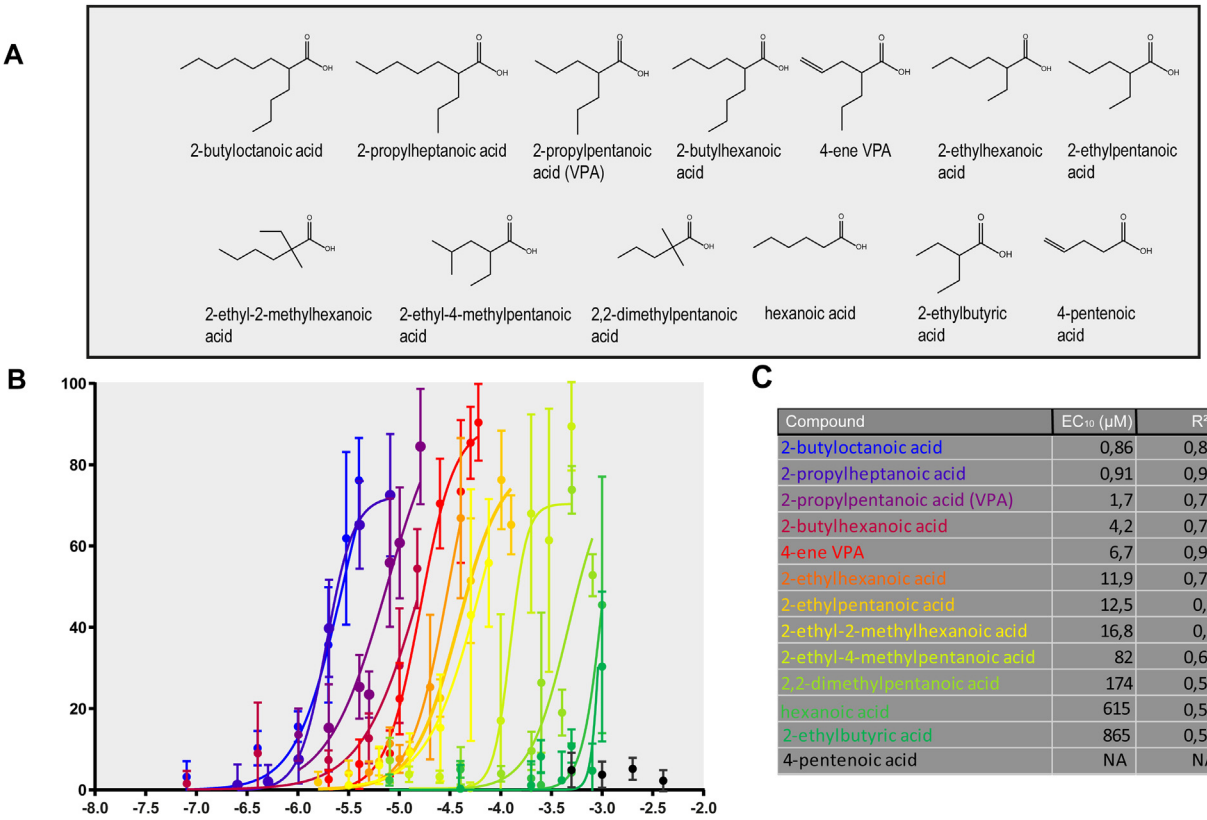
3.2. The CHA assay can be used to compare potencies of structurally homologous compounds

Having established that the CHA assay can be utilized to achieve potency assessments of craniofacial developmental toxicity of structurally diverse histone deacetylase inhibitors, we wished to examine the ability of the CHA assay to rank the craniofacial developmental toxicity potency of structurally closely related compounds. We based this assessment on the well-characterized craniofacial developmental toxicant valproic acid (VPA). VPA is a branched short-chain fatty acid, and we tested the impact of variations of the length of the aliphatic chains as well as further branching and unsaturation, on the potency of VPA and 12 structural homologs (Fig. 3A). The application of the CHA assay allowed us to quantify craniofacial developmental toxicity properties of the VPA structural homologs across several orders of magnitude, and demonstrate a clear trend in the dataset indicating the increasing potency of longer carbohydrate chains (Fig. 3B-C). Importantly, one compound, 4-pentenoic acid, exhibited no distorting effect on craniofacial development under the conditions tested and thus failed to exhibit effect levels sufficient to derive dose-response curves in the CHA assay, demonstrating the power of the CHA assay to distinguish positive from negative compounds.

4. Discussion

During the development of the CHA assay, we made use of the line *col2:mCherry* fluorescent reporter line, which allows for the rapid identification of cartilaginous facial structures through detection of the promoter activity of the collagen 2a gene. However, this reporter line and setup is not an absolute requirement for the application of the CHA assay. Simple and inexpensive classical staining techniques such as alcian blue staining, which has been applied in many studies to characterize mutations and developmental toxicants affecting craniofacial development (Neuhauss et al., 1996; Staal et al., 2018; Strecker et al., 2013), can be used to achieve cartilage staining as well. With the aid of an automated staging device such as the VAST (Union Biometrica, Hollister, United States), image acquisition can to be semi-automated to improve throughput, but such specialized equipment is also not essential, as embryos can be mounted in viscous material such as methyl cellulose or in agarose. Thus, the assay can be readily adopted without delay, if a lab is not in possession of the fluorescent reporter line with few additional steps added to the protocol. Using fluorescent reporter fish, it is possible to carry out the assay in a non-destructive manner with live embryos, but the assay is fully compatible with fixed samples which makes it more versatile, as multiple samples can be collected and analyzed at the operators' convenience.

The development of the craniofacial bony features relies on the proper migration of cranial neural crest cells from the neural tube



Compound	EC_{10} (μM)	R^2
2-butyloctanoic acid	0,86	0,81
2-propylheptanoic acid	0,91	0,95
2-propylpentanoic acid (VPA)	1,7	0,75
2-butylhexanoic acid	4,2	0,73
4-ene VPA	6,7	0,94
2-ethylhexanoic acid	11,9	0,77
2-ethylpentanoic acid	12,5	0,9
2-ethyl-2-methylhexanoic acid	16,8	0,7
2-ethyl-4-methylpentanoic acid	82	0,61
2,2-dimethylpentanoic acid	174	0,58
hexanoic acid	615	0,59
2-ethylbutyric acid	865	0,54
4-pentenoic acid	NA	NA

Fig. 3. A) 13 compounds with chemical structures similar to that of valproic acid was tested in the CHA assay for their relative potency in causing craniofacial developmental toxicity. **B)** Dose-response curves and **C)** EC_{10} and R^2 values derived for each of the VPA structural analogs. Error bars represent standard deviation.

to their appropriate location in the ventral part of the face, and subsequent differentiation into collagen-secreting chondrocytes. Such complex spatiotemporal regulation is inherently difficult to adequately recapitulate in *in vitro* systems, and the benefit of testing for adverse outcomes of chemical exposure in a naturally developing organism is self-evident. The intrinsically quantitative nature of a readout based on the angle formed by a clearly visible facial feature and relativizing it to age-matched sibling controls allows high sensitivity of the assay in detecting even very subtle effects pertaining to craniofacial development. The CHA assay can be applied as an add-on, forming a simple and sensitive auxiliary assay, to other non-destructive assays such as the General Developmental Score (GDS) test (Jomaa et al., 2014) or FET (OECD, 2013) in the same embryos. In most cases, a single adult pair of zebrafish will provide fertilized embryos in sufficient numbers perform a single biological replicate of several different compounds.

The apparent absence of craniofacial developmental toxicity of 4-pentenoic acid is interesting, as this compound has been demonstrated to have HDAC inhibitory properties *in vitro* (Gurvich et al., 2004), and as such would have been predicted to be among the positive compounds. This negative result, however, is in line with other assessments of the teratogenic risk associated with gestational exposure to 4-pentenoic acid in murine models (Nau and Löscher, 1986). Several explanations, *e.g.* the metabolic capacity of the embryo, could be proposed to explain this observation, though it goes beyond the scope of this communication to explore it in detail. It demonstrates the strength of including an *in vivo* vertebrate model in test strategies, rather than relying exclusively on *in vitro* assessment methods. The CHA assay quantifies effects on a late endpoint, which can be derived from multiple different stages in the long and complex developmental pathway, and, thus, this assay ensures that toxicity effects are not overlooked due to cell-type specificities in test systems. As such, the assay, as described here, will function best as a method to sensitively detect craniofacial developmental toxicity in a non-protected stage of vertebrate development, while specialized assays will still be necessary to identify key events at the molecular level. We propose that the assay can best be applied as part of a larger battery of test strategies including *in vitro* methods in cell lines of human relevance, to detect and quantify developmental toxicity effects in chemical risk assessment. Such battery testing could be organized according to an adverse outcome pathway (AOP) with independent key events quantified by the most appropriate and scalable models, and validation provided by the most complex and translatable models, such as organoids or non-protected *in vivo* models such as zebrafish embryos (Moné et al., 2020).

While a comprehensive structure-activity relationship analysis is beyond the scope of the present study, the CHA assay has the high-throughput potential to generate the wealth of data required to begin to develop craniofacial developmental toxicity in a quantitative manner akin to that of other existing QSAR models (Klüver et al., 2016). The results of our investigation of the structural analogs of valproic acid indicate a clear tendency towards elevated potencies for craniofacial developmental toxicity with elongation of the aliphatic chains. Combined with a dedicated pharmacokinetic analysis to provide correction factors for the test compounds (see (Siméon et al., 2020) for an example) and with other tests for different aspects of developmental toxicity, these data can make a valuable contribution to broad assessments of chemical safety concerns of similar branched-chain fatty acids.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2021.02.005.

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