Histopathological and proteomic responses in male Chinese rare minnow (Gobiocypris rarus) indicate hepatotoxicity following benzotriazole exposure*

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ABSTRACT

Benzotriazole (BT) and its associated derivatives are used ubiquitously in industrial processes, and can be detected in indoor temperature coolants and in chemicals designed to inhibit corrosion. This chemical has been widely detected in aquatic environments and shows some degree of environmental persistence. Evidence has shown that BT exposure can negatively affect endocrine systems and can result in neurotoxicity in fish. However, no study has examined whether this chemical exhibits hepatotoxicity in fish, and if so, what are the underlying mechanism associated with the damage. To address this knowledge gap, we measured the liver proteome of adult male Chinese rare minnow (Gobiocypris rarus) exposed to either 0.05, 0.5, or 5 mg/L BT for 28 days. Overall, 17 proteins were induced and 9 were reduced in abundance following BT treatment (ratio > 1.5, p < 0.05). Pathway analysis revealed that cellular processes affected by BT included xenobiotic clearance, oxidative stress response, apoptosis, and translation. Moreover, transcripts related to these toxic pathways were also significantly affected by BT. In addition, rare minnows exposed to BT showed signs of hypertrophy of hepatocytes, nuclei pyknosis, and higher levels of cellular vacuolization compared to the controls, thus these early proteomic responses in the liver may be related to pathology (i.e. adverse outcome pathway). Our data demonstrate that BT dysregulates molecular responses in the liver and tissue pathology indicative of damage. This study provides new insight into BT hepatotoxicity in Chinese rare minnow.

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1. Introduction

There has been a significant increase in the emergence of new chemicals of concern in the aquatic environment, as improved analytical methods are now able to detect chemical contamination with higher sensitivity (Herrero et al., 2013; Richardson, 2009). Many of these chemicals enter into aquatic ecosystems and can bioaccumulate in the aquatic food web (Liu et al., 2011; Wang et al., 2015). Benzotriazoles such as 1H-benzotriazole (BT) are industrial chemicals that are used in high volumes (~9000 tons/year worldwide) in both consumer products (e.g. additives to household dishwashing agents) and industrial compounds such as anti-corrosive and de-icing agents (Giger et al., 2006). Their wide application and resistance to biodegradation contribute to the dispersive occurrence of these compounds in the aquatic environment (Herzog et al., 2014; Liu et al., 2012). In fact, these chemicals can reach relatively high levels in some water systems. For example, BT has been detected in surface waters at concentrations ranging from 0.038 to 7.98 µg/L (Kiss and Fries, 2009; Loos et al., 2009; Voutsa et al., 2006; Wolschke et al., 2011), and higher
levels of BT have been reported in wastewater effluent, ranging from 10 to 100 μg/L (Voutsou et al., 2006; Weiss and Reemtsma, 2005). BT has also been detected in drinking water in the UK, at a concentration of approximately 30.9 ng/L (Janna et al., 2011). In addition to water contamination, BT has also been detected in indoor dust (Wang et al., 2013) and human urine (Asimakopoulos et al., 2013a, 2013b). Thus, these compounds appear to be relatively ubiquitous chemicals in aquatic environments and may pose potential risk to human health.

Despite the persistence of BT and widespread use, data on how it affects aquatic organisms and humans are limited. The acute toxicity of BT for fathead minnows (Pimephales promelas) is reported to be relatively low, with a LC50 value of 65 mg/L (Pillard et al., 2001). However, in contrast to acute dosing experiments, chronic exposure to BT at much lower levels (EC10 values varied from 0.97 to 3.94 mg/L) has been associated with the inhibition of both growth and reproduction in aquatic invertebrates (Seeland et al., 2012), indicating that there may be uncharacterized risks to aquatic organisms. BT may also be an endocrine disruptor, and studies show that BT elicits adverse responses in marine medaka (Oryzias melastigma) (He et al., 2012) and rare minnow (Gobiocephalirrus rarus) (Liang et al., 2014b). These studies are suggestive of an estrogenic mode of action, while antiandrogenic modes of action have been observed from studies conducted in zebrafish embryos (Fent et al., 2014). These data spur additional questions as to the mode of action for these compounds, and further studies are warranted to document the full range of any potential adverse effects.

We previously demonstrated that BT alters the mRNA levels of genes involved in the hypothalamic-pituitary-gonadal (HPG) axis and that BT can exert estrogenic disruption in rare minnow, based on alterations in the plasma E2 levels (Liang et al., 2014b). These studies are suggestive of an estrogenic mode of action, while antiandrogenic modes of action have been observed from studies conducted in zebrafish embryos (Fent et al., 2014). These data spur additional questions as to the mode of action for these compounds, and further studies are warranted to document the full range of any potential adverse effects.

We previously demonstrated that BT alters the mRNA levels of genes involved in the hypothalamic-pituitary-gonadal (HPG) axis and that BT can exert estrogenic disruption in rare minnow, based on alterations in the plasma E2 levels (Liang et al., 2014b). In addition, our previous proteomic study in the brain of female rare minnow has shown that BT may be related to neurotoxicity by interfering with cellular processes including cellular respiration, G-protein signal cascades, Ca2+-dependent signaling, cell cycle and apoptosis (Liang et al., 2016). However, hepatotoxicity and the mechanism underlying BT toxicity at the protein level have not been investigated, despite the fact that the liver is the major detoxification organ for chemicals.

The objectives of this study were to examine the proteomic response in the liver of rare minnows exposed to BT in dose-response experiments to learn more about the mechanisms of action of BT in fish. The rare minnow (Gobiocephalirrus rarus) inhabits the upstream waters of the Yangtze River in China. This small bodied fish is ideal for toxicity testing because it is relatively straight forward to maintain laboratory cultures, it has a short life cycle, and it can produce hundreds of eggs. Therefore, the rare minnow is considered a premier small fish model for the assessment of chemicals in the environment in China (Zha et al., 2008). Furthermore, omics-based approaches (i.e., genomics, proteomics, and metabolomics) to inform mechanism-based toxicology have been adapted in rare minnows to better characterize toxicity and molecular pathways perturbed by chemicals (Liang and Zha, 2016). In this study, the proteomic response in the liver of rare minnow, as well as hepatic histopathology, were carried out to assess the hepatotoxic potential of BT. Pathway analysis was conducted to synthesize proteome data to provide additional insight into the molecular processes that may underlie hepatotoxicity.

2. Materials and methods

2.1. Experimental design and sampling

Exposure experiments followed that as previously outlined in Liang et al. (2014b, 2016). Adult male Chinese rare minnows (~10 months old) used in this experiment were maintained at the State Key Laboratory of Environmental Aquatic Chemistry, which is housed at the Research Center for Eco-Environmental Sciences and Chinese Academy of Sciences. Fish were cultured in 10 L glass aquaria with flow-through dechlorinated tap water. Water parameters were as follows: pH = 7.2–7.6, water hardness = 44.0–61.0 mg CaCO3/L, and temperature = 25 ± 1 °C. Fish were allowed to acclimate to laboratory conditions for 2 weeks prior to the experiment. Healthy males based on visual inspection were randomly divided into 12 groups of 20 fish and were exposed to one of three concentrations of BT (0.05, 0.5, 5 mg/L) or to a water-only control as BT is readily soluble in water.

Benzotriazole (BT, CAS no. 95–14–7, purity >99%) was purchased from J&K Chemical Ltd. (USA). Stock solutions were first prepared by dissolving BT in distilled water. The stock solutions were kept in 4-L brown glass container and were diluted with aquarium water to obtain the final concentration for exposure. The stock solutions were slowly added to glass mixing vessels by means of a peristaltic pump at a rate of 60 mL/h. These solutions were then mixed with dechlorinated tap water and dispersed to the exposure tanks, at a continuous flow rate of 5 L/h. Stock solutions were renewed every second day and dosing rates were checked twice daily.

Each experiment condition included three replicates for a total of 12 tanks. During the exposure, the fish were subjected to a photoperiod of 16:8 h light: dark: and were fed newly hatched brine shrimp (Artemia nauplii) twice daily. After 28 days of experimentation, all fish were anesthetized on ice. The fish were dissected, and livers were removed and separated into two equal parts. One half of the liver was used for protein extraction and 2D gel electrophoresis, and the second half of the liver was used for RNA isolation and real-time PCR analysis. All samples at the time of dissection were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

A second experiment, longer than the first experiment, was conducted for 42 days. This second experiment was used to determine if liver damage occurred following a longer-term exposure to BT. No significant pathology was observed in low doses (0.05 and 0.5 mg/L) after 28 d of BT exposure in a preliminary experiment (data not shown), thus 42 d was used to assess pathology and to better describe an adverse outcome pathway for BT.

2.2. Proteomic analysis

2.2.1. Protein extraction and CyDye labeling

Protein extraction and CyDye labeling were performed according to Liang et al. (2014a). Briefly, ~50 mg of frozen hepatic tissue from control and BT-treated fish were homogenized and sonicated intermittently over 5 min in 0.6 mL of ice-cold lysis buffer I (7 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% w/v dithiothreitol (DTT), 30 mM Tris and a cocktail of protease inhibitors (Roche)). To remove remaining tissue fragments and insoluble material, the homogenates were centrifuged for 10 min at 18,000 × g, 4 °C. The supernatant was then collected and 100 μL of each sample protein extract was purified using a 2-D clean-up kit (GE Healthcare). Protein pellets were then resuspended in 50 μL lysis buffer II (lysine buffer 1, 0.5% pH 4–7 IPG buffer). Protein concentration was determined using a 2-D Quant kit (GE Healthcare) according to the manufacturer’s protocol. Purified protein samples were randomized and minimally labeled with Cy3 or Cy5 fluorescent dyes (50 μg protein/400 pmol dye) as per the manufacturer’s instructions (GE Healthcare). Cy3 was used to label a common protein pool that contained equal amounts of proteins from the samples and this acted as an internal standard for 2D gel electrophoresis. Labeling was performed on ice for 30 min in the dark and quenched with...
10 mM lysine for 10 min, on ice and in the dark.

2.2.2. Two-dimensional fluorescence difference gel electrophoresis and image analysis

The methods for the 2D-DIGE analysis proceeded as that outlined in Liang et al. (2014a). Briefly, Cy2-, Cy3-, Cy5-labeled samples were mixed together and dissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% w/v DTT, 0.5% pH 4–7 IPG buffer, and a trace of bromophenol blue). Samples (n = 3 biological replicates per group) were then loaded onto 18 cm immobilized pH gradient (IPG) strips (pH 4–7, GE Healthcare), and subjected to first dimension iso-electric focusing (IEF) overnight using Ettan IPGphor (GE Healthcare, USA). After IEF, each strip was equilibrated twice for 15 min with constant mixing (60 rpm). This was achieved by first using 1% DTT followed by 2.5% iodoacetamide (IAA) in order to reduce and alkylate the proteins. The SDS-PAGE was performed using an electrophoresis system (Ettan Dalt six, GE Healthcare) and 10% polyacrylamide gel. Following electrophoresis, the gels were scanned with Typhoon Trio variable mode imager (GE Healthcare, NJ) in each of the three channels that corresponded to the correct dye (488 nm for Cy2, 532 nm for Cy3, and 633 nm for Cy5). The intensity was adjusted to ensure that the maximum volume of each image was within 60–90 thousand. Image Master 2D Platinum 7.0 (GE Healthcare) software was used to analyze the images. A one-way ANOVA was used to determine if there were differences in abundance among spots. Protein spots were considered differentially expressed at a ratio \( \geq 1.5 \) (\( p < 0.05 \)) and were pursued for identification.

2.2.3. Protein identification

Protein identification was conducted in accordance with the method previously published (Liang et al., 2016). Following the image acquisition, gels were stained with silver nitrate and protein spots were manually excised, decolorized, dehydrated and whitened according to Liang et al. (2016). The digested peptides were resuspended with 0.8 µl of matrix solution (50% acetonitrile, 0.1% trifluoroacetic acid, and semi-saturated a-cyano-4-hydroxycinnamic acid). All mass spectra were acquired using a 4800 Plus MALDI-TOF/TOF™ Analyzer (Applied Bio-systems, USA) in positive reflectron mode. Signal-to-noise ratios of \( \geq 50 \) were required for tandem MS/MS analysis. The mass signals generated from the MS mode and the MS/MS mode were combined for protein identification. Mascot was set up to search an NCBI non-redundant (nr) database (zebrafish, 76,533 sequences, released Aug. 13, 2013; ray-finned fishes, 229,984 sequences, released Dec. 12, 2012) assuming the digestion enzyme trypsin. Mono-isotopic masses were used at a peptide mass tolerance of 100 ppm. Cysteine carboxymethylation was considered as a fixed modification, and the oxidation of methionine was specified as a variable modifications. Peptide and protein identifications were accepted if Confidence Interval % (C.I.) values were greater than 95%. The identified proteins were then matched to specific processes or functions by searching UniProt (http://www.uniprot.org/) and KEGG (http://www.genome.jp/kegg/) database.

2.2.4. Pathway analysis

For each differentially expressed protein, the official gene symbol was manually retrieved using gene card (http://www.genecards.org). The official gene symbols were then used to map proteins into the Pathway Studio 9.0 (Elsevier Life Science Solutions) and ResNet 10.0 for pathway analysis. This program uses expression, binding, and regulatory data among proteins to construct interaction networks. Pathways were built using direct connections with one neighbor. Using the “Name + Alias” function, thirty proteins mapped to the program. One protein did not successfully map as no mammalian homolog could be identified, while a second protein was excluded, as it is fish specific. In order to simplify the pathways, differentially expressed proteins across all three doses were considered as a whole for building pathways. As such, the median fold change of the protein was used to color pathways, and only proteins that were significantly altered in a BT treatment were used to calculate the median protein fold change. In the case of multiple spots (e.g. phosphorylation), the median value across all spots was used for the protein fold change relative to the control group. Sub-network enrichment analysis (SNEA) was also performed in the program to identify processes that were represented by the proteins. Criteria for an enriched cell process were that the network had to be significant (considered \( p < 0.05 \)) and there had to be more than 10 members in the network to represent the processes most likely altered by BT exposure at the proteome level. Further details on the program can be found in Langlois and Martyniuk (2013).

2.3. Relative quantification by real-time PCR

Total RNA was extracted from minnow liver (n = 6 biological replicates per group) using Trizol reagent (Life Technology), and this was followed by DNase (Promega, USA) treatment. RNA integrity was measured using a 1.2% agarose gel (0.5 × TBE buffer, 150 v, 15 min) and RNA was of sufficient quality (i.e. no significant degradation based upon visual inspection of each sample). The concentration and purity of the samples were determined using a Multiskan™ GO microplate spectrophotometer (Thermo Scientific, USA). For real-time PCR experiments, samples were adjusted to a 20 µl volume with 2 x GoTaq™ qPCR Master Mix (Promega, USA), 1 µl of 10 µM gene-specific primer, and ~100 ng first-strand cDNA synthesized from DNase-treated RNA. The relative abundance of \( \beta \)-actin was used to normalize the expression of target genes. All primer pairs used for real-time PCR are listed in Supplemental Table S1.

Real-time PCR reactions were assayed using the 7500 Real-Time PCR system (Applied Biosystems, USA). Each sample was analyzed in triplicate. The real-time PCR cycling parameters were as follows: initial denaturation step of 95°C for 10 min, 40 cycles of 95°C for 30 s, 57°C for 40 s, and 72°C for 30 s. To ensure amplification specificity, a melt curve was generated at the last cycle of 95°C for 30 s, 57°C for 30 s, and 72°C for 60 s. Data were analyzed according to the 2 \(-\Delta\Delta CT\) method. Statistical analysis of variance was performed in SPSS (v18, USA). The normality of data was assessed using the Kolmogorov-Smirnov test and logarithmic transformation was performed prior to ANOVA to ensure data conformed to assumptions. The Levene’s test of homogeneity of variance was used to test for adherence to ANOVA assumptions. A Dunnett’s post-hoc test was used to compare each treatment with the control. All quantitative data are expressed as mean ± S.E. A probability of \( p < 0.05 \) was considered statistically significant.

2.4. Histopathology

Histopathology analysis followed that outlined in Liang et al. (2014b) and Chen et al. (2016). A total of 12 groups of 10 fish for each group (0, 0.05, 0.5, 5 mg/L BT) were used for histopathology analysis. Following necropsy of all male fish in the experiment, livers were excised and fixed in 4% paraformaldehyde for 24 h. Liver samples were transferred to 70% ethanol, embedded in paraffin wax, and sectioned at 3–4 µm thick. Tissue sections were stained with hematoxylin and eosin (H and E) according to standard histological methods. Slides were visualized with an optical microscope BX53 (Olympus, Tokyo, Japan). Sections were imaged and analyzed using cellSens Standard software (Olympus, Tokyo, Japan).
To determine if the liver was compromised at the tissue level, nucleated cells and akaryotes were counted. Five fields of visions were selected at random and analyzed (n = 6 biological replicates per group were randomly selected from the original 10 fish). Statistical significance was considered to be P < 0.05 following a Student’s t-test.

3. Results

3.1. Proteome profiles of male rare minnow liver

In order to identify sets of proteins that respond to BT exposure in male rare minnow, we compared the liver proteome of control and treated animals using 2D-DIGE (Fig. 1). A total of 46 protein spots were found to be significantly altered in abundance (≥1.5-fold) in one or more BT-treated groups compared to that of controls and 32 proteins were successfully identified (Table 1). Based upon the p-value and ratio criteria, there were 17 up-regulated proteins and 9 down-regulated proteins following BT treatments (Table 1). Most of these proteins were changed remarkably in the lowest concentrations of BT. Only four proteins were significantly increased (phosphoglucomutase 1) or decreased (heat shock 60kD protein 1, glucose-6-phosphate dehydrogenase, and homogentisate 1,2-dioxygenase) in higher concentrations (0.5 mg/L and 5 mg/L groups), and these proteins did not show significant changes in abundance in the 0.05 mg/L group (Table 1).

Fig. 1. Representative two-dimensional gel image of the CyDye-labeled proteins from male rare minnow liver for control and BT treatments. Each gel is representative of three independent biological replicates. Numbers are allocated by the Image Master 2D Platinum 7.0 software and represent the spots with a significant variation in intensity (ratio ≥ 1.5, p < 0.05). The information for the proteins corresponding to the spot numbers is presented in Table 1.
Table 1
A detailed list of protein spots identified by MALDI-TOF/TOF MS from the liver of male rare minnow following BT exposure.

| Spot ID | GI accession no. | Protein name | MW/PI | Matched peptides | score | Fold change | Function category |
|---------|------------------|--------------|--------|------------------|-------|-------------|------------------|
|         |                  |              |        |                  |       | (treated vs control) |                  |
|         |                  |              |        |                  |       | 0.05 mg/L | 0.5 mg/L | 5 mg/L |

| Protein identified in multiple spots | | | | | | | | |
| 247 | 56078768 | Phosphorylase | 111.8/5.50 | 23 | 625 | 3.05 | 3.86 | 4.04 | carbohydrate metabolic process/glycogen phosphorylase activity |
| 251 | 56078768 | Phosphorylase | 111.8/5.50 | 19 | 425 | 1.53 | 1.37 | 1.43 |
| 252 | 56078768 | Phosphorylase | 111.8/5.50 | 19 | 279 | 2.22 | 2.41 | 2.34 |
| 254 | 56078768 | Phosphorylase | 111.8/5.50 | 18 | 535 | 2.89 | 1.92 | 2.62 |
| 258 | 56078768 | Phosphorylase | 111.8/5.50 | 22 | 549 | 2.30 | 1.46 | 1.91 |
| 398 | 56078768 | Phosphorylase | 111.8/5.50 | 18 | 369 | -1.93 | -2.09 | -2.06 |
| 405 | 56078768 | Phosphorylase | 111.8/5.50 | 17 | 445 | -1.70 | -2.69 | -3.41 |
| 408 | 56078768 | Phosphorylase | 111.8/5.50 | 15 | 344 | -1.39 | -2.12 | -2.09 |
| 361 | 71373043 | iron regulatory protein 1 | 99.4/6.45 | 10 | 295 | -2.33 | -2.73 | -3.29 | metabolic process/4 iron, 4 sulfur cluster binding |
| 537 | 39645909 | Glutamate dehydrogenase | 60.3/4.55 | 17 | 451 | 1.65 | 1.32 | 1.76 | cellular amino acid metabolic process/oxidoreductase activity |
| 559 | 39645909 | Glutamate dehydrogenase | 60.3/4.55 | 17 | 572 | 1.78 | 1.6 | 2.09 |
| 603 | 39645909 | Glutamate dehydrogenase | 60.3/4.55 | 15 | 721 | -2.26 | -3.07 | -6.88 |
| 615 | 39645909 | Glutamate dehydrogenase | 60.3/4.55 | 10 | 395 | -2.33 | -2.58 | -3.27 |
| 571 | 28278852 | Methionine adenosyltransferase I, alpha | 43.7/6.32 | 8 | 182 | 2.37 | 2.24 | 1.7 |
| 803 | 28278852 | Methionine adenosyltransferase I, alpha | 43.7/6.32 | 11 | 227 | -2.38 | -3.19 | -3.22 |
| 814 | 82213364 | Fructose-bisphosphate aldolase B | 39.7/4.89 | 4 | 98 | 1.61 | 1.57 | 2.06 | Glycolysis/fructose-bisphosphate aldolase activity |
| 885 | 82213364 | Fructose-bisphosphate aldolase B | 39.7/4.89 | 6 | 132 | -2.52 | -2.79 | -4.47 |
| 923 | 123918122 | glyceroldehyde-3-phosphate dehydrogenase | 36.0/8.20 | 9 | 458 | -2.31 | -5.77 | -7.73 | Glycolysis/NAD binding; NADP binding |
| 1374 | 123918122 | glyceroldehyde-3-phosphate dehydrogenase | 36.0/8.20 | 6 | 373 | 1.39 | 1.41 | 1.63 |

| Up-regulated proteins | | | | | | | | |
| 233 | 39645915 | Heat shock protein 90, beta (Grp94), member 1 | 91.4/4.77 | 19 | 361 | 1.6 | 1.15 | 1.61 | response to stress/ATP binding |
| 261 | 161612168 | Dmgdh protein | 97.9/6.54 | 12 | 218 | 2.07 | -0.95 | 0.93 | glucose catalytic process response to stress/ATP binding |
| 351 | 159155706 | Heat shock protein 8 | 71.4/5.32 | 23 | 824 | 1.95 | 1.93 | 2.01 | galactose catalytic process; glycogen biosynthetic process |
| 435 | 3276553 | Phosphoglucomutase 1 | 61.4/5.74 | 11 | 498 | 1.4 | 1.51 | 2.22 | aldehyde dehydrogenase (NAD) activity |
| 448 | 41393103 | aldehyde dehydrogenase family 9 member A1-A catalase | 56.1/6.18 | 9 | 95 | 1.6 | -1.01 | -1.01 | hydrogen peroxide catalytic process/catalase activity |
| 472 | 9622234 | aldehyde dehydrogenase family 9 member A1-A catalase | 56.1/6.18 | 9 | 95 | 1.6 | -1.01 | -1.01 | cellular aldehyde metabolic process |
| 550 | 20339358 | Aldehyde dehydrogenase 2 | 57.2/6.05 | 8 | 83 | 2.29 | 2.22 | 2.23 | gamma-aminobutyric acid metabolic process |
| 618 | 302191716 | 4-aminobutyrate aminotransferase, mitochondrial | 56.3/7.61 | 9 | 130 | 1.52 | 1.29 | 1.4 |
| 662 | 28278417 | S-adenosylhomocysteine hydrolase | 48.5/6.33 | 11 | 563 | 1.54 | 1.48 | 1.56 | S-adenosylmethionine cycle; one-carbon metabolism |
| 672 | 357394863 | Probable imidazolonepropionase | 47.3/6.05 | 7 | 231 | 1.94 | 1.6 | 1.05 | histidine catalytic process oxidoreductase activity |
| 677 | 822024124 | Hydroxysteroid dehydrogenase-like protein 2 | 44.7/7.08 | 9 | 141 | 1.59 | 1.49 | 2.19 | steroid binding catalytic activity oxidoreductase process/oxidoreductase activity |
| 931 | 45709875 | Zgc:85683 | 32.5/5.38 | 4 | 123 | 2.04 | 1.57 | 1.58 | Glycolysis/isophosphoglycerate mutase activitiy |
| 1015 | 528514207 | Uncharacterized protein | 25.3/5.2 | 4 | 130 | 2.36 | 2.18 | 1.24 | hypoxanthine metabolic process; pyridine ribonucleoside salvage transferase activity |
| 1018 | 44890340 | Phosphoglycerate mutase 1a | 29.1/6.19 | 8 | 86 | 2.87 | 2.58 | 1.76 |
| 1040 | 528500056 | Hypoxanthine phosphoribosyltransferase 1 | 24.9/6.21 | 11 | 363 | 1.54 | 1.52 | 1.5 |
| 1046 | 514483217 | Glutathione S-transferase theta 1a [Carassius auratus] | 26.4/6.09 | 5 | 186 | 1.52 | 1.47 | 1.28 |

(continued on next page)
In addition, among the protein spots identified, 6 proteins were detected in more than one spot (Table 1). For example, Protein spots 247, 251, 252, 254 and 258 were all identified as phosphorylase (PYGL), which appeared as a string of abundant spots on the gels (Supplemental Fig. S1). A shift in mass was also observed as protein PYGL, which appeared as a string of abundant spots on the gels.

3.3. Validation by qPCR

To validate the alteration of proteins identified after BT exposure, 6 proteins were selected for qPCR analysis (Fig. 4). The transcripts that were assessed included 4-aminobutyrate aminotransferase (abat), probable imidazolonepropionase (amidohydrolase domain containing 1, amidhd1), apolipoprotein A-I (apoa1a), glutamate dehydrogenase 1 (glud1), phospholipase (pygl), and sulfotransferase family 6, cytosolic sulfotransferase 6 (sult1st6). Transcripts in general showed correspondence with that of the protein abundance (Fig. 4). Here we point out that transcriptional and translation regulation is different, and discrepancies in the regulation of molecular targets by BT can be explained by these two different cell processes. Nevertheless, congruence between gene and protein responses following BT in multiple doses is stronger evidence for disruption of the target. Consistent with the proteomic data, the mRNA levels of abat and amidhd1 presented a marked up-regulation in lower concentrations of BT following BT treatments while proteins such as HPRT1, CAT and HSP90 were increased in this network (Fig. 3). All abbreviations for the protein networks are found in the Figure caption.

Table 1 (continued)

| Spot no. | GI accession | Protein name a | MW/PI | Matched peptides | score c | Fold changeb (treated vs control) | Function categoryc |
|----------|--------------|----------------|--------|------------------|---------|-------------------------------|-------------------|
| 1084     | 8575790      | Glutathione S-transferase [Pimephales promelas] | 22.0/7.64 | 4 | 127 | 2.54 | 2.17 | 1.5 | transferase activity |
| 445      | 528496446    | PREDICTED: fatty acid synthase isoform X2 | 276.8/6.01 | 17 | 255 | -2.83 | -5.7 | -4.38 | ND |
| 446      | 528489998    | Heat shock 60kD protein 1 (Chaperonin) | 61.4/5.56 | 15 | 884 | -1.24 | -1.52 | -1.03 | response to stress/ATP binding |
| 468      | 298201226    | glucose-6-phosphate dehydrogenase, partial [Gobioscypriis rarus] | 35.6/5.87 | 11 | 250 | -1.43 | -1.87 | -1.29 | pentose-phosphate shunt/NADP binding; glucose-6-phosphate dehydrogenase activity |
| 629      | 10441585     | Homogentisate 1,2-dioxygenase | 45.2/6.37 | 10 | 241 | -1.06 | -1.3 | -2.14 | l-phenylalanine catabolic process; tyrosine metabolic process |
| 724      | 41054972     | 405 ribosomal protein SA | 34.2/4.75 | 4 | 312 | -2.05 | -2.71 | -5.63 | cell adhesion; ribosomal small subunit assembly; translation/laminin receptor activity; structural constituent of ribosome hydrolyase activity; metal ion binding |
| 940      | 182889192    | Zgc:153353 | 40.1/7.51 | 7 | 130 | -1.62 | -1.61 | -1.16 | glutathione transferase activity |
| 954      | 182890800    | Zgc:101897 protein | 27.9/8.13 | 6 | 75 | -2.18 | -1.49 | -0.81 | estrogen sulfotransferase activity |
| 987      | 49900472     | Sulfotransferase family 1, cytosolic sulfotransferase 6 Apolipoprotein A-I, probable imidazolonepropionase (amidohydrolase domain containing 1, amidhd1), apolipoprotein A-I (apoa1a), glutamate dehydrogenase 1 (glud1), phospholipase (pygl), and sulfotransferase family 6, cytosolic sulfotransferase 6 (sult1st6). Transcripts in general showed correspondence with that of the protein abundance (Fig. 4). Here we point out that transcriptional and translation regulation is different, and discrepancies in the regulation of molecular targets by BT can be explained by these two different cell processes. Nevertheless, congruence between gene and protein responses following BT in multiple doses is stronger evidence for disruption of the target. Consistent with the proteomic data, the mRNA levels of abat and amidhd1 presented a marked up-regulation in lower concentrations of BT (Fig. 4). The pattern of apoa1a and sult1st6 mRNA in 0.5 and 5 mg/L exposure concentrations was in |
accordance with that of the protein data (Fig. 4). However, in the 0.05 mg/L group, the *apoA1* and *sult1st6* mRNA levels were not significantly changed while corresponding protein levels were decreased (Fig. 4). Again, this highlights the complexity underlying gene-protein regulation. In the case where proteins were detected in multiple spots in 2D-DIGE gels, the median value across all spots was used for the protein fold change relative to the control group. Both *glud1* and *pygl* were chosen to validate the protein abundance levels for multiple spot proteins. GLDU1 was down-regulated by 1.58-fold at 5 mg/L BT group while PYGL was not significantly altered following all three exposure concentrations of BT. The mRNA levels of *glud1* and *pygl* showed comparable response patterns when compared to protein data generated with DIGE (Fig. 4).

3.4. Histopathology

Normal livers in rare minnow typically show compact and tightly arranged hepatocytes with homogeneous cytoplasm, and a large spherical nucleus with one nucleolus (Fig. 5A). Notable histological changes were observed in the livers of the rare minnow following exposure to 0.05, 0.5 and 5 mg/L BT for 42 d (Fig. 5B–D) when compared to normal liver histology. Hypertrophy of the hepatocytes, nuclei pyknosis, and increases in cellular vacuolization were observed in all three treatment groups (Fig. 5B–D). Additionally, in the highest BT treatment group, the hypertrophy of the hepatocytes was even more prevalent than the other BT-treated groups (rectangle area in Fig. 5D).

4. Discussion

Benzotriazole is an emerging contaminant and is relatively widespread in aquatic environments. Evidence indicates that BT has endocrine disrupting potential (Fent et al., 2014; He et al., 2012; Liang et al., 2014b) and may be neurotoxic (Liang et al., 2016). To
Fig. 4. Protein and relative mRNA levels of selected proteins in the livers of male rare minnow following BT treatments. The magnified images of protein spots from the 2D-DIGE gels are shown in the upper part of each panel. The bar charts below show the protein levels based upon 2D-DIGE and mRNA levels using real-time PCR. The values represent the average fold changes. Significant changes of mRNA levels as compared to the controls (p < 0.05) are indicated by asterisks (*), and pound sign (#) refers to an average increase or decrease higher than 1.5-fold in protein levels compared to the controls.
characterize the liver proteome and to better understand the mechanism of hepatic toxicity of BT, 2D-DIGE coupled MALDI-TOF/TOF-MS was employed. A total of 32 differentially expressed proteins were identified in one or more of the treatment groups. Of these proteins, 6 were identified in the spot strings as the same protein, which suggests that BT may play a role in protein modification (McCarthy et al., 2003). Although no apparent shifts with MW or PI were observed in exposure gels compared to the control, the protein level of each spot was changed dramatically after BT treatment. We speculate that significant changes in protein modification for some proteins (e.g. phosphorylation) might be induced in response to BT (Lv et al., 2014; Thio et al., 2013), although further studies are needed to verify the extent of phosphorylation.

Pathway analysis revealed that BT-responsive proteins were mainly involved in the process of xenobiotic clearance, oxidative stress response, apoptosis, and translation. In the present study, three primary proteins related to xenobiotic clearance were identified, including SULT1ST6, ABAT, and GLUD1. Our data revealed that SULT1ST6 protein and mRNA were significantly decreased in abundance after BT exposure. SULT1ST6 is a member of sulfotransferases and exhibits sulfating activity toward endogenous compounds of both endocrine and nervous systems in fish (Gao et al., 2014; Liu et al., 2010). Thus, SULT1ST6 is active during detoxification and is also important in metabolizing environmental estrogens (Liu et al., 2008). Previous studies have also shown a significant decrease in sult1st6 mRNA levels in male rare minnow exposed to bisphenol A (Gao et al., 2014) and in zebrafish embryos exposed to chlorpyrifos (Jeon et al., 2016). These results suggest that the hepatic detoxification might be repressed by BT, and further support our previous study reporting on the estrogenic potential of this compound (Liang et al., 2014b).

Additionally, both the transcription and protein levels of ABAT and GLUD1 were significantly increased and decreased in response to 0.05 and 5 mg/L BT, respectively. As a signaling molecule and neurotransmitter, γ-Aminobutyric acid (GABA) is produced from glutamate by glutamate decarboxylase and is further metabolized into succinic semialdehyde by ABAT. During this process, GLUD1 converts glutamate to α-ketoglutarate, followed by further oxidation in the tricarboxylic acid (TCA) cycle to generate ATP (Bouché et al., 2003; McKenna et al., 1996). According to this metabolic process, we hypothesize that in the current study, the upregulation of ABAT increases GABA, resulting in the downregulation of GLUD1. Aberrant glutamatergic transmission has been associated with neuronal pathology and neurodegenerative diseases (Kim et al., 2011). Environmental chemicals have also been shown to disrupt the normal balance of these enzymes. For example, in the brain of methylmercury-exposed zebrafish, the transcript levels of glud1a and abat were decreased and increased, respectively (Cambier et al., 2012). In addition to the brain, GABA signaling also exists in vertebrate liver, and activation of this GABAergic system can protect the liver against toxic injury (Wang et al., 2017). Li et al. (2016) reported that a repression of this amino acid metabolism pathway is associated with liver impairment in zebrafish exposed to arsenic. In another study, GLUD1 was reported to be downregulated in male rare minnow (Gobiocypris rarus) liver after exposure to pentachlorophenol (Fang et al., 2010). Thus, altered...
protein and transcript levels of ABAT and GLUD1 indicate that BT are caused by BT via disturbing the apoptosis pathway; this damage impacts were examined after 0.05 and 0.5 mg/L BT treatments exposed to 5 mg/L BT for 28 days was observed while no significant impacts on each concentration in this study and these protein responses may disrupt hepatic GABA signaling and induce oxidative stress.

In conclusion, 2D gel electrophoresis identified proteins affected by BT that are involved in xenobiotic clearance, oxidative stress response, apoptosis, and translation. Furthermore, histological damage in the liver of male rare minnow was observed across all exposure groups following BT exposure. The results presented here provide novel insight into the mechanisms of hepatotoxicity of BT in male rare minnow. We hypothesize that BT induced cellular stress impairs protein synthesis and induces the apoptosis pathway, resulting in ROS production and liver lesions. However, additional experimental validation is required and the biological impacts of long-term BT exposure remain to be examined.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [grant numbers 21507064, 21677165]; and the Natural Science Foundation of Inner Mongolia Autonomous Region of China [grant number 2015MS0202].

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.06.013.

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Conversion of glutamate in TCA cycle increases the production of anti-oxidant molecule such as NADPH, thus GABA shunt pathway plays an important role in antioxidant defense (Ramond et al., 2014). Decreased GLUD1 coupled a general induction of heat shock proteins (HSP90B1 and HSPA8) and CAT indicates that there is a general and oxidative stress response in the liver following BT exposure. HSPs are well characterized molecular chaperones that assist in protein folding and are bio-indicators for their sensitivity to environmental stress (Gupta et al., 2010). Catalase is ubiquitous in tissues and acts to reduce hydrogen peroxide and to diminish the effects of reactive oxygen species (ROS) (Nishikawa et al., 2009). An increase in HSPs and catalase activity has been documented in the liver of fathead minnows (Pimephales promelas) responding to 17β-trenbolone (an androgen receptor agonist) and flutamide (an androgen receptor antagonist) (Martyniuk et al., 2009). Furthermore, a wide variety of chemicals affects proteins related to ROS production in fish species, including pesticides (Atamaniuk et al., 2013; Martyniuk et al., 2010), and heavy metals (Carlson et al., 2013; Lushchak, 2016). These studies support the hypothesis that BT can induce oxidative stress and adversely affect rare minnow liver at the molecular level.

As a consequence of oxidative stress, cell death and apoptosis may occur and this can result in the hepatotoxicity of BT. Cell death and apoptosis were two of the top pathways identified with sub-network enrichment analysis and therefore, appear to have a prominent role in BT toxicity. Notably, APOA1, the primary protein constituent of high-density lipoprotein (HDL) and a protein that mediates anti-apoptotic responses in mammals (Rütti et al., 2009), was decreased in each of the exposure concentrations of BT in this study. Inhibition of apoA1 transcripts has also been observed previously in the liver of male goldfish exposed to fluoxetine (FLX) and 17 alpha-ethinylestradiol (EE2) (Silva de Assis et al., 2013), as well as in male zebrafish livers following exposure to waterborne EE2 (Martyniuk et al., 2007). In addition, proteomic analysis in the male rare minnow liver identified proteins that are also associated with the process of translation. These proteins are also involved in apoptosis, suggesting impairment of both protein synthesis and cell death. For example, methionine adenosyltransferase I, alpha (MAT1A) is an essential enzyme that is responsible for the biosynthesis of S-adenosylmethionine. Reduced MAT1A expression in hepatocellular carcinoma contributes to increased apoptosis and enhanced tumorigenesis (Yang et al., 2013). Moreover, the histological damage in the liver of male rare minnow was observed at each concentration in this study and these protein responses may be associated with pathology of the liver in extended exposures. In a previous study, histological damage in the liver of rare minnow exposed to 5 mg/L BT for 28 days was observed while no significant impacts were examined after 0.05 and 0.5 mg/L BT treatments (Liang et al., 2014a). These results suggest that the hepatic lesions are caused by BT via disturbing the apoptosis pathway; this damage may be caused at relative low concentrations of BT after longer exposure period.
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