The effects of retinoic acid and valproic acid on gene expression in the zebrafishembryotoxicitytest: combining microarray analysis with whole mount in situ hybridization

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Abstract

The zebrafish embryo toxicity test (ZET) is a promising alternative for animal testing in developmental toxicity, reducing the great amounts of animals used at present. Before implementation of the ZET, precise characterization of the model, with regard to developmental processes, has to be performed before validation of the test is possible.

The classical method of interpretation of the ZET is morphological evaluation, which is consideredrather subjective. Transcriptomics holds great promise as a novel way to interpret the ZET in a much more accurate, sensitive and objective way. Furthermore, these techniques help to increase our understanding of the toxic mechanisms of different teratogens during embryological development.

In this study, two known developmentally toxic compounds, retinoic acid (RA) and valproic acid (VPA) were used. Gene expression patterns were analyzed by microarray analysis and in situ hybridization of eleven selected genes, and were compared to the morphological effects, making use of the general morphology scoring system.

The current study demonstrated comparable results for ISH and microarray analysis, and showed gene expression to be more sensitive than morphological effects seen in the zebrafish embryo after exposure to both VPA and RA. However, the technique of ISH predominantly showed to be suitable for research purposes, whereas microarray analysis is also applicable as a high-throughput screening method.

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Introduction

At present, the zebrafish is a widely used experimental animal in numerous fields of science and its use is still rising. This is also the case in developmental toxicology, where especially the zebrafish embryo is gaining interest because of its many benefits, when compared to conventional animal models. In particular, developmental toxicity testing comprises a major part of the total amount of experimental animals used every year (Katinka van der Jagt 2004). Regulations require the use of animals for developmental toxicity testing. These in vivo experiments include mainly rodent models, which are expensive and bare significantly more ethic objections in contrast with the use of zebrafish embryos in the zebrafishembryotoxicity test (ZET). That is why, over the last few years, several studies have been conducted to explore the possibilities for the zebrafishembryotoxicity test in developmental toxicology(Selderslaghs, Van Rompay et al. 2009), (Brannen, Panzica-Kelly et al. 2010). The zebrafish embryo is a small vertebrate embryo. The early embryological development of the zebrafish is completed within a week and hatching occurs alreadyat 72 hours post fertilization (hpf), which makes it a rapid method when compared to mammals with substantially longer duration of gravidity (Kimmel, Ballard et al. 1995). In addition, the zebrafish is not considered, according to European legislation, as a protected experimental animal for the first 120 hpf(Strahle, Scholz et al. 2012), EU Directive 2010/63/EU. In conventional developmental toxicology animal models, the classical endpoints are the only instants at which it is possible to evaluate the toxic effects on the foetus. However, in the ZET, it is possible to observe full embryological development, as the chorion is transparent.

When using the zebrafish embryo in evaluating developmental toxicity of a certain compound, the current procedure is based on the deviating morphology of the exposed embryo when compared to the normally developing embryo. Over the years, different morphology scoring systems have been developed (Hermsen, van den Brandhof et al. 2011)(Braunbeck, Boettcher et al. 2005; Brannen, Panzica-Kelly et al. 2010). A recurring difficulty in these methods of evaluation of possible developmentally toxic compounds is the subjectivity of the scoring, as the eventual scoring of the embryo is accomplished the sole interpretation of the assessor.

One of the drawbacks of morphology scoring, is the lack of detection of subtle effects caused by the compounds. To overcome this problem, implementation of gene expression analysis may be helpful. The detection of effects on gene level presents us with a novel way to evaluate toxic compounds, even when effects are not detectable with conventional methods of zebrafish embryo assessment. To detect those gene expression changes, there are several techniques available. In the current study, we will be focusing on the results of both microarray analysis and in situ hybridization. Microarray analysis provides us with the opportunity to measure the absolute levels

of gene transcript by measuring levels of mRNA in the entire embryo. With whole mount in situ hybridization (ISH), we can detect the specific sites of gene transcription and it is possible to determine, in a semi-quantitative way, the magnitude of gene transcript product of the specific gene of interest(Thisse and Thisse 2008).

In the current study, we investigated the effects of retinoic acid (RA) and valproic acid (VPA) in the ZET. RA, which is a metabolite of vitamin A, is an important morphogen during early embryonic development. The retinoic acid pathway is a prominent pathway in the embryological development where it is mainly involved in hindbrain development, axis formation, neural differentiation and the development of many organs (reviewed in (Gavalas and Krumlauf 2000) and (Schier 2001). RA achieves its effects through binding to nuclear receptors and consequently initiate the transcription of specific genes, i.e. it functions as a transcription regulator(Leid, Kastner et al. 1992). The concentrations of RA in the different cells have to be tightly controlled, as even a slight deviation from the normal levels has detrimental effects on embryologic development, with morphological abnormalities as a consequence (reviewed in (Petkovich 2001)).

The second compound we tested is VPA. This drug is widely used in the treatment of epilepsy, as well as in the treatment of several other neurological disorders (Johannessen 2000). The exact mechanism of action of VPA is still not completely clarified. Besides the desirable effects that VPA has, it also bares some negative side effects with it. One of the most prominent and most dreaded of these side effects are the effects seen on embryos and fetuses after VPA administration to the mother during pregnancy. A wide range of neural tube anomalies, as well as cardiac and skeletal defects have been describedin humans and zebra fish(Dalens, Raynaud et al. 1980), (Kozma 2001)(Herrmann 1993). The main target system of VPA and the system in which the neurological effects are accomplished, is the GABA (gamma amino butyric acid) inhibitory neurotransmitter system. Valproic acid reinforces the action of the neurotransmitter GABA, which results in a reduced excitability of the neuronal cells (Phiel, Zhang et al. 2001). In addition to the effects VPA has on the GABA system, it also inhibits histone deacetylases (HDACs), with altered demethylation of DNA and thus epigenetic gene regulation (Gottlicher, Minucci et al. 2001).

In this study, we compared the effects of RA and VPA on gene expression in the zebrafish embryo using microarray analyses and ISH. We combined the results of both of these molecular techniques, and in addition compared this with the classical method of morphological evaluation. Furthermore, suitability of the ZET in assessing the embryotoxicity of chemical compoundsin terms of mechanisms of action will be discussed.

Materials and methods

Zebrafishembryotoxicity test

The embryos used in this study were obtained from zebrafish, *Daniorerio,* maintained and bred in our in-house facility for several years at a temperature of 28˚C and with a light:dark regimen of 14:10 hours, respectively. Three days before spawning, male and female zebrafishwere separated and fed a high protein diet to ensure both high performance and high quality of eggs. The day before spawning, the males and females were paired in special spawning tanks in a 2:2 ratio.

Fertilized eggs of 4 to 6 different batches were collected,within 30 minutes after spawning, after inspection of the fertilization rate per batch, thoroughly mingled and rinsed from debris with Dutch standard water (DSW; demineralized water with 100 mg/L $NaHCO_3$, 20 mg/L $KHCO_3$, 200 mg/L $CaCl_2$ $2H_2O$ and 180 mg/L $MgSO_4 7H_2O$). Dead embryos were manually removed and embryos were selected, based on developmental stage and vitality of the eggs.

We started the exposure when the embryos resided in the 4- to 64 cell stadium by keeping the embryos in a beaker with the model compound. Just before distributing the embryos amongst the 24-wells plates, each well was filled with the same medium as which the embryos were exposed to. Every well of the 24-wells plate contained a single embryo, providing optimal exposure. The embryos were evaluated according to general morphology scoring (GMS) system for developmental delay and teratogenic effects, which is the commonly used method in our laboratory facility. The GMS focuses on the development of different, developmental hallmarks, vital organs and physiological activities of the embryo which is extensively described by Hermsen et al. (Hermsen, van den Brandhof et al. 2011). A certain amount of points is assigned to the embryo depending on the presence of several morphological endpoints; furthermore, these endpoints are also dependable on time progression. The maximal amount of points, indicating a normally developed embryo, is 7 at 24 hpf and 15 at 72 hpf.The GMS was performed at two time points to show the development of the deviating morphology in a time dependent manner; the time points selected are 24 hpf, which corresponds with the time point of ISH and microarray analysis, and 72 hpf, the classical endpoint. For each exposure compound concentration one 24-wells plate was scored. The scores for all embryos per compound were averaged, without taking into account the coagulated ones. In addition, teratogenic effects were recorded and the percentage of embryos with at least one effect was calculated for both timepoints. The teratogenic effects that were evaluated are pericardial, yolk sac or eye edema, malformation of the head, malformation/absence of sacculi/otoliths, malformation of heart or tail, modified chorda structure, scoliosis, rachischisis or yolk deformation.

Zebrafish embryos compound exposure

In our experiment, we have chosen a constant exposure of the zebrafish embryos with the two developmentally toxic compounds for the first 24 hpf,for the embryos used for the ISH and the microarray analysis. At this instant in time the greatest interspecies resemblances are present in both morphology as in gene expression profiles, which thus makes it the most accurate instant to use when applying the zebrafish embryo as a representative model(Irie and Sehara-Fujisawa 2007). The embryos used for morphological assessment were exposed until 72 hpf for classical morphology evaluation.

The embryos were exposed to all trans retinoic acid (RA; Cas no.:302-79-4) andvalproic acid sodium salt (VPA; Cas no.:1069-66-5). All substances were purchased at Sigma-Aldrich, unless stated otherwise. RA was dissolved in DMSO (dimethyl sulfoxide) with a final concentration of 0.2% (v/v) and VPA was dissolved in DSW with DMSO supplemented to the same concentration of 0.2% (v/v). 0.2% DMSO was included as solvent control. The concentrations of the toxic compounds used for ISH in this experiment were different for both compounds. The high concentrations were decided as the benchmark concentration at which a 20% decrease in GMS was accomplished (BMC_{20}) at 72 hpf based on previous morphology data (Hermsen, van den Brandhof et al. 2011; Sanne A.B. Hermsen 2012). For both RA and VPA this BMC_{20} concentration corresponds with the highest concentration of the microarray analysis and result in equipotent morphological effects. The concentration of RA used for ISH is8nM and for VPA 1500 µM. Because of the broad morphological malformations of the embryo after exposure to 1500 µM of VPA as early as 24 hpf, the ISH was also performed after exposure to VPA at a concentration of 320 µM with an additional DMSO exposed embryo included, corresponding to a BMC of 5% for GMS.

Microarray analysis

Microarray analysis was performed and expression of selected genes was investigated at 24 hpf. A detailed description of the RNA isolation and microarray hybridization can be found elsewhere (unpublished work, (Sanne A.B. Hermsen 2012)). Microarray images were visually inspected and quality control of the data was done using the Affymetrix array QC pipeline at ArrayAnalysis.org (www.arrayanalysis.org, BiGCaT Maastricht University, the Netherlands) together with RMA normalization using the Brainarray custom CDF version 15 for annotation (http://brainarray.mbni.med.umich.edu). Expression values were log2-transformed. Maximal fold change (FC) per gene was determined between the experimental and control group using the average normalized signal values per group.

The average fold change of the different genes was determined by comparison with the basal levels of gene transcript, as is present in the solvent control embryos (level in exposed/level in controls).
The microarray that was used in the current experiment does not contain the transcript for hspb12 and therefore microarray data on this specific gene will not be presented.

In situ hybridisation

We used whole mount in situ hybridisation (ISH) to detect the shifts in the localization of gene expression and determine, in a semi-quantitative way, the shifts in gene expression levels. The ISH was conducted in two instances, for each experiment a different DMSO exposure groupwas included. After exposure, the embryos were rinsed twice in phosphate buffered saline (PBS), fixed overnight in 4 % paraformaldehyde (PFA) at 4˚C and transferred to 1xPBS. Thereafter, the chorionwas removed manually, with the usage of two forceps, in order for the ISH to be conducted.
First the peeled embryos were thoroughly washed; once with 1 ml 50:50 PBS/methanol, four times with 1 ml of PTW (PBS with 0.1% Tween20),followed bypermeabilizationwith 1 ml of 5 µg/ml Proteinase K (purchased at Roche Applied Science) at 37˚C for 10 minutes, to enable tissue penetration of the labeled probe. To ensure tissue integrity after the Proteinase K treatment, the embryos were re-fixated using 4% PFA.
Subsequently,five washes were conducted with PTW at room temperature and a quick wash with 200µL of pre- hybridisation mix (50% deionized formamide (v/v), 5xSSC, 0.1% Tween20, 1 mM citric acid).
The actual hybridisationwas preceded by a pre-hybridisation of 3 hours at 70˚C with 200 µL of hybridisation mix (pre-hybridisation mix completed with 500 µg/ml tRNA and 50 µg/ml heparin). The hybridisation, during which the DIG-labeled probe will bind to complementary RNA products, was done with the hybridisationmix supplemented with the DIG-labeled (digoxigenin-labeled) probe (1 or 2µL of probe, dependent on the probe). The probes, for the genes mentioned in table 1, were synthesized as described by Thisse(Thisse and Thisse 2008). The hybridisationwas completed overnight at 70˚C.
After the hybridisation the embryos were washed; once with 50:50 PBT/0.2xSSC (PBT; PTW with 2% of sheep serum, 0.2% bovine serum albumin), once with 2xSSC and twice with 0.2xSSC all at a temperature of 70˚C. At room temperature, the embryos were washed once with 50:50 PBT/0.2xSSC and once with PBT.
To prevent the presence of non-specific binding sites with the antibody Anti-Digoxigenin-alkaline phosphatase (Anti-DIG-AP), we blocked the embryos with PBT at room temperature for about 3 hours. Next, we incubated the embryos overnightwith 0.5mL of 0.5µg Anti-DIG-AP per 1 mL of PBT, at 4˚C under gentle agitation.

To purify the embryos from the Anti-DIG-AP, six wash steps with PBT at room temperature were done.Before we started the staining, the embryos were washed three times with the staining buffer (100 nMTris (pH9.5), 50 mM$MgCl_2$, 100 mMNaCl, 0.1% Tween20 (v/v), 1 mM levamisole). The staining was started by incubating the embryos with 1mL staining buffer supplemented with magnesium chloride, in which 20µL of NBT/BCIP (Roche Applied Science) was dissolved, and was conducted in the absence of light. The time needed for optimal staining is dependent upon the gene under investigation, i.e. every gene has its own staining duration. Periodic inspection of the staining, every 15 minutes, was necessary to ensure optimal staining. If the appropriate staining was accomplished, the embryos were washed three times with PTW to discard the staining buffer and consequently stop the staining process. Hereafter the stained embryos were fixed with 1 mL of 4% PFA for 20 minutes at room temperature.Three short washing steps with PTW were performedto discard the PFA.

Before capturing the embryos on photo, we transferred the stained embryos from the PTW into 100% glycerol to facilitate correct positioning of the embryo.

Genes of interest

The genes that have been chosen in the current study are primarily chosen because of their prominent role in the retinoic acid pathway or because of their involvement in the embryological development. The genes analyzed with ISH all showed a significant concentration dependent response in the microarray analysis performed on embryos exposed to flusilazole(Hermsen, Pronk et al. 2012). Furthermore, genes were also chosen based on their known expression pattern.

The genes selected together with a description of their function and pathway they are involved in, are shown in table 1 on the next page.

Gene	Entrez gene ID no	Function/ pathway
Aldh1a2	116713	l/r symmetry determination, anterior/posterior patterning, retinoic acid pathway
Dhrs3b	450050	Retinoic acid pathway
Cyp26a1	30381	Brain development, retinoic acid pathway
Hspb12	324021	Response to stress
Ca2	387526	Carbonate dehydratase activity
Hoxc13a	58059	DNA binding, regulation of transcription, multicellular organismal develoment
Hoxb6b	58053	DNA binding, regulation of transcription
Stm	386700	Detection of gravity, oticplacode formation
Vmhc	30616	Embryonic heart tube development, ventricular cardiac myofibril development
Znf703	114429	Brain development, Wnt pathway, regulation of transcription
Hoxb6a	30341	DNA binding, regulation of transcription

Table 1 List of selected genes for ISH, accompanied with Entrex gene ID and the gene functions and/or pathways the genes are involved in.

Results

General Morphology Scoring/ morphological assessment

Figure 1 shows the average morphology scoring of the exposed embryos at both 24 hpf and 72 hpf, using the GMS system (Hermsen, van den Brandhof et al. 2011). The results of the VPA and RA exposed embryos are shown, each with their corresponding solvent controls. The DSW exposed embryos showed a similar response as DMSO (not shown).

A decline in total scoring is evident in all three groups of VPA and RA exposed embryos, when compared to the DMSO and DSW controls at 72 hpf. The RA exposed embryos had an average scoring of almost 12, while VPA 320 µM exposed embryos had a scoring of 12.7 and VPA 1500 µM only had 8.6 points. At a concentration of 1500 µM of VPA, the scoring at both 24 hpf and 72 hpf was lower than at a 320 µM concentration of VPA on both time points. Only VPA tested at 1500 µM showed a relevant decrease in GMS at 24 hpf; the scoring was reduced with 2.5 points. The other VPA concentration and RA showed no decrease in scoring compared to the respective controls.

The teratogenic effects, as described in the GMS system, present in the exposed and control embryos at the two different time points are presented in figure 2. In the graph, the number of embryos observed with one or more teratogenic effects are depicted as a percentage of the total amount of living embryos for each compound.

Evident was the significant increase in the percentage of embryos with teratogenic effects when comparing allcompound exposed embryos to the controls. Furthermore, a clear time dependent increase in percentage of embryos affected with teratogenic effects was observed for the RA exposed embryos, from 11% to 78% at 24hpf and 72 hpf, respectively. This effect was not visible in both of the VPA exposed groups of embryos, where the percentage remained38% for the VPA 320 µM group and 73% for the VPA 1500 µM exposed embryos at both time points.

The most abundant effects that were seen in this experiment varied between the two developmentally toxic compounds. VPA exposure most often led to malformation of the head, pericardial edema, malformation of the tail, yolk sac edema and malformation of the heart. The most elaborate malformations after RA exposure were scoliosis, pericardial edema, yolk sac edema and yolk deformation.

Microarray analysis

The results of the microarray, unpublished work by SA Hermsen, for ten selected genes are presented in figure 3 and 4.

In both compound exposure groups, cyp26a1 showed a considerable concentration dependent increase in gene expression. The concentration dependent increase was the greatest after RA exposure, showing a fold changeof almost five at the highest concentration. Stm and hoxb6b were bothconcentration dependently up regulated as well by the two compounds. The

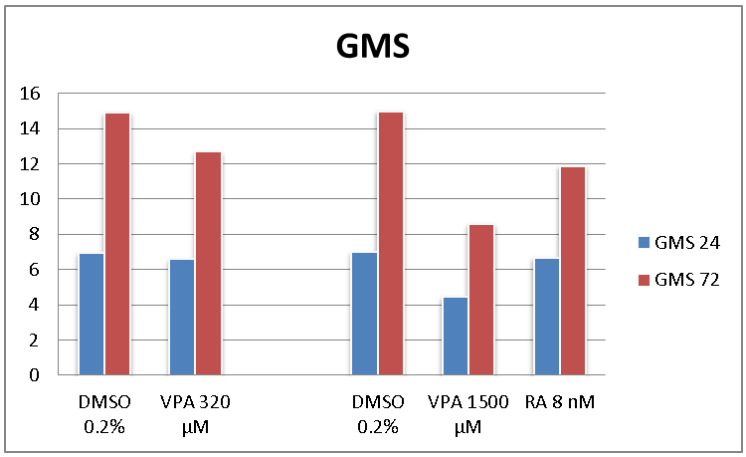

Figure 1General morphology score at both 24 hpf (blue) and 72 hpf (red) for the DMSO control, VPA 320µM, VPA 1500µM and RA 8nM.

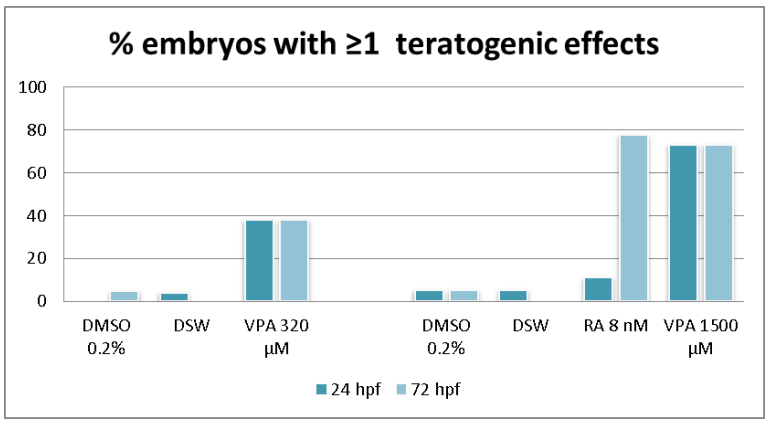

Figure 2The percentage of embryos with one or more teratogenic effects after exposure to VPA and RA at both 24 hpf and 72 hpf.

Figure3 Left lateral view showing morphology of the embryos at 72 hpf of the control and exposed embryos. Morphological malformations are pointed out with arrowheads; heart edema (he), bent tail (bt) and chorion (ch)

transcription of stm was most prominently up regulated in the VPA exposed embryos, when compared to the RA exposed embryos, showing a FC of 1.3 at the highest exposure concentration. Znf703 was elevated in a concentration dependent manner by both exposure compounds, however, it only showed a FC greater than one in the group of VPA exposed embryos.

The genes hoxc13a and vmhc were down regulated by both RA and VPA.However, after VPA exposure, the concentration dependent decrease in hoxc13a transcript levels started with aFC greater than one at the lowest two concentrations. For both exposure substances, vmhc had a fold change just above one at the lowest concentration.

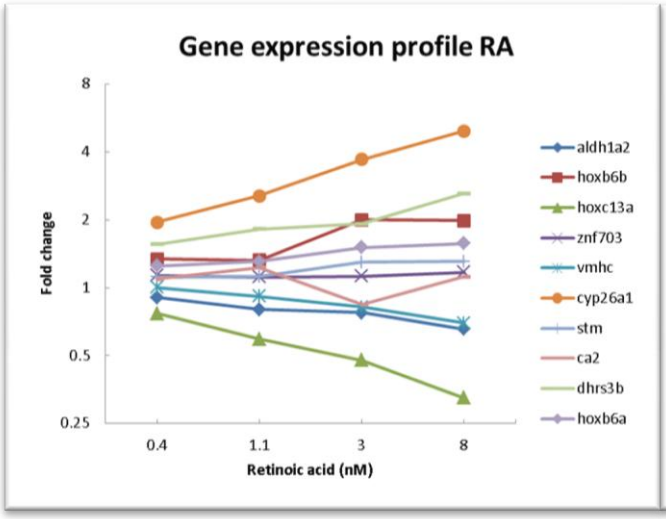

Figure 4 Concentration dependent gene expression of ten genes after RA exposure as found in the microarray analysis.

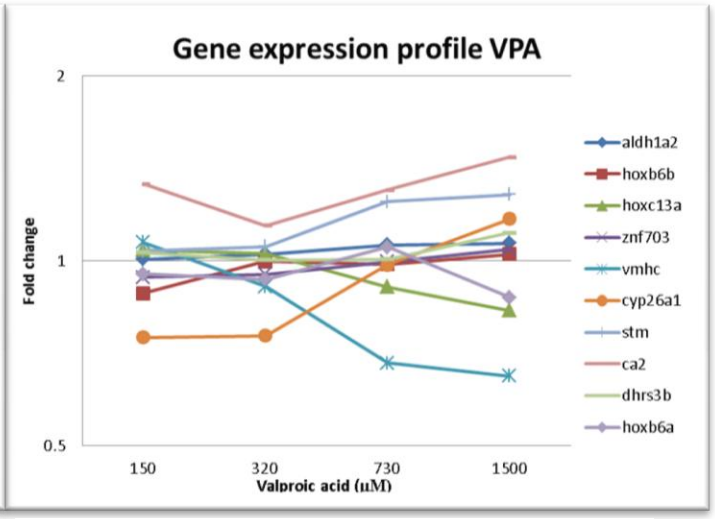

Figure 5 Concentration dependent gene expression of ten genes after VPA exposure as found in the microarray analysis.

Aldh1a2 showed opposing behavior with regard to both exposure substances. When exposed to RA, a concentration dependent down regulation was observed, whereas VPA exposure caused a minor up regulation of aldh1a2 with concentration.

Ca2 transcription was up regulated after VPA exposure in a concentration dependent manner. Except for the 320 µM concentration group which was less up regulated compared to the other concentrations tested.

Hoxb6a showed a concentration dependent up regulation in the RA exposed embryos for all concentrations. In the VPA exposed embryos this gene did not show a consistent concentration dependent response.

Dhrs3b was clearly up regulated by RA, again in a concentration dependent manner witha FC of2.6 at the highest concentration. This same gene in VPA exposed embryos only showed a slight up regulation with concentration.

In situ hybridization

The pictures obtained after performing the ISH are shown in figures 6a-c.The VPA and RA high dose exposed ISH embryos are shown to the right with its DMSO solvent control. On the left side of the picture, the 320 µM VPA exposed ISH embryos are shown, again together with their corresponding DMSO control. The staining visible in the exposed embryos was compared to the staining intensity and localization in the DMSO embryos.

Cyp26a1 was clearly up regulated after exposure to RA and after exposure to the two concentrations of VPA. The staining was most evident in the tail bud. In both the high

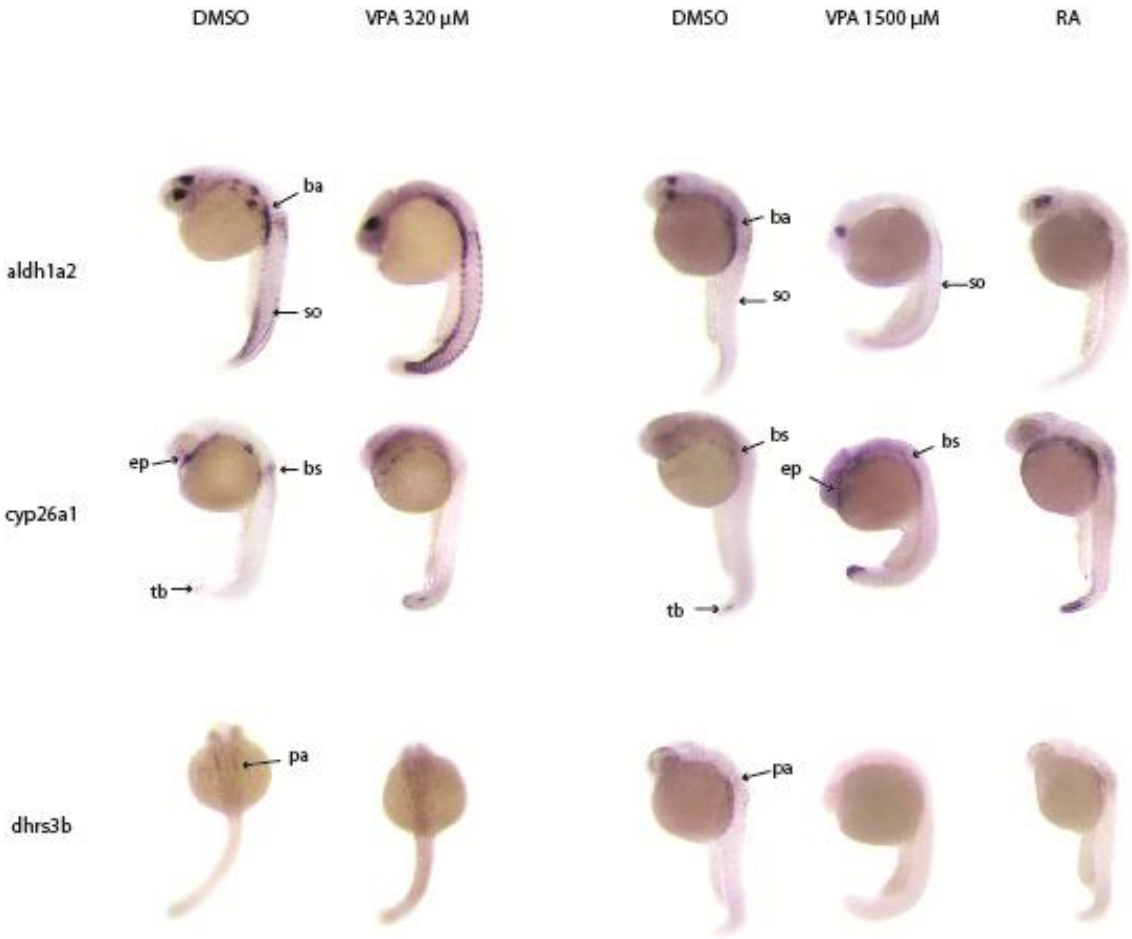

Figure 6a Representative pictures of embryos stained with ISH for *aldh1a2*, *cyp26a1* and *dhrs3b*. Pictures are made from aleft lateral view except for*dhrs3b* which is from a dorsal view for DMSO and VPA 320 µM. Specific structures are pointed out with the use of arrowheads; branchial arches (ba), somites (so), epidermis (ep), branchial structures (bs), tail bud (tb) and pharyngeal arches (pa).

concentration VPA embryos and the RA embryos, an additional subtle increase in gene transcription in the branchial structures and the epidermis was visible.

Aldh1a2 showed a clear concentration dependent up regulation after exposure to VPA. In the control embryos staining intensity in the somites is very low and after VPA exposure the staining in the somites increased. In the other stained structures no significant change in level of staining intensity was observed. For the RA exposed embryo the increase in gene expression levels was less visible, only a slight up regulation can be detected in the branchial arches. Aldh1a2 is under

normal circumstances highly expressed in the retina, however at this location a difference between the control and both exposure compounds was not observed. Remarkable was the decrease in staining intensity in the branchial arches in the high concentration VPA embryos when compared to the control embryos, while in general the staining in the embryo had increased.

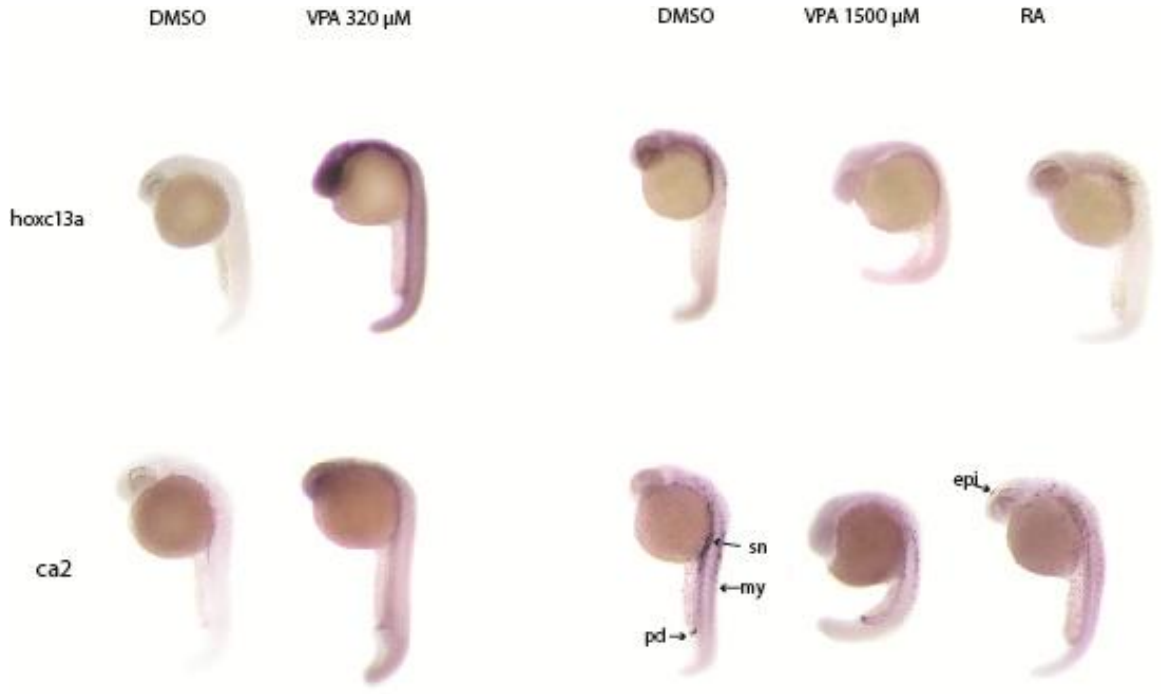

Figure 6b Representative pictures of embryos stained with ISH for *hoxc13a* and *ca2*. Pictures obtained from aleft lateral view of the stained embryos. The following structures are pointed out with arrowheads: pronephric ducts (pd), myotomes (my), spinal cord neurons (sc) and epiphysis (epi).

For dhrs3b non-specific staining was visible and therefore result may be unreliable. . However, it could be suggested that only for the highest concentration of VPA a down regulation of the dhrs3b expression was visible in the pharyngeal arches compared to the DMSO control. For the 320 µM of VPA and the RA exposed embryos, no significant differences were recognized.

Bothhoxb6a and hoxb6b were mainly expressed in the neural tube. Only hoxb6a showed a slight down regulation after VPA exposure, especially in the cranial region of the neural tube. For hoxb6b there was no change in staining intensity detected after VPA exposure. The hoxb6a gene showed a clear up regulation after RA exposure in the neural tube region. ISH data was only available on the hoxb6b gene for the 320 µM VPA exposed embryos and thus the 1500 µM VPA and RA exposed embryos ISH pictures are absent.

The third homeobox gene analyzed with ISH was hoxc13a. The exposed embryos all showed non-specific staining, consequently we cannot draw any conclusions from these ISH pictures.

The low concentration VPA exposed embryos showed a similar error with the gene ca2 as was seen with hoxc13a. The entire embryos were non-specifically stained and we cannot draw further conclusions.The 1500 µM VPA exposed embryos together with the RA exposed embryos did show a specific staining for ca2. The gene expression changes were in particular visible in the spinal cord neurons, myotomes, pronephric ducts and in the epiphysis. Striking is the almost completeabsence of ca2 expression in the epiphysis in the control, but especially in the RA

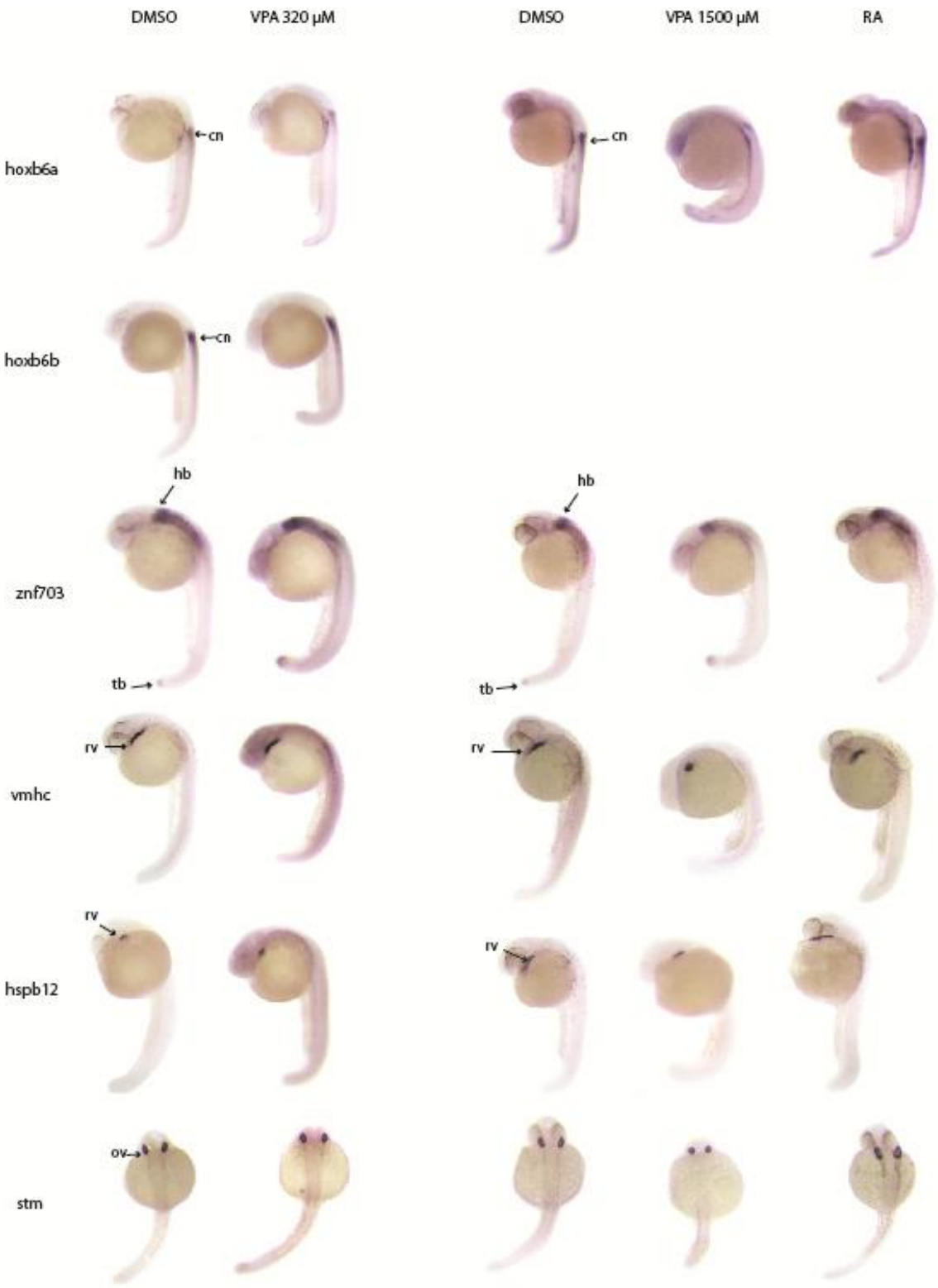

Figure 6cRepresentative pictures of embryos stained with ISH for *hoxb6a, hoxb6b, znf703,vmhc, hspb12* and*stm*.Picuters obtained from a left lateral view of the embryos, except for the embryos stained for *stm*, which are dorsally viewed. Stained structures are pointed out with the use of arrowheads: cranial neural tube (cn), hindbrain (hb), tail bud (tb), right heart ventricle (rv) and otic vesicle (ov).

exposed embryos this expression was evident. In the somites, a slight down regulation was visible for bothexposure compounds. In the pronephricducts these downward changes were only detectable in the RA exposed embryos and not in the VPA exposed ones.

In the controls, znf703 was predominantly expressed in the hindbrain and the tail bud. At both concentrations of VPA a slight increase in staining was seen in the hindbrain and the tail bud. RA also showed an increase in staining intensity in the hindbrain, but no difference could be detected in the tail bud.

A decrease in staining intensity was detected in the VPA exposed embryos after staining of the gene vmhc for both concentrations of VPA. Furthermore, the highest concentration of VPA showed relatively an even greater decrease in staining intensity when compared to the lower VPA concentration. RA showed the same inhibiting effect on vmhcexpression as VPA did. This gene was expressed in the ventricle of the developing heart, where a clear change in staining intensity and in change of staining area was visible. Especially at the 1500 µM concentration of VPA there was a characteristic change of shape visible in the expression pattern of vmhc. In the control embryos, it was tubular shaped and after exposure to VPA it showeda more rounded shape. This same effect was visible in the RA exposed embryos; however, when compared to the VPA exposed embryos, this shape change was less prominent.

Hspb12 was expressed in the developing ventricle of the heart as well and, at this specific site, the effects of VPA were detected. At 320 µM VPA, a significant down regulation was observed and at the concentration of 1500 µM an even greater decrease was demonstrated. In the RA exposed embryos no significant difference with the solvent control could be detected. Again a change of expression pattern could be recognized, instead of the original tubular shape in the controls it exhibited a more rounded shape.

Staining of the stm gene showeda difference in expression pattern, regarding localization and quantitative levels of expression, at the high concentration of VPA. The expression of stm was limited to the otic vesicles in all embryos. The effects of 1500 µM VPA,which were perceived in the otic vesicles, were both a decrease in staining intensity as well as a decrease in size of the expression area. The embryos exposed to 320 µM of VPA and to RA did not show any difference in expression pattern of the stm gene when compared to the controls.

Discussion

With whole mount in situ hybridization and microarray analysis,combined with classical morphological effects, we investigated the effects of RA and VPA on the development of the zebrafish embryo. RA and VPA are two known compounds causing effects on embryonic development in different species(Herrmann 1993), (Dalens, Raynaud et al. 1980), (Robinson, Verhoef et al. 2012).

Morphology

The effects of an excess of RA during embryological development, has been shown to lead to a series of abnormalities concerning neuronal tissues, amongst several other organs (Mulder, Manley et al. 2000), (Duester 2008). In our study, after exposure of the zebrafish embryos to RA, one of the most often observed abnormalities was scoliosis, which corresponds with the effect RA has on neuronal tissue observed in rodents (Yu, Levi et al. 2012), (McCaffery, Adams et al. 2003). We also found pericardial edema in the RA exposed embryos, which is supported by previousfindingsconcerning the essential role RA has in heart development (Liu and Stainier 2012), (Lin, Dolle et al. 2010).

Exposure to VPA has led to both malformation of the heart and pericardial edema, what is in accordancewith previous conducted research in rodents(Menegola, Broccia et al. 1996), (Na, Wartenberg et al. 2003) and humans after in utero exposure with VPA (Ozkan, Cetinkaya et al. 2011). Malformation of cranial neural tissue found in micemight explain the effects seen on the morphology of the head in our experiments(Mulder, Manley et al. 2000).The tail deformation we observed is in agreement with the findings of Khera et al. showingsimilar effects of VPA on neural tissue in the caudal part of the embryo found in rats (Khera 1992). In addition, VPA disrupts neural differentiation of embryonic stem cells into neural cells in vitro, indicating the effect of VPA on neural tissues(Theunissen, Pennings et al. 2012).

Retinoic acid

As stated before, RA is an important morphogen during development(Duester 2008). The tight control on distribution of RA is dependent upon its degradation by cyp26a1 as well as on the synthesis of RA by aldh1a2, and the balanceof levels of these processes throughout the embryo(Ross, McCaffery et al. 2000; Kam, Deng et al. 2012; Rhinn and Dolle 2012).

The up regulation of cyp26a1 was evident in both ISH results as in the microarray data after RA and VPA exposure. The enzymes from the cyp26 family are responsible for the degradation of retinoic acid in the embryo; as a consequence the levels of gene transcripts of the cyp26enzymes have to be tightly controlled with the use of feedback loops involving many other genes, including some of the genes under investigation in the current study, such as dhrs3b and especially aldh1a2. When RA levels rise, the transcription of cyp26a1 is consequently also up regulatedin

order to reduce the levels of RA to a physiological level. This mechanism of RA induced cyp26a1 up regulation is present to cope with the physiologically occurring variations in endogenous RA levels (Pennimpede, Cameron et al. 2010).

Most frequently, after in utero exposure to pathological high levels of RA, malformations of several neural tissues were observed(McCaffery, Adams et al. 2003). RA induced abnormalities, can partly be explained by remaining high levels of RA in spite of the cyp26a1 up regulation; the RA induced cyp26a1 up regulation is not satisfying to cope with the pathologically high levels of RA.

During axis extension in embryological development, cyp26a1 is an important regulator in the caudal part of the embryo, where it prevents premature exposure of the caudal stem zone to RA (MacLean, Abu-Abed et al. 2001), (Abu-Abed, Dolle et al. 2001). The up regulation, visible in the ISH pictures at the site of the tail bud after RA exposure, confirms the function with regard to protecting the stem zone against incorrect RA signaling. A frequent malformation seen in our experiment was scoliosis, in which an abnormal curving of the spine is present. The abovementioned effects of RA on neural tissue, explains the presence of this neuromuscular scoliosis.

The synthesis of RA is accomplished by aldh1a2. As opposed to cyp26a1, aldh1a2 is mostly expressed in areas where the RA signal is required for the development of certain structures (Pennimpede, Cameron et al. 2010). According to the ISH results obtained for the aldh1a2 gene, the gene expression is restricted to the retina, branchial arches and somites. Formation of the spinal cord and hindbrain in embryological development requires RA signaling(Maden 2006), the signal required to ensure neural differentiation, and thus high expression of aldh1a2 in this specific area can be expected. The level of mRNA throughout the entire embryo of aldh1a2, reflected in the microarray results, showed a prominent decrease after RA exposure. This observation is supported by studies previously conducted in mice(Niederreither, McCaffery et al. 1997) and can be explained by the same feedback loops in which cyp26a1 is involved. Studies conducted with mutant mice, deficient of the RA synthesizing enzyme aldh1a2,were affected with spina bifida (Niederreither, Abu-Abed et al. 2002), what illustrates that not only excess RA causes neural tube defects (NTDs) but also a complete absence of RA signaling results in pathologic malformations of neural tissues.

In our study, a subtle decrease in mRNA signal was observed in the somites and in the branchial arches. The somites are essential in the establishment of a segmented spinal column and the associated spinal nerves, and are located lateral to the notochord and neural tube. The localization of the aldh1a2 expression and the changes accomplished after RA exposure, as demonstrated with ISH, may explain the known teratogenic effects RA has with regard to the neuronal tissues(Mulder, Manley et al. 2000; Ozkan, Cetinkaya et al. 2011). Furthermore, down

regulation of aldh1a2, as detected after RA exposure in the microarray data was supported by the ISH pictures.

The third gene under investigation in the current study that is involved in retinol metabolism is dhrs3b, a dehydrogenase/reductase that is responsible for an early metabolic conversion in the retinol metabolism. The up regulation as measured in the microarray, after exposure to both VPA and RA, is not recognized in the ISH pictures, a shift in localization of the staining also is not perceivable. This can be explained by failure of the staining procedure resulting in non-specific staining.

In the current study design, three Homeobox (Hox) genes were included for analysis;hoxb6b, hoxb6a and hoxc13a. Homeobox genes are essential in the formation of the cranial-caudal axis in vertebrates and accordingly also the posterisation of the notochord. The Hox genes are therefore expressed in a region-specific manner that is tightly controlled(Stobe, Stein et al. 2009). Previously, it has been proposed that RA signaling is closely involved with the expression patterns of the Hox genes (Huang, Chen et al. 2002)(Shimizu, Bae et al. 2006)(Keynes and Krumlauf 1994)(Kmita and Duboule 2003; Noordermeer, Leleu et al. 2011). Another gene, involved in neural development, is znf703. The formation of the antero-posterior axis in neural tissue is regulated in several feedback mechanisms, in which the regional levels of RA and the transcription of Hox genes are related to each other. This explains the prominent effect high levels of exogenous RA have on the transcription of hoxb6a, as seen in the current study. Hoxb6a showed a concentration dependent up regulation after RA exposure in the microarray analysis, which corresponds well with the findings of the ISH. We found the expression of hoxb6a restricted to the cranial neural tube, further caudal to this point, the expression regresses in a gradual way. The morphological findings are in accordance with the localization of the two hoxb6 genes and with the response they show in ISH and microarray.

Not only our results concerning hoxb6a are in accordance with this hypothesize, also our results concerning hoxb6b provides more information. The microarray results of hoxb6b showed a similar concentration dependent increase after RA exposure as hoxb6a. Hoxb6b furthermore showed an even greater fold change, suggesting hoxb6b to be more sensitive in relation to RA levelsthan hoxb6a.

Hoxc13a did not show a clear response in the ISH after RA exposure. This is contradictive of what is seen in the microarray results; they showed a significant concentration dependent down regulation. This can be explained by a staining error leading to inappropriate discoloration.

Znf703, expressed in the hindbrain and the tail bud, showed an increased expression due to RA exposure in the ISH, the microarray data of the same gene showed only a slight increase. As mentioned earlier, RA is known to play a critical part in the formation of the neural tissues, including the hindbrain. It is presumed that znf703 transcription is promoted by increasing RA

levels (Hoyle, Tang et al. 2004), which supports our findings. It could be hypothesized that this correlation between RA levels in vivo and znf703 gene transcription contributes to the morphological defects seen by RA overexposure. The normal feedback loops involving RA and znf703 transcription are disturbed by exogenous excess levels of RA and morphological effects are seen in the regions where znf703 is transcribed, that is predominantly the hindbrain.

Two of the genes under investigation are involved in the formation of the heart ventricle, namely vmhc and hspb12. Hspb12 is a heat shock protein and accordingly acts to prevent protein aggregation in the cell, it thus has a protective function in the cell (Narberhaus 2002). The ISH for hspb12 of the RA exposed embryos did not show a significant change in gene expression. This suggests that there is not a direct or indirect relation between RA and hspb12 at this time point in the embryological development. Because of the absence of microarray data on hspb12 it is not possible to clarify the ISH results further. Alternatively, RA does indeed have somewhat of an effect on hspb12, but the effect is merely of a minor extent and consequently the effect is not visible in the semi-quantitative ISH, what would be in line with what is known in literature about the role of RA signaling in cardiac development(Keegan, Feldman et al. 2005).
Our ISH data showedvmhc expression to be restricted to the ventricle of the heart, which is in accordance with what is described in literature(Yelon, Horne et al. 1999). The concentration dependent decrease, seen in the microarray analysis was confirmedbythe data obtained by ISH.One of the prominent morphological effects seen in our experiment after RA administration was malformation of the heart. The gene transcription data concerning vmhc is thus consistent with the eventual morphological manifestations, according to both our morphological and transcriptomics results.

The effects of RA on stmcould not be detected in the ISH but did show a minor up regulation in the microarray analysis. These ambivalent results may be due to the minor expression area of the gene, consequently it is not possible to detect the up regulation of 2 FC, as seen in the microarray, as the initial staining intensity is already very high.

The known effects of RA on developmental processes were demonstrated in the zebrafish with the current study. Furthermore, it showed to be possible to correlate morphological effects with the effects measured usingtranscriptomics. Few genes, however, did not show conclusive results, and optimization of the procedure might be required.

Valproic acid

The two genes of major importance with regard to retinol metabolism, cyp26a1 and aldh1a2, were both, however only minor, up regulated by VPA in the microarray analysis and ISH. The ISH

changes were primarily localized in the somites for aldh1a2, and in the tail bud and epidermis for cyp26a1.The effects seen on the morphology, that is malformation of tail and head, correspond well with the localization of the changes in cyp26a1 and aldh1a2 expression as seen in the ISH. As mentioned before, VPA is a known HDAC inhibitor through which it alters the expression of several genes in a epigenetic way and it is known to regulate gene transcription through affecting different transcription factors.Interference of HDAC inhibitorswith the functioning of retinoic acid receptors (RARs) are proposed in literature(Weston, Chandraratna et al. 2002, (Pillai, Coverdale et al. 2004)(Weston, Chandraratna et al. 2002; Menegola, Di Renzo et al. 2006), which supports the abovementioned effects of VPA seen in our study on the levels of the RA metabolizing genes.

The Homeobox genes were only affected to a limited extend by VPA exposure in our study. The findings of the current study are not conclusive with what is expected according to literature (Faiella, Wernig et al. 2000), where VPA induced either an up or down regulation of different *hox* genes. The inconclusiveness of our results with previous studies conducted, might be explained by the exposure duration or timing, or the concentrations used.Other studies showed inconclusive results of the effect of VPA on Hox genes as well(Kawanishi, Hartig et al. 2003), (Williams, Mann et al. 1997).
Additionally, the third Hox gene, hoxc13a, showed a significant concentration dependent down regulation in the microarray analysis, which supports the earlier mentioned literature findings.

Remarkable are the results of ISH concerning the heart development related genes hspb12 and vmhc, and themicroarray results of vmhc. A significant down regulation of vmhcwas detected in the microarray analysis, which was supported by the staining in the ISH pictures. There was a notable change in shape of the transcription area visible in the ISH pictures. Pericardial edema and malformation of the heart were two of the main morphological malformations seen after VPA exposure in the current study. The significant down regulation of both of the genes involved in heart development are in agreement with the morphological findings. Our results, on both morphology and transcriptomics, are supportedby known effects of VPA on cardiac development(ten Berg, van Oppen et al. 2005), (Kozma 2001), (Wu, Nan et al. 2010).

Stm, a gene of which the expression is confined to the otic vesicles (Sollner, Burghammer et al. 2003), showed a concentration dependent increase after VPA exposure in the microarray data. In the ISH pictures however, the staining area decreased in size after exposure to 1500 µM of VPA, contradicting the microarray results.

In conclusion, the development related effects of VPA,were clearly demonstrated with the use of ISH and microarray analysis. Toxic targets of VPA were illustrated and related to morphological effects seen after VPA exposure.

With the technique of in situ hybridization, the level of mRNA of a certain gene can be visualized throughout the entire embryo, combining the localization of transcription with the level of gene transcript.

Furthermore, it is not possible to quantify the expression of the gene and consequently interpretations of changes in levels of transcription have tobe made based on empiric evaluation of the assessor. Quantification of the levels of mRNA in the ISH, however, is possible when making use of fluorescence staining. In the current study, we compared the staining with the FC of the same gene in the microarray data. By these means, we demonstrated that even a minor fold change was detectable with the use of ISH. It must beemphasized that microarray analysis is a very sensitive technique, when keeping in mind that the expression in an entire embryo is measured. However, any information about local transcription levels is missed using this technique. ISH acts thus complementary to the microarray, as this technique shows the distribution of the gene transcript across the intact embryo. The two techniques have showed to be a great tool in elucidating information about expression patterns of genes of interest in the zebrafish embryo.

Another downside to the use of ISH, is the labor intensity.The handling of the embryos during the procedure cannot be performed by robots and thus has to be conducted manually. This disqualifies ISH as a high throughput detection method and use will be restricted to research purposes. The microarray analysis however,is very suitable as a high throughput testing method.

Combining the results obtained from both ISH and microarray analysis has demonstrated to obtain valuable information about the toxicological mechanisms of VPA and RA in the zebrafish embryo. Furthermore, it has provided us with a tool to detecttoxic effects before observedon the morphology. However, further research is still necessary before implementation of transcriptomics in the ZET as a drug screening method can be accomplished. Microarray analysis shows great potential as a high throughput screening method in developmental toxicity testing, whereas ISH has a greater function as a research tool.

The effects of exogenous high levels of RA were demonstrated to interfere with several embryologic developmental processes by making use of transcriptomics. The teratogenic effects of VPA were further clarifiedwith the use of transcriptomics. To make the prediction as accurate as possible and identify toxic mechanisms, expansion of the gene set used in the ISH. The gene set of the current study was focused on retinol metabolism, hox genes and heart development,

genes involved in other important developmental pathways might give us an even greater insight into toxicological mechanisms during embryological development.

Concluding the findings of the current study we can state that molecular techniques concerning gene expression are efficient tools in the field of developmental toxicology. ISH can provide us with additional information on mechanisms of action compared to microarray and morphological scoring alone.Future research regarding improvement of the ISH would be useful.

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