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The role of *cyp1a* and heme oxygenase 1 gene expression for the toxicity of 3,4-dichloroaniline in zebrafish (*Danio rerio*) embryos

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Abstract

Expression profiling of exposed cells or organisms can reveal genes sensitive to environmental contaminants or toxic compounds. However, the mechanistic relevance of altered gene expression often remains to be elucidated. Toxicant-dependent differential gene expression may indicate protection to or mediation of toxicity. Previous studies revealed a number of differentially transcribed genes in zebrafish embryos exposed to the model compound 3,4-dichloroaniline (3,4-DCA). To evaluate the significance of two of the most sensitive genes, cytochrome P 450 1a (*cyp1a*) and heme oxygenase 1 (*hmox1*), for 3,4-DCA toxicity, RNA interference-mediated knockdown and overexpression studies have been conducted. Knockdown of gene transcription by siRNA for *cyp1a* and *hmox1* enhanced the frequency of developmental disorders in embryos exposed to 3,4-DCA. Vice versa, injection of *cyp1a* and *hmox1* mRNA reduced the number of disorders. The opposite effects of siRNA and mRNA injection clearly indicate a protective role of the corresponding proteins. Functional studies such as the one presented could be applied to a wide variety of genes. They would be ideally suited to study the role of genes identified from toxicogenomic studies in the zebrafish embryo model.

Keywords: *Danio rerio* embryo test; Differential gene expression; RNA interference; Overexpression

1. Introduction

The response of an organism to chemical exposure is in many cases brought about by changes at the level of gene expression (e.g., de Longueville et al., 2004; Nuwaysir et al., 1999). These changes may reflect a protective response but could also mediate toxic effects. Toxicogenomic studies are often used to identify sensitive genes, but with limited information on their role for the expression of toxicity. However, if alterations in gene expression are used as indicators or predictors of adverse effects, the knowledge on the effect of gene expression for an organism's health is of particular interest.

The role of gene expression on toxicity has been studied in various test systems by using knockout mutant strains (e.g., Smith et al., 2001; Girardot et al., 2004) or transient gene silencing (e.g., Kaizaki et al., 2006). Methods for transient gene knockdown have been also established in the zebrafish, a widely used aquatic model organism in ecotoxicology (e.g., Carney et al., 2004; Dodd et al., 2004). In particular zebrafish embryos have been shown to be a useful model, since repression of genes or corresponding protein levels, respectively, can be achieved by injection of, e.g., antisense siRNAs or morpholinos in embryos. It was the goal of the current study to elucidate the effect of cytochrome P 450 1a (*cyp1a*) and heme oxygenase 1 (*hmox1*) induction for the toxicity of 3,4-dichloroaniline (3,4-DCA) in embryos of the zebrafish. In a previous study using microarrays and quantitative RT-PCR, *cyp1a* and *hmox1* were two of six genes that we identified as differentially transcribed in zebrafish embryos exposed to 3,4-DCA (Voelker et al., 2007). 3,4-DCA is a model environmental contaminant, which was used as reference compound in the development of the zebrafish embryo toxicity test (Nagel, 2002). It is the major breakdown product of the phenylamide herbicides diuron, linuron and propanil. It has a higher toxicity and is detected more frequently in environmental samples than its parent compounds (Claver et al., 2006; Giacomazzi and Cochet, 2004). Furthermore, 3,4-DCA has been listed as a priority hazardous substance by the European Commission (Commission of the European Communities, 1994). Quantitative structure activity analysis indicates that the mode-of-action of 3,4-DCA can be defined as polar narcosis (Arnold et al., 1990). Thus, it may resemble many other toxic chemicals that are anticipated to

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act by an unspecific so called baseline toxicity (Escher and Schwarzenbach, 2002).

The genes that have been selected for the current study – *cyp1a* or *hmox1* – or their corresponding proteins, respectively, have been reported to be sensitive for a wide variety of structurally diverse compounds and/or stress factors (Alam et al., 2004; Bock, 1994; Denison and Heath-Pagliuso, 1998; Waller and McKinney, 1995). This has also been confirmed in a subsequent study in which the effect of exposure of zebrafish embryos to 13 different test compounds was analysed by microarray methodology. *cyp1a* and *hmox1* were found to be the most sensitive and widely induced genes (Weil et al., manuscript in preparation).

In the present study we used a dual approach to study the role of *cyp1a* and *hmox1* gene expression on the toxicity of 3,4-DCA in zebrafish embryos. Gene expression was transiently repressed by injection of short interfering RNAs (siRNAs). siRNAs have been widely used in mammalian cell culture (Elbashir et al., 2001) but have recently been shown to efficiently block gene function in zebrafish embryos as well (Dodd et al., 2004; Kanungo et al., 2007; Liu et al., 2005). Furthermore, we used transient overexpression to confirm the results of gene inactivation. We expected that overexpression would lead to reciprocal results with respect to toxicity. The toxicity was quantified by calculation of the frequency of delayed or impaired development.

2. Material and methods

2.1. Fish culture

The zebrafish wildtype strain WiK was obtained from the Max Planck Institute for Developmental Biology (Tübingen, Germany). Fish were cultured at 26 ± 1 °C at a 14:10 h light:dark cycle in a recirculating tank system using local tap water (pH 8–8.2, water hardness 1.2–2.4 mM bivalent ions, conductivity 520–560 μS/cm). Fish were fed *ad libitum* three times daily, once with *Artemia* and twice with commercial flake food (Tetra, Melle, Germany). Production of embryos was performed according to Nagel (2002) with modifications allowing the control of the fertilization time. Each spawning tank contained two males and one female, which was kept in an inserted tank. Prior to the desired spawning time, the male fish were placed into the insert.

2.2. Preparation of sense-mRNA

mRNA for overexpression studies was transcribed from cDNA full length clones of *cyp1a* (AB078927) and *hmox1* (NM_199678). The clones were obtained from the German Resource Centre for Genome Research (RZPD, Berlin, Germany). The cDNA clone of the gene *hmox1* was obtained as insert of the overexpression vector pCMV Sport 6.1, which is flanked by a 5′Sp6 polymerase promoter region and a 3′ early polyadenylation sequence (SV 40 polyA). The full length cDNA clone of the gene *cyp1a* was obtained as insert of the vector pME 18S-FL and was subcloned into the pCMV Sport 6.1 vector.

For the synthesis of sense mRNA, the linearized and purified plasmid DNAs were transcribed *in vitro* using the Sp6 mMessage mMachine kit (Ambion, Huntington, UK). Yields of mRNA were determined by photometric absorbance measurement at 260 nm. The size of the *in vitro* transcription products was assessed by running a denaturing formamide agarose gel and confirmed the expected length. As a negative control for overexpression studies, total mRNA was purified from total RNA of 48 h old zebrafish embryos using the Qiagen Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). The amount of total mRNA was adjusted to concentrations slightly above the specific mRNA concentrations (108–151%).

2.3. Preparation of siRNA

For silencing of *cyp1a* and *hmox1* gene expression, three different 21 nucleotide long target mRNA sequences starting with an AA dinucleotide were selected for each gene using the siRNA target finder (http://www.ambion.com/techlib/misc/siRNA_finder.htm). To control gene specificity, the target sequences were compared to the zebrafish genome by nucleotide BLAST in order to avoid homology to any other sequence. As negative controls, mismatch-siRNAs (mm-siRNAs) with the same nucleotide composition, but a randomly altered sequence was used. The lack of homology to other zebrafish sequences was confirmed by nucleotide BLAST against the zebrafish genome. The amount of mm-siRNAs was adjusted to concentrations slightly above the specific siRNA concentrations (105–120%). Synthesis of siRNAs was performed using the *Silencer*® siRNA Construction Kit (Ambion). siRNAs against the target sequences 5′-AAATCGGAAACAACCCACATT-3′ for *cyp1a* and 5′-AAGATCACACAGAAATCACTG-3′ for *hmox1* were identified to specifically repress mRNA levels.

2.4. Microinjection of zebrafish embryos

Injection needles were prepared from borosilicate glass capillaries (1.0 mm × 0.58 mm, with inner filament, Harvard Apparatus, Edenbridge, UK) using a puller device (Narishige PC-10, Narishige Ltd., London, UK). Each injection capillary was calibrated by injections into paraffin oil and calculation of the injection volume based on the diameter of the injected droplet.

Fertilized eggs were arranged for injection at the edges of a microscope slide in a petri dish. For each injection type (overview of all conducted injections: see Table 1) 40–70 embryos were used per experiment. Experiments were repeated three to seven times (Table 2). Prior to injection, all residual fluid was removed in order to fix the embryos to the microscope slide. Sense mRNA or siRNA were co-injected with 0.3% fluorescein isothiocyanate dextran (FITC dextran 10 kDa, Sigma–Aldrich, Seelze, Germany) into zebrafish one-cell-stage by pressure injection (Eppendorf Transjector 5246, Eppendorf, Germany). FITC Dextran was used as an indicator for successful injection into the one-cell-stage and visualized using an epifluorescence microscope (Leica MZ16F, Leica Microsystems, Wetzlar, Germany). RNA was diluted in 0.25 M KCl and the

Table 1
Type of injections that were used to study the effect of gene manipulation on toxicity of 3,4-DCA in zebrafish embryos

Type of injection	Exposure to 3,4-DCA	Aim
mRNA, siRNA	−	Demonstration that injected amounts of RNA are non-toxic.
	+	Effect of knockdown/overexpression on 3,4-toxicity.
Total mRNA, mm-siRNA	−	Demonstration that injected amounts of RNA are non-toxic.
	+	Negative control for specific gene manipulation.
FITC dextran	+/−	Control for successful injection, demonstration that FITC dextran did not cause unspecific effects.

FITC = fluorescein isothiocyanate.

injection volume was adjusted to give final amounts of sense and antisense RNA of 0.75–9 ng (for details see Table 2) per embryo. The amount of injected RNA did not cause any developmental disorders in non-exposed control embryos. Total mRNA or mismatch-siRNAs for control experiments were injected in the same amount as corresponding mRNA or siRNA.

2.5. Exposure of zebrafish embryos to 3,4-DCA

Exposure of zebrafish embryos to 3,4-dichloroaniline (3,4-DCA, Riedel-de Haen, Seelze, Germany; purity 99.9%) was performed according to Nagel (2002). Analysis of the purity of 3,4-DCA by GC–MS analysis indicated very weak contaminations by other compounds (analysis performed by Dr. F. Sacher, Technologiezentrum Wasser, Karlsruhe, Germany). Several other dichloroanilines could be identified as the major contaminants (all below 0.1%). These have not been shown to induce *cyp1a* gene or are very weak inducers (data not shown). No potent AHR (aryl hydrocarbon receptor) agonists have been detected among the contaminations.

Following the injection of siRNA or mRNA into the one-cell-stage, 40–70 embryos per experiment and treatment were exposed for 48 h to 12.4 μM 3,4-DCA in Petri dishes of 70 mm diameter in 10 ml test medium (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.75 mM $NaHCO_3$, 0.08 mM KCl). 3,4-DCA was added to the test medium by an aqueous stock solution (308.5 μM).

3,4-DCA-induced malformations were analysed and recorded using a Leica MZ 16 F stereomicroscope and a LEICA DMI 4000 B microscope with DIC contrast method (camera: Leica DFC 350 FX). Severity and frequency of developmental disorders in embryos injected with siRNA and mRNA and exposed to 3,4-DCA were compared to non-injected control and 3,4-DCA exposed embryos at 26 and 50 h post fertilization (hpf). At the end of exposure, embryos were shock frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

2.6. RT-PCR (reverse transcriptase polymerase chain reaction)

Total RNA was extracted from 50 hpf zebrafish embryo using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed according to standard protocols and manufacturer's instructions with DNAse I- (Roche, Grenzach, Germany) treated RNA, RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), *GoTaq* Polymerase (Promega, Mannheim, Germany) and an annealing temperature of 55 °C. For the confirmation of overexpression the following primers were used: 5′-ATTCATCCTTCCTTCCCTTCAC-3′ and 5′-ACCTTCTCGCCTTCCAACTTAT-3′ for *cyp1a* (AY398333); 5′-GCTTCTGCTGTGCTCTCTATACG-3′ and 5′-CTCTCAGTCTCTGTGCATATCG-3′ for *hmox1* (NM_199678). For the confirmation of siRNA-mediated suppression of *cyp1a* and *hmox1* primers downstream the targeted sequences of siRNAs were used: 5′-GGTCCGATTTCTGTGTCTGAG-3′ and 5′-AACTCTTCGCCCTGTTTTAGG-3′ for *cyp1a*; 5′-GCTTCTGCTGTGCTCTCTATACG-3′ and 5′-CTCTCAGTCTCTGTGCATATCG-3′ for *hmox1*. As a constitutive control, cyclophilin A (*cycA*, NM_212758) was amplified using the primers 5′-GACTTCACAAACCACAATGGAA-3′ and 5′-

Table 2
Injection volume and amount of mRNA and siRNA for overexpression and repression of the genes *cyp1a* and *hmox1* in zebrafish embryos

Type of injection	Number of experiments	Concentration (ng/nl)	Injection volume (nl)	Injected amount (ng)
mRNA				
cyp1a	7	0.07	15	1.05
hmox1	5	0.05	15	0.75
Total polyA mRNA	6	0.025	45	1.13
siRNA				
cyp1a	3	0.6	15	9
hmox1	4	0.35	15	5.25
mm-siRNA				
cyp1a	3	0.42	22.45	9.43
hmox1	4	0.58	10.9	6.32

For each experiment 40–70 embryos were used.

CCAAAGCCCTCTACTTTCTTGA-3′. The number of cycles (30 cycles for *cycA* and *cyp1a*, 35 cycles for *hmox1*) were adjusted for each gene to obtain amplification products below the saturation of the reaction. PCR-fragments were analysed by agarose gel electrophoresis and ethidium bromide staining. Densitometric analysis was performed using the software ImageJ 1.33u (NIH, Bethesda, ML, USA). Data were normalised by using cyclophilin A expression as reference. Due to conserved trends in each experiment but deviations in absolute values a further normalisation was performed by calculation of relative gene expression as percent of non-injected embryos exposed to 3,4-DCA.

2.7. Western blot analysis of CYP1A

Zebrafish embryos were treated from 2 to 50 hpf with 3,4-DCA (12.4 μM) and CYP1A protein was analysed by western blot at 26 and 50 hpf. For embryos collected at 26 hpf, the chorion and the yolk sac were removed manually using forceps. Previous analysis had shown that in 26 hpf embryos CYP1A can only be detected after removal of yolk proteins probably due to an increase in the concentration of cellular proteins in the embryo homogenate. Western blot was performed using standard protocols on 75 embryos per analysis. 50 μg of protein samples were resuspended in SDS loading buffer and loaded on a 10% SDS polyacrylamide gel. CYP1A proteins on the blot membranes were detected with an anti-rainbow trout CYP1A (Biosense Laboratories AS, Bergen, Norway) and anti-mouse IgG HRP-linked antibody (Cell Signaling Technology, Danvers, MA, USA). The binding of the CYP1A antibody was visualized by chemiluminescence using Lunigen TMA-6 solution (GE Healthcare, Buckinghamshire, UK) and FluorChem8900 (Alpha Innotech, San Leandro, CA, USA).

2.8. Data analysis and statistics

The toxic effect of 3,4-DCA exposure was quantified by counting the number of embryos with impaired development. These comprised alterations in development (indicated by reduced size of head, tail and eyes) and/or deformations of the body axis. Alterations in the frequency of developmental aberrations were described by calculation of a developmental disorder index:

$$\text{Developmental disorder index} = \frac{\text{Percentage of injected embryos with impaired development}}{\text{Percentage of non-injected embryos with impaired development}}$$

For both the developmental disorder index and the relative gene transcription, the same statistical analysis was used. First, normal distribution of residues was confirmed by the Shapiro–Wilks-*W*-test ($p < 0.05$) using the software Statistica 7.1. (StatSoft Europe GmbH, Hamburg, Germany.) Then, a significance analysis using an unpaired two-tailed *t*-test ($p < 0.05$) was performed by the software GraphPad Instat 3.01 (GraphPad software, San Diego, CA, USA).

3. Results

3.1. Toxicity of 3,4-DCA

Toxicity was monitored by analysing the severity and frequency of impaired development at 26 and 50 hpf in embryos exposed to 3,4-DCA. We anticipated that effects of gene repression or overexpression on the toxicity of 3,4-DCA would reveal the most striking effects at threshold concentrations, i.e. concentrations, at which only a proportion of the embryos exhibit toxic effects. Therefore, we selected the lowest observed effect concentration (LOEC, 12.4 μM, Voelker et al., 2007) for functional analyses. At the LOEC, about 45% of the embryos showed a reduction of head size and tail length and exhibit smaller eyes than control fish. (Fig. 1A, see Fig. 1B–I for details). Furthermore, embryos exhibited a partial or complete lack of pigmentation at 50 hpf. These alterations indicate a general delay in development but a distortion of the body axis was observed for many embryos as well. The effect of gene manipulation was quantified by calculation of a developmental disorder index which summarises the proportion of the frequency of all embryos with delayed or disturbed development of siRNA-/mRNA-injected embryos with respect to non-specific controls. Frequencies for specific disorders have not been calculated.

3.2. Detection of CYP1A protein in 3,4-DCA treated zebrafish embryos

In order to demonstrate that *cyp1a* induction in embryos is reflected by elevation of corresponding protein levels, western blot analysis was performed. CYP1A protein could not be detected in controls but in embryos exposed to 12.4 μM 3,4-DCA. The mouse anti-rainbow trout CYP1A antibody recognized the expected 55 kDa protein already in embryos at 26 hpf. Increasing CYP1A levels were observed at 50 hpf (supplementary material Fig. S1). HMOX1 protein levels could not be analysed due to the lack of availability of antibodies for fish HMOX1.

3.3. Effect of injections on the mRNA level

To enhance the level of *cyp1a* and *hmox1* transcripts and their corresponding proteins, zebrafish one-cell-stage embryos were injected with mRNA. Vice versa, for transient knockdown of the gene expression, embryos were injected with siRNA. In order to control the efficiency of these injections, relative mRNA levels were analysed by RT-PCR followed by densitometric analysis of agarose gels. RT-PCR confirmed that mRNA levels were still significantly elevated 50 h after injection (Fig. 2). This elevation was about 84% for *cyp1a* and 18% *hmox1* in 3,4-DCA-exposed embryos. No significant alteration in specific mRNA levels was observed in embryos injected with control mRNA (total mRNA). For embryos injected with siRNA a reduction of relative mRNA levels of 14% (*cyp1a*) and 26% (*hmox1*) with respect to control injections with mismatch siRNA was observed. Embryos exposed to 3,4-DCA and injected with mismatch-siRNA did not show changes in mRNA abundance (Fig. 2). The changes in

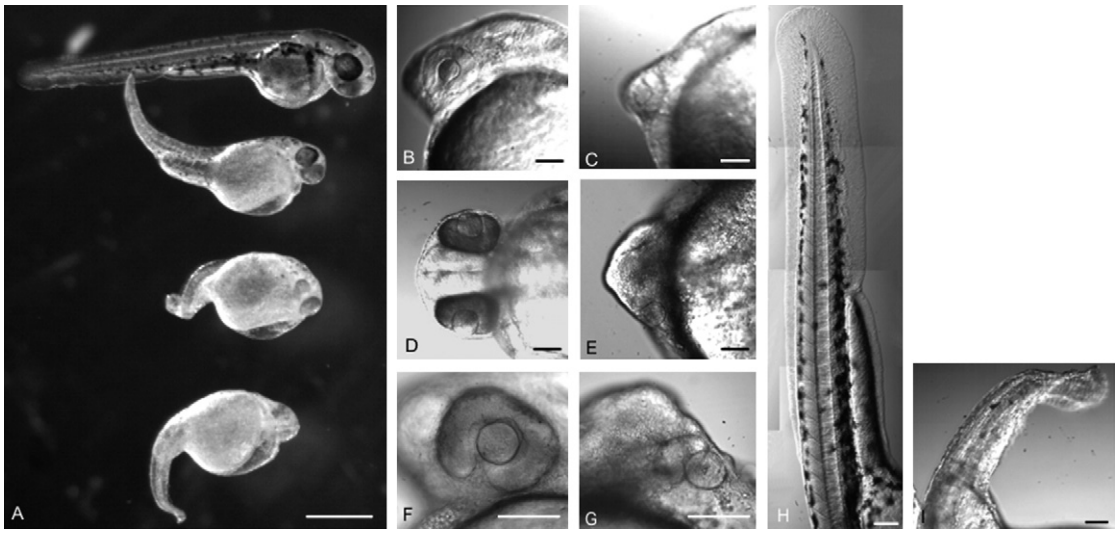

Fig. 1. Developmental aberrations in zebrafish embryos exposed from 2 to 50 hpf to 3,4-dichloranililine (3,4-DCA, 12.4 μM): (A) Overview of 3,4-DCA dependent phenotypes, view from lateral: top: untreated embryo at 50 hpf, down: treated embryos at 50 hpf with different degrees of malformations. Aberrations comprised a shrunken tail, distorted body axis, reduced head size as well as a possible impact on proper development of the eye (bar = 500 μm). B–G: detailed documentation of phenotypes (bar = 100 μm): (B) untreated embryo, view from lateral: normal head development at 26 hpf, (C) treated embryo, view from lateral: reduced head size at 26 hpf, (D) untreated embryo, view from lateral: eyes at 50 hpf (d = 203 μm), (E) treated embryo, view from lateral: eyes at 50 hpf (d = 118 μm), (F) untreated embryo, view from ventral: normal eye development at 50 hpf (diameter (d) = 165 μm), (G) treated embryo, view from ventral: reduced eye size (d = 118 μm) and loss of pigmentation at 50 hpf, (H) untreated embryo, view from lateral: tail length of 1990 μm at 50 hpf, (I) treated embryo, view from lateral: tail length of 577 μm at 48 hpf. The presented phenotypes have been used to quantify the proportion of developmental disorders in 3,4-DCA-specific affected embryos.

mRNA abundance appear weak. However, it must be noted that siRNA injection is most effective during early stages of development (Dodd et al., 2004; Liu et al., 2005). Therefore, it can be anticipated that average repression or elevation of mRNA levels have been above those recorded at 50 hpf. Relative mRNA levels could not be measured before 50 hpf, since they were analysed in the same embryos that were used for the calculation of the developmental disorder index.

3.4. Effect of mRNA and siRNA injection on toxicity of 3,4-DCA

In embryos that were injected with *cyp1a* and *hmox1* mRNA, the severity of impaired development in individual embryos was similar, but – if compared to control injections with total mRNA – the frequency of embryos showing a developmental delay or disorders was significantly reduced to 63 ± 24 and

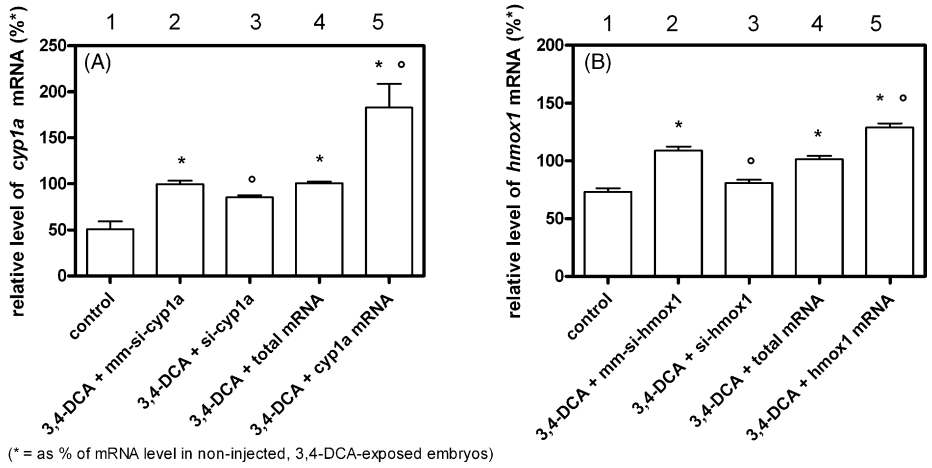

Fig. 2. Impact of siRNA/mRNA injection on transcript abundance. Semiquantitative (gel densitometry) RT-PCR was used to quantify *cyp1a* (A) and *hmox1* (B) transcript levels in zebrafish embryos (50 hpf, see Section 2 for further details), exposed to 3,4-DCA. mRNA levels were specifically manipulated by either injection of mRNA (*cyp1a* = 1.05 ng, *hmox1* = 0.75 ng) or siRNA (*cyp1a* = 9 ng, *hmox1* = 5.25 ng). Control injections were performed with either total mRNA (1.13 ng) or mismatch-siRNA (mm-siRNA; *cyp1a* = 9.4 ng, *hmox1* = 6.3 ng). 'Control' refers to non-injected, non-exposed embryos. Cyclophilin A was used as reference gene ((*) significant induction compared to control ($p < 0.05$), (°) significant alteration of transcript level compared to the unspecific injections with either mm-si-RNA or total mRNA ($p < 0.05$). Number of cycles for amplification: 30 for *cycA* and *cyp1a*; 35 for *hmox1* cycles. Number of experiments used for semiquantitative analysis of RT-PCR: 3 (*cyp1a* and *hmox1*) for siRNA injection, 3 (*cyp1a*) and 4 (*hmox1*) for mm-siRNA injection, 4 (*cyp1a*) and 5 (*hmox1*) for mRNA injection and 3 (*cyp1a* and *hmox1*) for total mRNA injection. Each experiments consisted of 40–70 embryos per treatment type).

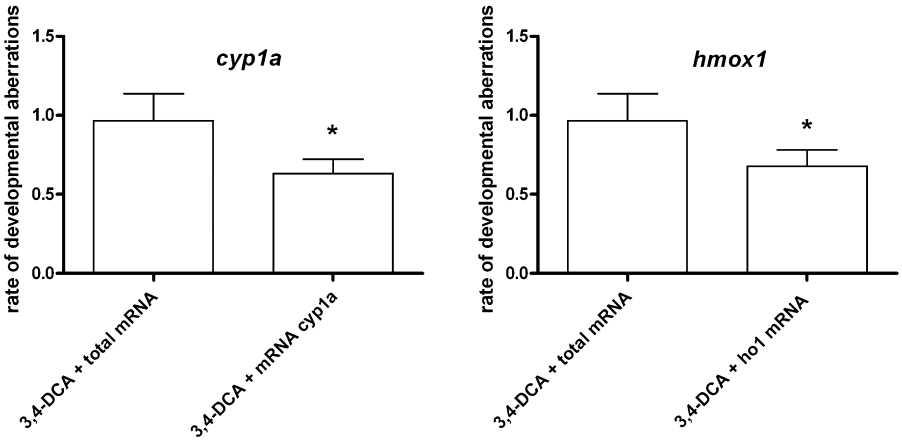

Fig. 3. Overexpression of the genes *cyp1a* and *hmox1* in 50 hpf zebrafish embryos exposed from 2 to 50 hpf to 12.4 μM 3,4-DCA caused a significant reduction of developmental disorders (right bars). Total mRNA control injections did not influence the frequency of 3,4-DCA-malformations (left bars). The frequency of developmental disorders was described by calculation of a developmental disorder index (see Section 2 for details, (*) significantly different to embryos injected with total embryonic mRNA, $p < 0.05$. $N = 7$ (*cyp1a*) or 5 (*hmox-1*) experiments with 40–70 embryos each).

67 ± 23%, respectively (Fig. 3). Total mRNA control injections did not show any changes in malformation frequency (96 ± 17%, Fig. 3). Likewise, injection of mRNA into non-exposed control embryos did not interfere with development (data not shown).

In embryos injected with siRNA for *cyp1a* and *hmox1*, severity of delayed or impaired development was enhanced in 3,4-DCA-exposed embryos. The frequency of impaired development was significantly elevated by si-*cyp1a* (171 ± 14%) and si-*hmox1*-injection (146 ± 41%) (Fig. 4). Some embryos injected with siRNA showed a completely non-detached tail, and strongly reduced size of head and eyes (Fig. 5D). Injection of mismatch-si-RNA into 3,4-DCA-exposed embryos did not affect the frequency of disorders (mm-si-*cyp1a*: 97 ± 9.7%; mm-si-*hmox1*: 96 ± 6.6%). Injection of siRNA into non-exposed control embryos did not induce any malformations (data not shown).

4. Discussion

For genes that play a role in protection to or mediation of toxicity, their knockdown or overexpression can be expected to provoke opposite effects on the expression of toxicity. In the present study, a protective function of *cyp1a* and *hmox1* was demonstrated for 3,4-DCA exposure by the reciprocal impact of siRNA and mRNA injection on the toxicity in zebrafish embryos. This protective function was indicated by alterations in the frequency of impaired development: knockdown increased, overexpression decreased the frequency of delayed embryos or malformations.

The genes *cyp1a* and *hmox1* have been reported to be sensitive for a wide variety of structurally diverse compounds and/or stress factors (Alam et al., 2004; Bock, 1994; Denison and Heath-Pagliuso, 1998; Waller and McKinney, 1995). They have been selected for functional analysis in this study because they have

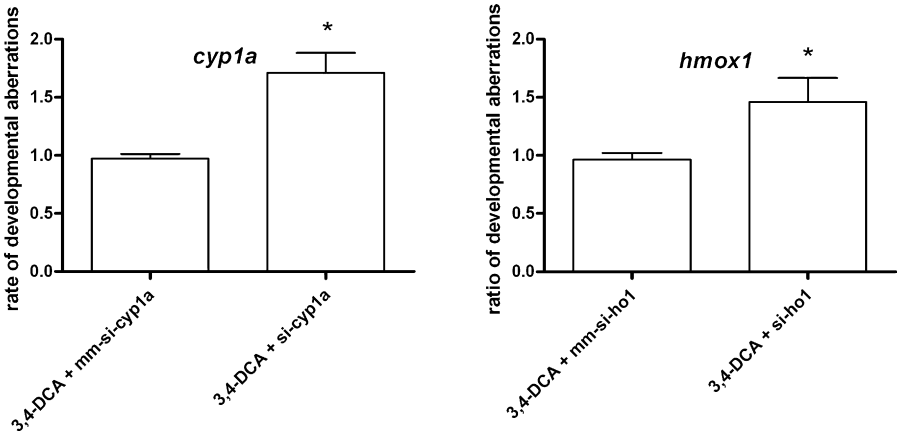

Fig. 4. Repression of the genes *cyp1a* and *hmox1* in 50 hpf embryos exposed from 2 to 50 hpf to 12.4 μM 3,4-DCA caused a significant increase in the frequency of developmental disorders (right bars). Mismatch-siRNA (mm-siRNA) injections did not alter frequency of 3,4-DCA-dependent malformations (left bars). The frequency of developmental disorders was described by calculation of a developmental disorder index (see Section 2 for details, (*) significantly different to embryos injected with the corresponding mismatch-siRNAs, $p < 0.05$, $N = 3$ (*cyp1a*) or 4 (*hmox-1*) experiments with 40–70 embryos each).

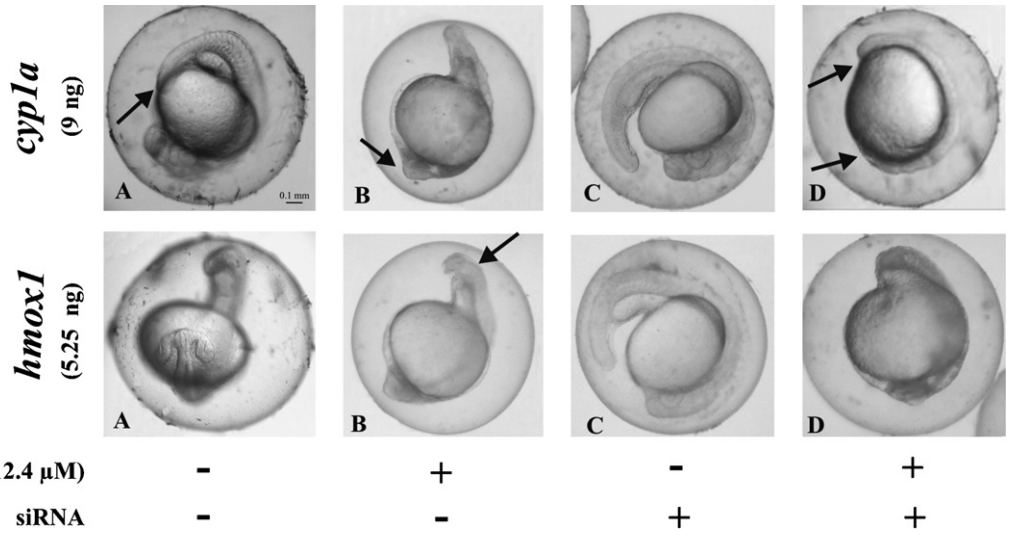

Fig. 5. Overview on the effect of *cyp1a* and *hmox* si-RNA injections on severity of malformations in zebrafish embryos exposed to 3,4-dichloroaniline (3,4-DCA). siRNA injections lead to an increase of severity of malformations (compare B and D). Injection of siRNA into non-exposed control embryos did not cause any phenotypical alterations (A and C). Arrows indicate prominent disorders of head and tail during embryonic development or the corresponding control phenotype (A = non-exposed, non-injected control embryo; bar represents 0.1 mm, B = exposed, non-injected control embryo, C = non-exposed, injected embryo, D = exposed and injected embryo). Embryos were exposed from 2 to 26 hpf to 12.4 µM 3,4-DCA and were analysed at 26 hpf. For details on phenotypes see also Fig. 1.

previously been shown to be sensitively induced in zebrafish embryos by a wide range of chemicals (Voelker et al., 2007; Weil et al., manuscript in preparation). CYP1A is one of the major enzymes involved in phase1 detoxification processes, including biotransformation and deactivation of xenobiotics (Goldstein and Faletto, 1993; Nebert et al., 1989; Riviere and Cabanne, 1987). The *cyp1a* gene is induced via binding of xenobiotics to the AHR, leading to an enhanced transcription rate. The subsequent increase in biotransformation can result in an enhanced biotransformation rate (Goldstein and Faletto, 1993; Nebert et al., 1989; Riviere and Cabanne, 1987). However, CYP1A induction can also be responsible for toxic effects by promoting the generation of active metabolites (Guengerich and Liebler, 1985; Mandal, 2005; Payne et al., 1987; Incardona et al., 2006). Thus, the effect of *cyp1a* induction on toxicity is dependent on the target compounds. Consequently, experimental knockdown of *cyp1a* expression can have opposing effects as has been shown by injection of antisense morpholinos into zebrafish embryos exposed – for instance – to beta-naphtoflavone and pyrene (Billiard et al., 2006; Incardona et al., 2006).

Toxic effects of xenobiotics can also be mediated by interference with cellular homeostasis and signaling pathways caused by deregulation of gene expression. A prominent example is the toxicity of the potent CYP1A inducer TCDD, which is assumed to provoke toxicity via induction of AHR-controlled target genes (Mandal, 2005; Rifkind, 2006; Carney et al., 2006). Initial results indicated a possible role of CYP1A for toxicity of TCDD (Teraoka et al., 2003). However, subsequent studies in CYP1A morphants of zebrafish have shown AHR2 but not CYP1A expression to be required for mediation of toxic effects (Carney et al., 2004). Similar findings have been reported for the toxicity of benz[*a*]anthracene (Incardona et al., 2006).

The protective function of *cyp1a* induction for 3,4-DCA exposure reported here is in line with the metabolism pathways known for (chlorinated) anilines. In zebrafish, chloroanilines are metabolised to acetanilids (Zok et al., 1991). It has been shown that CYP1A2 is responsible for the N-oxidation of arylamines with a subsequent *O*-esterification by *N*-acetyltransferases in rat and human cells (King et al., 1999). Furthermore, the metabolism of 3,4-DCA to hydroxylated dichloroanilines and a weak induction of EROD (ethoxy-resorufin-*O*-deethylase) activity in rat liver (McMillan et al., 1990a, 1990b) indicates that *cyp1a* induction by 3,4-DCA may elevate its metabolic rate. Consequently, excretion of 3,4-DCA would be enhanced and reduce its internal concentration. Since the mode-of-action of 3,4-DCA has been defined as polar narcosis (Arnold et al., 1990), the attenuation of toxicity could result from reduced membrane concentrations. However, this hypothesis is currently not supported by experimental data and further studies on the metabolism of 3,4-DCA in zebrafish embryo would be necessary.

The observed effects of gene knockdown and overexpression suggest that *cyp1a* is translated into an active protein already in prehatched embryos. In contrast, Mattingly and Toscano (2001) have reported *cyp1a* to be transcriptionally silenced in TCDD-treated zebrafish embryo until 3 dpf. However, CYP1A could be detected by immunohistochemistry by Andreasen et al. (2002) in 36 hpf embryos exposed from to TCDD from fertilization and by us in ≥26 hpf embryos exposed to 3,4-DCA from fertilization. The different findings might be explained by technical limitations. Mattingly and Toscana have analysed CYP1A levels by the catalytic measure EROD. This measure is well established for liver microsomal preparations and cell cultures but obtaining a signal in zebrafish embryos before hatching appears difficult, a circumstance that we also found. Immunohistochemical detection combined with the functional analysis, however, provides strong evidence for CYP1A translation in pre-hatched embryos.

The enzyme heme oxygenase 1 is involved in the response of cells to various external stimuli (Alam et al., 2004). The regulation of *hmox1* is very complex and depending on the inducing agent, a participation of transcription factor NRF2 (NFE212), heat shock response elements, stress response elements as well as the mitogen activated protein kinase pathway (Alam et al., 2004, 1999) have been reported. Furthermore, via the regulation of the transcription factor NRF2, the AHR pathway might be involved in the control of *hmox1* transcription (Miao et al., 2005; Voelker et al., 2007). The effects of gene knockdown in mammalian cells suggest a protective role of *hmox1* in the case of oxidative stress (Kaizaki et al., 2006). In the current study a protective role has been experimentally demonstrated for the first time for the zebrafish embryo using 3,4-DCA as a model compound. The underlying mechanism is not known but could be explained by the general cytoprotective function of HMOX and the generation of catalytic products with antioxidant, anti-inflammatory, anti-apoptotic and cell-signaling properties. HMOX1 catalyses the breakdown of heme to bilirubin and its reduced form biliverdin. Both bilirubin and biliverdin exhibit antioxidant activity (Mazza et al., 2003) and may protect the organism against reactive oxygen species (ROS). However, an oxidant function of 3,4-DCA has yet not been reported. Thus, future investigations on the role of *hmox1* should explore if ROS indeed is produced upon exposure of 3,4-DCA. Second, the degradation of heme catalyzed by HMOX1 leads to the generation of carbon monoxide and iron, which are biologically active and could regulate secondary downstream adaptive genes (Jin and Choi, 2005; Maines, 1997). Translation of *hmox1* in zebrafish embryos has not been demonstrated in this study due to the lack of availability of suitable antibodies. However, induction of gene expression has been demonstrated for other stress proteins (Martin et al., 2001) and no evidence for a transcriptional silencing for stress proteins has been reported thus far.

In summary, this study demonstrates that siRNA and mRNA injection in zebrafish embryos can be used to study the function of single genes with respect to the expression of toxicity. To our knowledge, this is the first time that a combination of knockdown and overexpression has been applied to elucidate gene function with respect to toxicity. The opposing observations made by knockdown and overexpression verified the specificity of functional manipulations. Functional studies such as the one presented here could be applied to a wide variety of genes and would be ideally suited to study the role of genes identified from toxicogenomic studies in order to link gene transcription to organism health.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2007.10.007.

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