

UC Davis

UC Davis Previously Published Works

Title

Chronic toxicological effects of β -diketone antibiotics on Zebrafish (*Danio rerio*) using transcriptome profiling of deep sequencing

Permalink

<https://escholarship.org/uc/item/26j6c05w>

Journal

Environmental Toxicology, 31(11)

ISSN

1520-4081

Authors

Wang, Huili
Yin, Xiaohan
Li, Fanghui
[et al.](#)

Publication Date

2016-11-01

DOI

10.1002/tox.22141

Peer reviewed

Chronic Toxicological Effects of β -Diketone Antibiotics on Zebrafish (*Danio rerio*) Using Transcriptome Profiling of Deep Sequencing

Huili Wang,¹ Xiaohan Yin,^{2*} Fanghui Li,² Randy A. Dahlgren,³ Yuna Zhang,² Hongqin Zhang,¹ Xuedong Wang²

¹Department of biochemistry and molecular biology, College of Life Sciences, Wenzhou Medical University, Wenzhou 325035, China

²Department of Environmental Sciences, Wenzhou Medical University, Wenzhou 325035, China

³Department of Land, Air and Water Resources, University of California, Davis, California 95616

Received 27 December 2014; revised 10 March 2015; accepted 18 March 2015

ABSTRACT: Transcriptome analysis is important for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues. Herein, differentially transcribed genes were identified by deep sequencing after zebrafish (*Danio rerio*) were exposed to β -diketone antibiotics (DKAs); 23,129 and 23,550 mapped genes were detected in control and treatment groups, a total of 3238 genes were differentially expressed between control and treatment groups. Of these genes, 328 genes (213 up- and 115 down-regulation) had significant differential expression ($p < 0.05$) and an expression ratio (control/treatment) of >2 or <0.5 . Additionally, we performed Gene Ontology (GO) category and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, and found 266 genes in the treatment group with annotation terms linked to the GO category. A total of 77 differentially expressed transcriptional genes were associated with 132 predicted KEGG metabolic pathways. Serious liver tissue damage was reflected and consistent with the differences in genetic classification and function from the transcriptome analysis. These results enhance our understanding of zebrafish developmental processes under exposure to DKA stress. © 2015 Wiley Periodicals, Inc. *Environ Toxicol* 31: 1357–1371, 2016.

Keywords: transcriptome; β -diketone antibiotics; zebrafish; deep sequencing; immune system

INTRODUCTION

Antibiotics are an important class of pharmaceutical and personal care products that are emerging pollutants in our environment. Fluoroquinolone and tetracycline antibiotics are β -diketone antibiotics (DKAs) owing to their β -diketone functional group. Biological resistance to DKAs is of great concern in the environment due to their large production and wide usage (Yoon et al., 2010). DKAs are absorbed by humans and animals in low amounts and then released to the environment as active metabolites. In recent years, DKA residues in the $\mu\text{g/L}$ to mg/L concentration range have been

Correspondence to: X. Wang; e-mail: zjuwxd@163.com

Contract grant sponsor: National Natural Science Foundation of China.

Contract grant number: 31270548 and 21377100

Contract grant sponsor: Key Project of Zhejiang Provincial Environmental Protection Department.

Contract grant number: 2012B014

Additional Supporting Information may be found in the online version of this article.

*Co-first author.

Published online 6 April 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/tox.22141

© 2015 Wiley Periodicals, Inc.

detected in fish livestock, poultry farms and sewage treatment plants (Mellon et al., 2001; Bueno et al., 2007). Although their half-lives are not very long in the environment, DKAs can create a “pseudo-persistent” phenomenon due to large doses and frequent application (Yahiat et al., 2011), which causes a serious threat to aquatic organisms and human health (Dong et al., 2010).

DKAs can lead to serious environmental biological effects, such as severe liver and kidney toxicity and neurotoxicity. For example, quinolone antibiotics may cause severe central and peripheral neurotoxicity resulting in medicine-source disease symptoms, such as fibromyalgia, respiratory myasthenia gravis, myocarditis, and rheumatism (Yu et al., 2009). Although some DKAs have weak hormone or mutagenic activity, they act on hormone-sensitive pathways and have synergistic effects with other factors, which can lead to severe ecological risk (Sendzik et al., 2010). *In vivo* and *in vitro* experiments have validated that some DKAs interfere with endocrine system, including estrogen activity, causing immune toxicity, feminization, reproductive disorder, abortion, and cancer of the reproductive organs (Lemus et al., 2009). In detoxification metabolism studies, research found that a variety of DKAs affected not only the activity of cytochrome P450, but also those of acetylcholinesterase (AChE) and superoxide dismutase (SOD) (Yu and Yang, 2010; Wang et al., 2014).

DKAs have strong genetic toxicity, such as DNA damage, cross-linking and adduct formation, DNA local hypermethylation of kidney cells in *Carassius auratus*, and teratogenic effects on *Cyprinus carpio* (Li et al., 2010; Khadra et al., 2012). These molecular-level results greatly enhanced toxicity prediction accuracy among different species, different toxicological endpoints, and even different chemical structures (Goto et al., 2010).

In general, most of the previous research on antibiotic toxicology was concerned with acute toxicity under high concentration ($\mu\text{g/L}$ to mg/L range) of a single drug as related to their effects on biological behavior, morphological development, hatching and survival, angiogenesis, etc. Previous molecular toxicological studies focused primarily on the expression level of individual genes and the detection of toxicity biomarkers in a single system. In recent years, the systems toxicology concept was put forward to describe the use of omics technologies to provide information for integrated toxicity testing strategies via the creation of signatures toxicity. These signatures are then linked to underlying pathways of toxicity and pathway information determined from biochemistry and molecular biology to improve the assessment of compound toxicity. The more advanced omics technologies allow acquisition of more reliable data for systems toxicology studies (Heijne et al., 2005).

The zebrafish (*Danio rerio*) genome is now in its ninth version but remains incompletely annotated. Bioinformation approaches have been used to identify important zebrafish genes and the majority of research involving zebrafish RNA-

sequencing has focused on immunity fields (Hegedűs et al., 2009; Ordas et al., 2010). In recent years, the use of deep sequencing to study other biological subjects in zebrafish has increased (Aanes et al., 2011; Aday et al., 2011). At present, we can gain excellent insight into novel genes by deep sequencing, which has proven its utility for zebrafish toxicology studies. Transcriptomic analysis, both descriptive and quantitative, is important for interpreting the functional elements of the genome and for revealing the molecular constituents of cells and tissues. In this investigation, we used the next generation sequencing platform for high throughput analysis of zebrafish transcriptome under DKA antibiotic stress.

Based on our previous research examining biomarkers and behavior of zebrafish exposed to DKAs (Wang et al., 2013), representative DKA species were selected for exposure to zebrafish from the embryonic stage to adulthood at mg/L concentrations. A high-throughput RNA sequencing technique was used to compare transcriptional data of zebrafish in control and DKA-exposure groups. We then obtained differentially expressed genes, compared the transcription differences between control and treatment samples, and determined specific gene markers for correlation with drug-induced diseases. From these data, the molecular mechanisms for chronic toxicity effects and the ecological risk to DKA exposure were assessed. Our data showed that the differentially expressed genes under DKA-exposure were closely related with immunotoxicology, and that they played a key role in immunoregulation by zebrafish. Moreover, the toxicological effects of DKAs were involved in neurobehavioral, metabolic and developmental toxicity, and cancer. Based on the transcriptomic results, we searched for the immune-relevant genes and metabolic pathways using gene ontology (GO) slim classification and KEGG analysis. At the same time, we studied the effects of DKAs on zebrafish immune system according to histopathological analyses, and the changes of superoxide dismutase enzyme (SOD) and glutathione (GSH). SOD is a metal-containing active proteinase and an antioxidant enzyme with a substrate of free radicals. It can effectively clear free radicals resulting from biological oxidation, and thus is a direct indicator for aging and death. GSH is non-protein thiol compound synthesized in the liver and plays an important role in the body's defense system to remove free radicals and enhance immunity. The above indicators were used to verify the results of the transcriptome and to further demonstrate the toxicity effects of DKAs on zebrafish.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the recommendations in the guide for care and use of laboratory animals of Wenzhou Medical University. The protocol was

approved by the Committee on the Ethics of Animal Experiments for Wenzhou Medical University. All zebrafish surgery was performed on ice and all efforts were made to minimize suffering.

Test Chemicals

Six representative DKAs were selected in this study: ciprofloxacin (Amresco, CAS No. 85721-33-1, 99%), ofloxacin (Amresco, CAS No. 82419-36-1, 99%), norfloxacin (Amresco, CAS No. 70458-96-7, 99%), chlortetracycline (Amresco, CAS No. 64-72-2, 95%), enrofloxacin (Amresco, CAS No. 93106-60-6, 99%), and doxycycline (Amresco, CAS No. 24390-14-5, 99%). These drugs were purchased from Ke Jian Biology Science and Technology Co., Ltd, Shanghai, China. As reported previously by our group, the 120-hpf NOEC (no observed effect concentration) of DKAs was 18.75 mg/L (Wang et al., 2014). Therefore, the 9.38 mg/L mixture of DKAs was selected for transcriptome sequencing and histopathological analysis, and 2.34, 9.38, and 37.5 mg/L mixtures of DKAs for biochemical assay according to our previous study (Wang et al., 2014).

Zebrafish Husbandry and Exposure Experiment

Fertilized embryos (6-h postfertilization, 6 hpf) with good quality were collected and exposed in embryonic medium for control, and in embryonic medium with 9.38 mg/L DKAs for treatment. Embryos were randomly distributed in Petri dishes (100 mm) and exposed (50 embryos with 40 mL solution per treatment) for 6 days. All embryos were hatched and survived without malformation at this stage. Zebrafish larva with no malformations were then transferred into 2 L tanks for the period of 6 to 30 days postfertilization (dpf). After 30 days, the zebrafish larva were transferred to 12 L tanks until the end of the experiment at 90 dpf. Zebrafish were kept in a static system that received charcoal-dechlorinated tap water with a constant temperature of $28 \pm 0.5^\circ\text{C}$ and 50% of the water renewed daily with freshly prepared solutions. Dead fish were removed from each tank on a daily basis. Feeding was initiated at day 6 and larva between 6 and 30 dpf were fed three times daily with zebrafish larvae diet (Aquatic Habitats, Inc.); after 30 dpf zebrafish were fed twice daily with freshly hatched live *Artemia* (Jiahong Feed Co., Tianjin, China).

At 90 dpf, zebrafish were placed in an ice bath for 2 to 3 min until they were gently anesthetized. Ten fish from different parental stock and tanks were pooled as one representative sample for RNA isolation.

RNA Isolation, Library Construction, and Sequencing

For RNA isolation, fish were homogenized in liquid nitrogen using a glass homogenizer, and centrifuged at 40,000

$\times g$ for 10 min at 4°C . Total RNA was extracted from zebrafish using TRIzol Reagent (Invitrogen, Carlsbad, CA) and further purified using DynaBeads Oligo(dT)uiz25 following manufacturer's instructions (Invitrogen). Before cDNA synthesis, mRNAs were hydrolyzed by RNA Fragmentation Reagent (Ambion, Austin, TX). DNA contamination was removed with a DNA-freeTM kit (Ambion). RNA quality was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to meet the following requirements: $\text{OD}_{260}/\text{OD}_{280} > 1.8$ and $\text{OD}_{260}/\text{OD}_{230} > 1.5$. RNA concentration was determined with a spectrophotometer (Biotek Instruments, Winooski, VT). The RNA from adult zebrafish for control and treatment groups was removed with a MICROBExpressTM kit (Ambion). The enriched mRNA was converted to a RNA-Seq library using the mRNA-Seq library construction kit (Illumina Inc, San Diego, CA) following manufacturer's protocols. The two samples were pooled for paired-end sequencing using the Sanger-modified Illumina protocol (Quail et al., 2008). Detection results showed that the sequencing depth was sufficient, and the length of sequencing reads was 151 bp. Pyrosequencing generated base-space reads by sequencing-by-synthesis on single stranded templates. Released pyrophosphate (PPi) was indirectly detected by luminescence production in a base-specific manner (Rothberg et al., 2008).

RNA-Seq Data Analysis

For resequencing, data analysis was performed directly on the Illumina MiSeq integrated computer, requiring no specialized servers or computing facilities. The generated 151 bp reads were mapped to Ensembl *Danio rerio* Zv9 (ftp://ftp.ensembl.org/pub/release-66/fasta/danio_rerio/dna/) using Tophat (<http://tophat.cbcb.umd.edu/>); those reads that did not align uniquely to the genome were discarded (Sultan et al., 2008). The quality parameter was set to 30 for use in the Tophat pileup. Each base was assigned a value based on the number of mapped sequence coverage. The quantitative unigene expression value and RPKM metric (reads per kilobase of exon per million mapped reads) were calculated with custom Perl scripts by normalizing the sequence coverage over the gene length and total unique mapped reads in the library (Mortazavi et al., 2008). Analysis of differential expression was determined to obtain the differentially expressed genes between control and treated samples as calculated on the basis of total read counts per transcript using Bayesian methods. The number of uniquely mapped read-pairs for each gene in each sample was stored. The total number of mapped reads in each lane was normalized using the total mapped reads in each lane. Finally, the differentially expressed genes were defined as those showing at least a 2.0-fold change in transcription expression level as determined by Bayesian statistical evaluation.

TABLE I. Summary of RNA-Seq and data analysis results

Sample	Total Reads	Read Length (bp)	Total Nucleotides (nt)	Mapped Reads	Range in Expression Levels (RPKM)
Control	9,013,934	151	1,361,104,034	6,977,499	$2.6 \times 10^{-2} \sim 6.7 \times 10^3$
Treatment	13,519,220	151	2,041,402,220	8,719,694	$2.1 \times 10^{-2} \sim 2.9 \times 10^3$

Gene Ontology and KEGG Pathway Analysis

Based on similarity searches with known genes, as well as their protein functional annotation, unigene sequences were annotated according to a Blast hit in the non-redundant (nr) and Swiss-Prot protein database results with an *E*-value of less than 10^{-5} . Unigenes that did not have a homolog in these databases were scanned using ESTScan. After nr annotation, GO analysis of all differentially expressed genes was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) software to obtain GO annotations. These results were used to perform GO functional classification in order to understand the distribution of gene functions at the macrolevel (Conesa et al., 2005). The KEGG database contains systematic analysis of inner-cell metabolic pathways and functions of individual gene products. The biochemical pathway information was collected by downloading relevant maps from the KEGG database (Kanehisa and Goto, 2000).

qRT-PCR Analyses of the Differentially Expressed Genes

Primer sets were designed using Premier 5.0 software and synthesized by Shanghai Sangon Biotechnology Company (Shanghai, China). To validate the genes (*LOC100334180* and *sdcl1b*) of zebrafish at the transcription level *in vivo*, qRT-PCR was performed using the StepOne™ RT-PCR System (Applied Biosystem, Foster, CA). Total RNA was extracted from zebrafish and added into RNA later to prevent degradation. The quality of RNA was analyzed as reported above (Zebrafish husbandry and exposure experiment section). After the RNA was determined to be free of protein and salt contamination, cDNA was obtained by reverse transcription using a PrimeScript RT-PCR Kit (TaKaRa, Japan), following an initial denaturation time of 5 min at 95°C, and 35 cycles of amplification comprised of a denaturation step for 1 min at 95°C. Each experimental group was performed using three technical replicates. Relative quantification of the targets in each sample was determined using the signal of β -actin as a stable reference gene.

Histopathological Analysis

The concentration for DKAs was set on the basis of no obvious malformation of zebrafish when exposed at 9.38 mg/L DKAs for 90 dpf. After anatomy, the liver of zebrafish was removed, and fixed in precooled glutaraldehyde at 4°C. Then,

a series of pretreatment procedures (poaching, fixing, dehydrating, embedding, and sectioning) were performed according to Sendzik et al. (2010). The treated tissue samples were observed by transmission electron microscope (TEM).

Statistical Analysis

The experimental data were reported as mean $\pm$ SD (standard deviation, $n = 3$). Outliers were removed from each set of measured values, which were defined as values falling outside the range of mean $\pm$ 2SD. One-way ANOVA was performed to calculate statistical significance followed by post hoc Dunnett's or Tukey's tests to independently compare each exposure group with the control group. All statistical analyses were conducted using SPSS 18.0 software (SPSS, Chicago, IL) to assess the significance at $p < 0.05$ level, unless otherwise stated.

RESULTS

Transcriptome in Control and Treatment Groups

The control and treated samples were each pooled for deep sequencing analysis using Illumina high throughput second generation paired-end sequencing. The deep sequencing produced 9,013,934 and 13,519,220 clean reads representing a total of 1,361,104,034 (1.36 Gb) and 2,041,402,220 (2.04 Gb) nucleotides (Table I). All sequence reads were mapped to known genes in Ensembl Danio rerio Zv9 in order to reveal the molecular characteristics of the transcriptome between control and treatment groups. A total of 26,100 genes were identified with as few as one mapped read pairs, constituting 23,129 (88.6%, control) and 23,550 (90.2%, treatment) genes with detectable expression within the total known zebrafish genes in the Ensembl database. As indicated in Figure 1, both the transcriptomes in control and treatment groups showed a relatively continuous distribution of gene expression levels. Similar to previous RNA-seq studies in other tissues, there were only a few genes which had high expression levels, while most genes were expressed at very low levels.

The dominant characteristics of the mRNA in the cell were heterogeneous and redundant, with a few types having extremely high abundance, but the majority having very low expression. In the clean reads database, the number of read

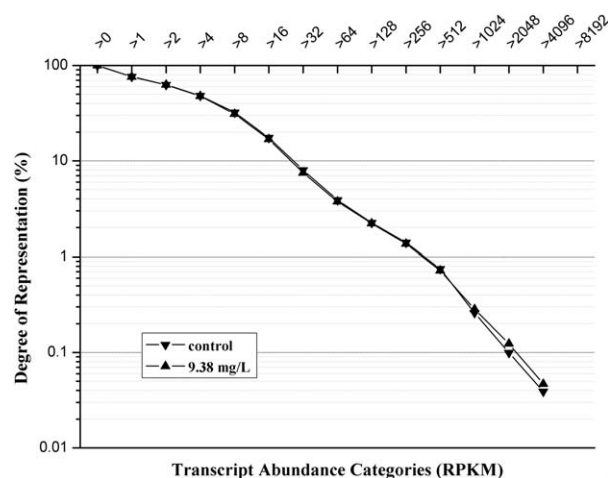


Fig. 1. Distribution of transcript entries and total transcript counts over different tag abundance categories. The percentages of total transcript counts and number of different transcript entries per category are plotted on a logarithmic scale (base 10).

copies reflected the quantitative level of gene expression. It has been documented in previous studies that RNA-seq can readily detect gene expression levels across a broad dynamic range (Wang et al., 2009). The gene expression levels in zebrafish ranged from 0.021 to 6748 RPKM, showing a dynamic range of more than five orders of magnitude in RNA concentration. The average read size in two samples was 151-bp with Q30 percentage up to 87.0% and GC content up to 49.3%. All annotated genes were analyzed for evidence of differential expression. A total 3238 genes in the treatment group were differentially expressed when compared with the control group. Of those genes, 328 genes (213 up- and 115 down-regulated genes) were considered to be significant with $p < 0.05$ and an expression ratio (control/treatment) > 2 or < 0.5 . The top 15 differentially expressed genes and five no differential expression genes are summarized in Table II; these genes were further applied to studies of gene function, protein product, and comparative genomics.

GO Slim Classification

To determine the biological relevance of the gene pool, a GO analysis was performed using DAVID software for annotation and identification of novel genes expressed in zebrafish. A total of 266 differentially expressed genes from zebrafish were assigned for GO analysis based on matches with sequences of known functions. Comparing the distribution of GO categories from the total Ensembl *Danio rerio* Zv9 database (9631 Unigene clusters), the differentially expressed genes with unknown functions in the treatment group fell within three classifications: biological process, molecular function, and cellular component (Fig. 2). Within

the 266 significantly differentially expressed genes, a large proportion of genes was involved in cell, cell part (cellular component), binding, catalytic (cellular component), cellular process, metabolic process (biological process). In the cellular component classification, most genes were involved in housekeeping functions, such as cell, extracellular, and organelle. For the molecular function classification, binding was significantly enriched ($p < 0.05$) in the treatment group. For the biological process classification, genes functioning in immune system processes, cellular process, metabolic process, response to stimulus and biological regulation were enriched in the treatment group. For DKAs-exposed zebrafish, the enriched categories of molecular function and biological process suggest that the abundance of immunological reaction traits is of particular interest, which may infer an immune response.

Metabolic Pathways by KEGG Analysis

A total of 77 differentially expressed genes were associated with 132 predicted KEGG metabolic pathways, and the number of genes in different pathways ranged from 1 to 16. The four major pathways (metabolic pathways, human diseases, environmental information processing, and organismal systems) included over 51 genes. The most important pathways that may be relevant to bacterial (*Staphylococcus aureus*) infection included infectious diseases (13), immune diseases (5), endocrine system (7), immune system (3), PPAR signaling pathway (2), MAPK signaling pathway (2), transport and catabolism (3), carbohydrate metabolism (8), and other antihyperthermia stress and anti-oxidative stress pathways or gene families. These predicted pathways are likely to be useful for further investigation of their functions in DKA-exposed zebrafish. We Listed two differentially expressed genes: tubulin, alpha 7-like and prostaglandin E receptor 2a (subtype EP2) (Supporting Information Fig. 1).

qRT-PCR Results

As listed in Table III, one gene (multidrug resistance-associated protein 1-like) was found to be down-regulated when compared with control group, while another (sidekick cell adhesion molecule 1b) was up-regulated. The up-regulated gene showed more than twofold up-regulation (5.11-fold for *sdcl1b*), and *LOC100334180* was no obvious expression changes but associated with ATPase activity, coupled to transmembrane movement of substances.

Histopathological Analysis

The liver is the main immune and metabolic organ in humans and animals, and it regulates the body metabolic balance through large numbers of both innate and adaptive immune cells. Thus, the histopathological observation of zebrafish liver tissue was conducted by TEM for control [Fig. 3(A,B)] and DKA-exposed treatment groups [Fig. 3(C–

TABLE II. Top 15 significantly differential expression genes and five no differential expression genes by DKAs exposure in RNA-seq dataset

Ensembl Version	Gene ID	Gene Name	Official Symbol	Fold Change	log2Fold Change	Location Chromosome	p-Value	GO Annotation
ENSDARG000000078472	100004222	Heat shock transcription factor family member 5	hsf5	56.74	5.82	Un	0.00015	Molecular function: DNA binding; sequence-specific DNA binding transcription factor activity; cellular component: nucleus; biological process: regulation of transcription, DNA-templated; response to stress;
ENSDARG000000004221	266676	Dynein, axonemal, heavy polypeptide 9	dnah9	48.63	5.60	12	0.00059	biological process: cilium or flagellum-dependent cell motility, determination of left/right symmetry; molecular function; cellular component
ENSDARG000000039657	100005699	Fibronectin type III and ankyrin repeat domains 1	fank1	47.01	5.55	12	0.00077	Molecular function: protein binding
ENSDARG000000035862	563417	Insulin-like 3 (Leydig cell)	insl3	35.12	5.13	2	6.28E-07	Molecular function: hormone activity; cellular component: extracellular region; biological process
ENSDARG000000000516	368636	Ankyrin and armadillo repeat containing	ankar	33.64	5.07	9	8.06E-05	Molecular function: binding, protein binding; cellular component;
ENSDARG0000000094668	100034589	si:ch211-51a19.5	si:ch211-51a19.5	29.99	4.90	20	6.40E-06	Molecular function: nucleotide binding, microtubule motor activity,
ENSDARG0000000061486	569992	si:dkeyp-86b9.1	si:dkeyp-86b9.1	29.83	4.89	5	8.27E-09	ATP binding, ATPase activity, nucleoside-triphosphatase activity; cellular component: dynein complex
ENSDARG000000002480	431777	Tubulin, alpha 7 like	tuba7l	29.29	4.87	1	2.99E-11	Molecular function: nucleotide binding, GTPase activity, structural molecule activity, GTP binding; cellular component: cytoplasm,

TABLE II. Continued

Ensembl Version	Gene ID	Gene Name	Official Symbol	Fold Change	log2Fold Change	Location Chromosome	p-Value	GO Annotation
ENSDARG00000011434	393608	Prostaglandin E receptor 2a (subtype EP2)	ptger2a	29.18	4.86	17	0.015	cytoskeleton, microtubule, protein complex; biological process: GTP catalytic process, microtubule-based process, microtubule-based movement, protein polymerization
ENSDARG00000009443	445049	zgc:92137	zgc:92137	0.079	-3.65	17	8.69E-32	Molecular function: signal transducer activity, receptor activity, G-protein coupled receptor activity, coupled receptor activity, prostaglandin E receptor activity; biological process: signal transduction, G-protein coupled receptor signaling pathway; cellular component: integral component of membrane
ENSDARG00000070206	368748	si:busm1-160c18.6	si:busm1-160c18.6	0.067	-3.88	8	0.046	Molecular function: protein binding Biological process: antigen processing and presentation of peptide or polysaccharide antigen via MHC class II, immune response, antigen processing and presentation; molecular function: protein binding; cellular component-membrane, integral component of membrane, MHC class II protein complex
ENSDARG000000045164	556906	Neuronal pentraxin IIb	nptx2b	0.062	-4.00	12	0.033	Biological process: regulation of circadian rhythm, regulation of neuronal synaptic plasticity

TABLE II. Continued

Ensembl Version	Gene ID	Gene Name	Official Symbol	Fold Change	log2Fold Change	Location Chromosome	p-Value	GO Annotation
ENSDARG000000075626	565611	zgc:172090	zgc:172090	0.050	-4.30	3	0.012	Molecular function: GTP binding; cellular component; biological process
ENSDARG000000093796	565204	im:7150573	im:7150573	0.044	-4.48	7	7.46E-10	Molecular function: DNA binding, sequence-specific DNA binding transcription factor activity, sequence-specific DNA binding; cellular component: nucleus; biological process: transcription, DNA-templated, regulation of transcription, DNA-templated, multicellular organismal development
ENSDARG000000059263	100006598	Homeobox D12a	hoxd12a	0.028	-5.11	9	0.0002	
ENSDARG000000009582	100334180	Multidrug resistance-associated protein 1-like	LOC100334180	1.79	0.84	3	0.14	Molecular function: ATPase activity, coupled to transmembrane movement of substances, ATP binding
ENSDARG000000055652	794259	ATP citrate lyase b	aclyb	0.86	-0.20	12	0.77	Molecular function: cofactor binding, ATP citrate synthase activity, transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer, succinate-CoA ligase (ADP-forming) activity, catalytic activity, ATP binding cellular component: cytoplasm biological process: cellular carbohydrate metabolic process

TABLE II. Continued

Ensembl Version	Gene ID	Gene Name	Official Symbol	Fold Change	log2Fold Change	Location Chromosome	p-Value	GO Annotation
ENSDARG000000010130	564392	Sphingomyelin phosphodiesterase, acid-like 3B	smpd3b	0.88	-0.18	16	0.82	Biological process: sphingomyelin catabolic process molecular function: hydrolase activity, sphingomyelin phosphodiesterase activity cellular component: extracellular space
ENSDARG000000074670	567405	Megakaryocyte-associated tyrosine kinase	matk	0.81	-0.30	22	1.00	Biological process: phosphorylation, protein phosphorylation, molecular function: protein kinase activity, nucleotide binding, ATP binding, transferase activity, transferring phosphorus-containing groups
ENSDARG000000062854	555553	Sidekick cell adhesion molecule 1b	sdclb	0.69	-0.54	1	0.63	

TABLE III. qRT-PCR Analysis Results

Ensembl Version	Gene ID	Gene Name	Official Symbol	Primer Sequence	qPCR Results
ENSDARG00000009582	100334180	Multidrug resistance-associated protein 1-like	LOC100334180	F: CCTAAGCTGGATTGTGCG R: AACTGGGTTCGGATGGTG	-1.69
ENSDARG000000062854	555553	Sidekick cell adhesion molecule 1b	sdclb	F: GACACCGCTCCGTACTTT R: AACCACGATTCTCTTGTATTAT	+5.11

“+” indicates the up-regulated expression of genes in qRT-PCR. “-” indicates the down-regulated expression of genes in qPCR.

(Lü et al., 2012). Transcriptomic analysis provides a valuable tool for further study of the specific mechanisms resulting from DKA exposure on the zebrafish immune system

during its complex life cycle. So we discussed the immune effect of DKAs on zebrafish emphatically in this experiment.

According to the transcriptome results, we found some genes functioned in immune system processes and oxidoreductase activity. In our previous study (Wang et al., 2014), GSH concentrations in the 2.34 and 9.38 mg/L treatments were significantly higher ($p < 0.01$ and $p < 0.05$) than the control at 72 hpf, suggesting that the response was primarily induced at the end of the hatching period. With prolonged exposure time, GSH concentrations recovered to a level similar to the control at 96 hpf, with the exception of the 37.5 mg/L treatment. However, GSH concentrations were significantly inhibited at 120 hpf, especially for the 9.38 and 37.5 mg/L treatments ($p < 0.01$). At 120 hpf, SOD activities were reduced in all DKA-exposed groups ($p < 0.05$), a dose-dependent relationship between SOD activity and DKA concentration (Supporting Information Figs. 2 and 3). SOD and GSH are key enzymes providing antioxidant protection to organisms for preventing immune damage. SOD is closely related to immune processes of aquatic organisms and plays an important role in improving the phagocytic defense capabilities and the body's immune function. Therefore, SOD is commonly used as an immune-evaluating index for aquatic animals (Elchuri et al., 2005). GSH is an important tripeptide providing detoxification functions and plays a direct or indirect role in cell life activities including regulation of gene expression, enzyme activity, metabolic regulation, protection of cells, amino acid transport, and immune function accommodation (Romeu et al., 2002).

The balance of reactive oxygen species (ROS) generation and clearing is important to life processes. If the generation of ROS increases rapidly, it will result in oxidative stress and antioxidant defense balance disorders, leading to all sorts of biological macromolecular oxidative damage (Elchuri et al., 2005). The main function of SOD is removing O_2^- to avoid the inhibition of catalase and glutathione peroxidase activity and ensure proper functioning of the enzymatic antioxidant system. Across all investigated DKA concentrations (2.34–37.50 mg/L), the SOD activities at 120 hpf were significantly inhibited, suggesting that DKA exposure led to severe oxidative damage to zebrafish larvae. At 72 hpf, a significant rise in GSH was induced by 2.34 mg/L DKAs possibly implying more ROS production. With

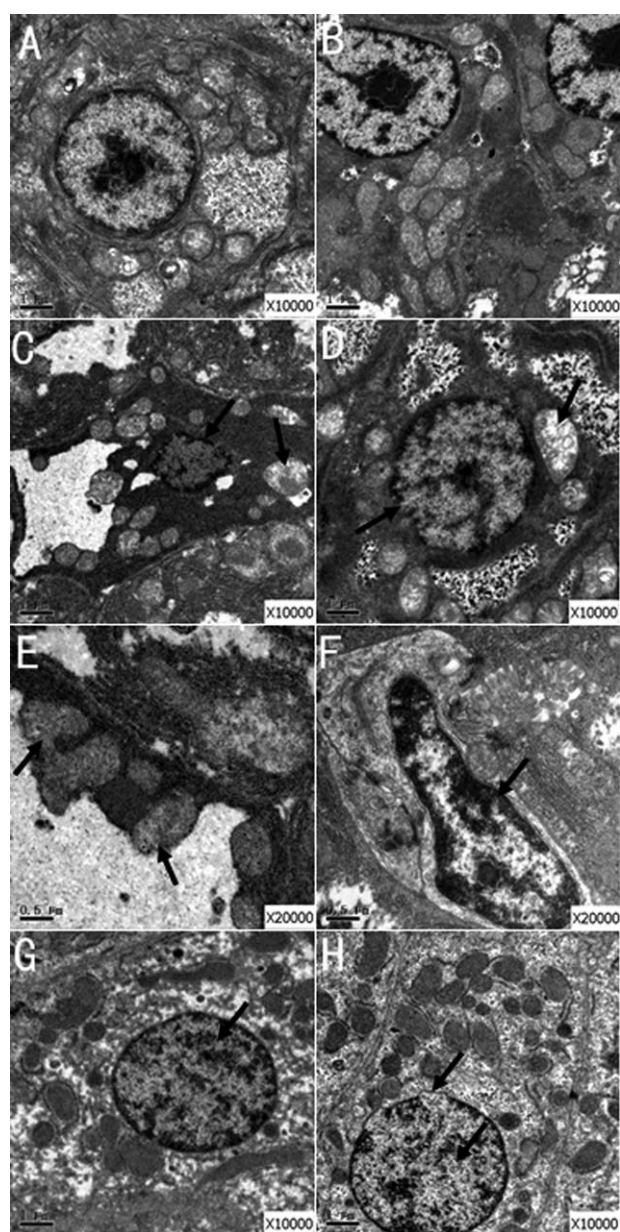


Fig. 3. Histopathological analysis of liver tissue.

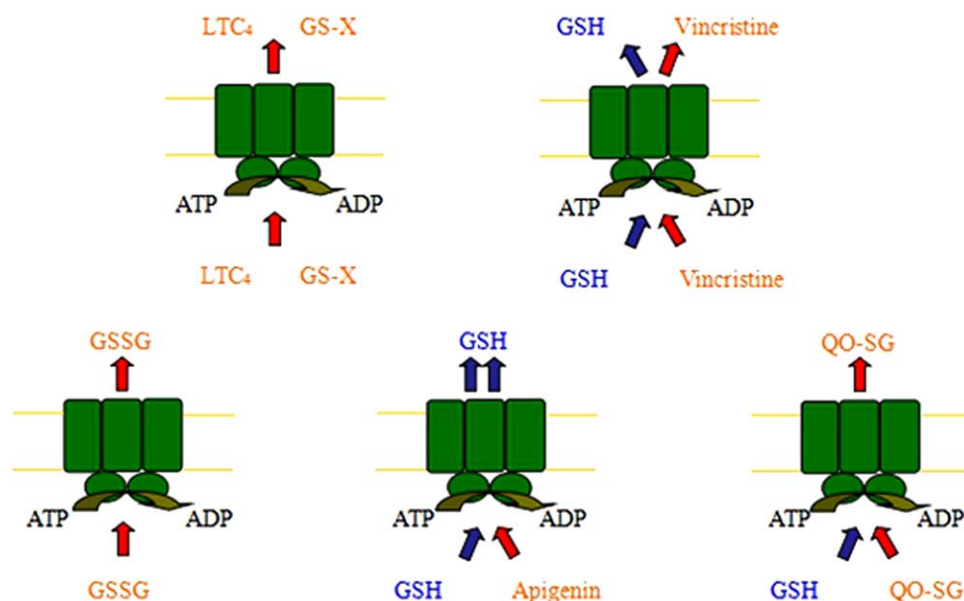


Fig. 4. Multiple mechanisms of ATP-dependent transport by MRP1. [Color figure can be viewed at wileyonlinelibrary.com]

increasing exposure time from 96 to 120 hpf, the GSH concentration returned to levels similar to that of the control group. This may be explained by activation of other antioxidant systems to maintain normal levels of GSH. After DKA exposure, zebrafish liver generated histopathologic lesions and inhibition of SOD activity, suggesting decreasing immune function and disruption of the immune system balance. Reduced GSH concentration and SOD activity along with liver injury suggested that long-term exposure to DKAs resulted in immune system disorder and a decrease in zebrafish heterologous poison resistance. These SOD and GSH responses to DKA stress were consistent with the results of the transcriptome, i.e., some significantly up- or down-regulated genes were closely related to the immune system and anti-oxidative stress pathways. In addition, the expression quantity changes in multidrug resistance protein 1 (MRP1 or ABCC1) indicated possible damage of the immune system according to qRT-PCR results, despite of no significantly differential expression in the transcriptome.

Multidrug resistance protein 1 (MRP1) expresses in tumor cells caused by drug resistance, as well as in normal tissues. It outflows a variety of drugs as a member of the ATP-binding cassette family by using energy from ATP binding and hydrolysis. Apart from outside resistance to drugs, which results in a reduction in drug concentration, MRP1 effluxes many endogenous conjugated organic anions and metabolites of xenobiotics, which play a physiological and a protective role in both normal and harmful tissues (Wijnholds et al., 2000; Leslie et al., 2005). A majority of chemotherapeutic drugs are transported by MRP1, which can mediate resistance mechanisms related to the specificity of intracellular GSH levels (Fig. 4) (Cole and Deeley, 2006).

The reduced cellular GSH concentration affects MRP1-mediated drug transport, and MRP1 can cause multidrug resistance by means of promoting GSH conjugation with drugs and improving discharge of drugs from cells. Thus, GSH not only acts as an antioxidant but also plays an important role in xenobiotic elimination reactions. GSH promotes the export of various xenobiotics in MRP1-overexpressing cells (Renes et al., 1999). The depletion of intracellular GSH by copper *N*-(2-hydroxyacetophenone) glycinate reduced MRP1 expression (Mookerjee et al., 2006).

After long-term and low-dose exposure to DKAs, multidrug resistance-associated protein 1 was down-regulated, and a similar decreasing trend was observed for the GSH concentration with prolonged exposure time after 120 hpf. The decreased multidrug resistance of zebrafish possibly results from the declined chance of antibiotics, in their form of archetype or metabolites, conjugation with GSH, and the further transport barrier of exogenous substances mediated by MRP1. The transport barrier can lead to difficult discharge of toxic cellular substances, immune system disorder and oxidative stress injury. MRP1 also plays a role in the cellular efflux of reduced and oxidized forms of GSH, and thus contributes to many physiological and pathophysiological processes influenced by these small peptides under conditions of oxidative stress (Cole, 2014). Several studies have demonstrated that MRP mediates GSH efflux during apoptosis (Hammond et al., 2007; Fico et al., 2008). Therefore, it can be inferred that the decreased GSH concentration is possibly related to down-regulation of MRP1 expression. However, it was reported by Franco et al. (2014) that GSH depletion was independent of MRP1 activity, i.e., the down-regulation of MRP proteins and MRP transport activity did not regulate

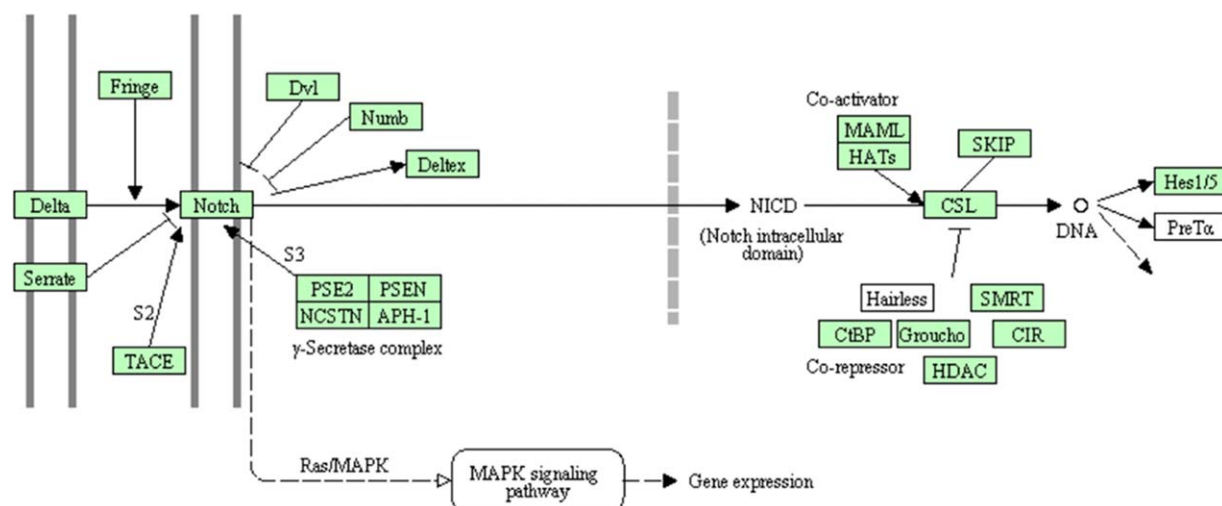


Fig. 5. Notch signal pathway. [Color figure can be viewed at wileyonlinelibrary.com]

GSH depletion and/or apoptosis. Thus, further investigation is required concerning the relationship between the down-regulation of MRP1 expression and the decreased concentration of GSH under DKA stress. MRP1 expression may also be affected by the Notch signal pathway (Fig. 5) (<http://www.kegg.jp/kegg/>), previous research has shown a relationship between expression of MRP1 and Notch1 in cancers (Meani et al., 2005; Yauch et al., 2008). Therefore, further research is required to investigate whether or not the expression of MRP1 is still under the influence of the Notch signaling pathway when exogenous toxic substances invade normal tissue.

Overall, RNA-seq presents an effective and high throughput strategy for capturing molecular snapshots of chemical-induced biological states of a whole adult vertebrate. It also provides a sensitive and unbiased alternative to analysis with major advantages for detection of a broad range of transcriptional responses that occur during drug exposure, as exemplified in this study using the DKA-exposed zebrafish. Many candidate genes were proposed for their potential involvement in immune system reactions, and future work will be able to build upon these findings to provide deeper insights into their functions and to understand their molecular basis for immunology in model organisms.

CONCLUSIONS

Data obtained in this study provide abundant evidence that long-term DKA exposure to zebrafish caused significant damage to its immune systems. By deep sequencing, thousands of genes were found to be differentially expressed between control and DKA treatments, among which 328 genes (213 up- and 115 down-regulation) had significant differentially expressed genes ($p < 0.05$) and an expression ratio (control/treatment) of > 2 or < 0.5 . Through GO category and KEGG pathway enrichment analysis, 266 genes in

the DKA treatment group were found with annotation terms linked to the GO category, and 77 differentially expressed transcriptional genes were associated with 132 predicted KEGG metabolic pathways. Changes in concentrations of SOD and GSH under DKA stress were consistent with the results of transcriptome, i.e., some significantly up- or down-regulated genes were closely related to the immune system and antioxidative stress pathways. Serious liver tissue damage further verified the differences in genetic classification and function in the transcriptome analysis. These results provide a foundation for establishing systems toxicology models, explicating their ecological risk and treatment of medicine source disease.

REFERENCES

- Aanes H, Winata CL, Lin CH, Chen JP, Srinivasan KG, Lee SG, Lim AY, Hajan HS, Collas P, Bourque G, Gong Z, Korzh V, Aleström P, Mathavan S. 2011. Zebrafish mRNA sequencing deciphers novelties in transcriptome dynamics during maternal to zygotic transition. *Genome Res* 21:1328–1338.
- Aday AW, Zhu LJ, Lakshmanan A, Wang J, Lawson ND. 2011. Identification of cis regulatory features in the embryonic zebrafish genome through large-scale profiling of H3K4me1 and H3K4me3 binding sites. *Dev Biol* 357:450–462.
- Bueno MJ, Aguera A, Gomez MJ, Hernando MD, Garcia-Reyes JF, Fernandez-Alba AR. 2007. Application of liquid chromatography/quadrupole-linear ion trap mass spectrometry and time-of-flight mass spectrometry to the determination of pharmaceuticals and related contaminants in wastewater. *Anal Chem* 79:9372–9384.
- Cole SP. 2014. Targeting multidrug resistance protein 1 (mrp1, abcc1): Past, present, and future. *Annu Rev Pharmacol Toxicol* 54:95–117.
- Cole SP, Deeley RG. 2006. Transport of glutathione and glutathione conjugates by mrp1. *Trends Pharmacol Sci* 27:438–446.

- Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. 2005. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21:3674–3676.
- Dong LX, Xie XJ, Zhou QX, Huang PP. 2010. Molecular ecotoxicology of antibiotics, an emerging type of environmental contaminants: A review. *Chin J Ecol* 29:2042–2048.
- Elchuri S, Oberley TD, Qi W, Eisenstein RS, Jackson Roberts L, Van Remmen H, Epstein CJ, Huang TT. 2005. CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* 24:367–380.
- Fico A, Manganelli G, Cigliano L, Bergamo P, Abrescia P, Franceschi C, Martini G, Filosa S. 2008. 2-Deoxy-d-ribose induces apoptosis by inhibiting the synthesis and increasing the efflux of glutathione. *Free Radic Biol Med* 45:211–217.
- Franco R, Bortner CD, Schmitz I, Cidlowski JA. 2014. Glutathione depletion regulates both extrinsic and intrinsic apoptotic signaling cascades independent from multidrug resistance protein 1. *Apoptosis* 19:117–134.
- Goto K, Yabe K, Suzuki T, Jindo T, Sanbuissho A. 2010. Chondrotoxicity and toxico-kinetics of novel quinolone antibacterial agents DC-159a and DX-619 in juvenile rats. *Toxicology* 276:122–127.
- Hammond CL, Marchan R, Krance SM, Ballatori N. 2007. Glutathione export during apoptosis requires functional multidrug resistance-associated proteins. *J Biol Chem* 282:14337–14347.
- Hegedűs Z, Zakrzewska A, Ágoston VC, Ordas A, Rácz P. 2009. Deep sequencing of the zebrafish transcriptome response to mycobacterium infection. *Mol Immunol* 46:2918–2930.
- Heijne WH, Kienhuis AS, van Ommen B, Stierum RH, Groten JP. 2005. Systems toxicology: applications of toxicogenomics, transcriptomics, proteomics and metabolomics in toxicology. *Expert Rev Proteomics* 2:767–780.
- Kanehisa M, Goto S. 2000. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28:27–30.
- Khadra A, Pinelli E, Lacroix MZ, Bousquet-Melou A, Hamdi H, Merlina G, Guisresse M, Hafidi M. 2012. Assessment of the genotoxicity of quinolone and fluoroquinolones contaminated soil with the vicia faba micronucleus test. *Ecotoxicol Environ Saf* 76:187–192.
- Lemus JA, Blanco G, Arroyo B, Martínez F, Grande J. 2009. Fatal embryo chondral damage associated with fluoroquinolones in eggs of threatened avian scavengers. *Environ Pollut* 157:2421–2427.
- Leslie EM, Deeley RG, Cole SP. 2005. Multidrug resistance proteins: role of P-glycoprotein, mrp1, mrp2, and BCRP (abcg2) in tissue defense. *Toxicol Appl Pharmacol* 204:216–237.
- Li QQ, Peng SQ, Sheng ZG, Wang YM. 2010. Ofloxacin induces oxidative damage to joint chondrocytes of juvenile rabbits: Excessive production of reactive oxygen species, lipid peroxidation and DNA damage. *Eur J Pharmacol* 626:146–153.
- Lü AJ, Hu XC, Xue J, Zhu JR, Wang Y, Zhou GZ. 2012. Gene expression profiling in the skin of zebrafish infected with *Citrobacter freundii*. *Fish Shellfish Immunol* 32:273–283.
- Meani N, Minardi S, Licciulli S, Gelmetti V, Coco FL, Nervi C, Pelicci PG, Müller H, Alcalay M. 2005. Molecular signature of retinoic acid treatment in acute promyelocytic leukemia. *Oncogene* 24:3358–3368.
- Mellon M, Benbrook C, Benbrook KL. 2001. Hogging it Estimates of Antimicrobial Abuse in Livestock. Washington, DC: Union of Concerned Scientists Publications. pp 7–9.
- Mookerjee A, Basu JM, Majumder S, Chatterjee S, Panda GS, Dutta P, Pal S, Mukherjee P, Efferth T, Roy S, Choudhuri SK. 2006. A novel copper complex induces ROS generation in doxorubicin resistant Ehrlich ascitis carcinoma cells and increases activity of antioxidant enzymes in vital organs in vivo. *BMC Cancer* 6:267.
- Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. 2008. Mapping and quantifying mammalian transcriptomes by RNA-seq. *Nat Methods* 5:621–628.
- Ordas A, Hegedus Z, Henkel CV, Stockhammer OW, Butler D, Jansen HJ, Raczy P, Mink M, Spaink HP, Meijer AH. 2010. Deep sequencing of the innate immune transcriptomic response of Zebrafish embryos to Salmonella infection. *Fish Shellfish Immunol* 31:716–724.
- Quail MA, Kozarewa I, Smith F, Scally A, Stephens PJ. 2008. A large genome center's improvements to the illumina sequencing system. *Nat Methods* 5:1005–1010.
- Renes J, de Vries EG, Nienhuis EF, Jansen PL, Müller M. 1999. ATP and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein mrp1. *Br J Pharmacol* 126:681–688.
- Romeu M, Mulero M, Giralt M, Folch J. 2002. Parameters related to free radicals in erythrocytes, plasma and epidermis of the hairless rat. *Life Sci* 71:1739–1749.
- Rothberg JM, Leamon JH. 2008. The development and impact of 454 sequencing. *Nat Biotechnol* 26:1117–1124.
- Sendzik J, Shakibaei M, Schäfer-Korting M, Lode H, Stahlmann R. 2010. Synergistic effects of dexamethasone and quinolones on human-derived tendon cells. *Int J Antimicrob Agents* 35:366–374.
- Sultan M, Schulz MH, Richard H, Magen A, Klingenhoff A, Scherf M, Seifert M, Borodina T, Soldatov A, Parkhomchuk D, Schmidt D, O'Keeffe S, Haas S, Vingron M, Lehrach H, Yaspo ML. 2008. A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. *Science* 321:956–960.
- vander Vaart M, van Soest JJ, Spaink HP, Meijer AH. 2013. Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. *Dis Model Mech* 6:841–854.
- Wang HL, Che BG, Duan AL, Mao JW, Dahlgren RA, Zhang MH, Zhang HQ, Zeng AB, Wang XD. 2014. Toxicity evaluation of β -diketone antibiotics on the development of embryonic larval zebrafish (*Danio rerio*). *Environ Toxicol* 29:1134–1146.
- Wang Z, Gerstein M, Snyder M. 2009. RNA-seq: A revolutionary tool for transcriptomics. *Nat Rev Genet* 10:57–63.
- Wijnholds J, deLange EC, Scheffer GL, van den Berg DJ, Mol CA, van der Valk M, Schinkel AH, Scheper RJ, Breimer DD, Borst P. 2000. Multidrug resistance protein 1 protects the

- choroid plexus epithelium and contributes to the blood cerebrospinal fluid barrier. *J Clin Invest* 105:279–285.
- Yahiat S, Fourcade F, Brosillon S, Amrane A. 2011. Removal of antibiotics by an integrated process coupling photocatalysis and biological treatment - Case of tetracycline and tylosin. *Int Biodeterior Biodegradation* 65:997–1003.
- Yauch RL, Gould SE, Scales SJ, Tang T, Tian H, Ahn CP, Marshall D, Fu L, Januario T, Kallop D, Nannini-Pepe M, Kotkow K, Marsters JC, Rubin LL, de Sauvage FJ. 2008. A paracrine requirement for hedgehog signaling in cancer. *Nature* 455:406–410.
- Yoon Y, Ryu J, Oh J, Choi B-G, Snyder SA. 2010. Occurrence of endocrine disrupting compounds, pharmaceuticals, and personal care products in the Han river (Seoul, South Korea). *Sci Total Environ* 408:636–643.
- Yu HY, Wang BL, Zhao J, Yao XM, Gu Y, Li Y. 2009. Protective effect of bicyclol on tetracycline-induced fatty liver in mice. *Toxicology* 261:112–118.
- Yu LZ, Yang XL. 2010. Effects of fish cytochromes p450 inducers and inhibitors on difloxacin N-demethylation in kidney of Chinese idle (*Ctenopharyngodon idellus*). *Environ Toxicol Pharmacol* 29:202–208.