Letter

Acute hepatotoxicity induced by quetiapine fumarate in larval zebrafish

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(Received April 18, 2016; Accepted April 27, 2016)

ABSTRACT — Quetiapine fumarate (QF) is a widely used antipsychotic agent for the first-line treatment of schizophrenia, with good tolerability and compliance in humans and no observed adverse effects against liver. Taking advantages of zebrafish, which can be utilized in rapid drug screening and acute toxicity assessment, our study determined a certain hepatotoxicity of QF for the first time, indicating a potential safety hazard of this commonly used drug to humans.

Key words: Quetiapine fumarate, Larval zebrafish, Hepatotoxicity, Liver, Yolk sac

INTRODUCTION

The liver is a major target of drug-induced toxicity or injury owing to the crucial role of this organ in drug biotransformation and metabolism processes. Drug-induced hepatotoxicity (DIHT) or liver injury is now recognized as a significant primary cause of acute/chronic liver diseases and the subsequent deaths in Western countries, such as fulminant hepatic failure leading to jaundice, coagulopathy, and multisystem organ failures with an estimated 3.5 deaths per million people in the US (Lee, 2003; Khashab *et al.*, 2007; Hoofnagle *et al.*, 1995). The incidence of such liver disorders has been estimated as ~19 new cases per 100,000 persons each year and accounts for 11 and 32% of all acute liver failure cases in the US and Europe (Bjornsson *et al.*, 2013; Reuben *et al.*, 2010; Hadem *et al.*, 2012). DIHT remains the most common reason of drug failure in clinical trials and of post-market warnings and withdrawals of approved drugs (Norris *et al.*, 2008; MacDonald and Robertson, 2009). The hepatotoxic drugs or secondary metabolites may exert toxic effects on critical organelles (e.g. mitochondria) in the

hepatocytes or on drug transporters (e.g. P-glycoprotein), by changing structures of liver proteins, depleting glutathione (GSH) and inducing lipid peroxidation (Guengerich and Shimada, 1991; Jollow *et al.*, 1973). The consequences are hepatocellular necrosis, apoptosis and sensitization to cytokine or inflammatory mediators. Currently, DIHT occurrence is attributable to the failed predictability of preclinical assays using traditional *in vitro* and *in vivo* models. The *in vitro* models fail to recapitulate the complexity of the intact organism and have less than 25% sensitivity for detection of hepatotoxins, whereas *in vivo* models are very laborious, low throughput, costly and time-consuming (Eckardt *et al.*, 1998; O'Brien *et al.*, 2006; O'Brien *et al.*, 2003). Accordingly, new models which can improve our ability to identify the DIHT earlier in drug development process are urgently needed. In the past few years, zebrafish has attracted increasing attention as a promising tool for such modeling purpose (Milan *et al.*, 2003).

Zebrafish (*Danio rerio*), a cyprinid teleost, is emerging as a predictive vertebrate model animal used for toxicity assay of drugs (Selderslaghs *et al.*, 2012). The mor-

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phological and molecular basis of tissues and organs in zebrafish is either identical or similar to humans, possessing orthologs for ~85% of human genes and > 86% of human drug targets (Chen and Fishman, 1996; Granato and Nüsslein-Volhard, 1996; Gunnarsson *et al.*, 2008; Howe *et al.*, 2013). It has been confirmed that zebrafish has a strikingly similar toxicity profile to mammalian and is more appropriate for identifying endpoints of toxicity and elucidating the toxicity mechanisms (McGrath and Li, 2008; Hill *et al.*, 2012; Hill, 2005). Zebrafish complete primary liver morphogenesis by 48 hr post-fertilization (hpf) and the liver is fully formed and function by 72 hpf. Physiologically, at 120 hpf, the liver functions of larval zebrafish, including bile formation and excretion, serum protein secretion, digestion, metabolism, and storage of nutrients, synthesis of enzymes and other cofactors, lipogenesis, and xenobiotic metabolism are fully operational, are the same as that of mammals (Chu and Sadler, 2009). Most cell types in human liver are present in the larval zebrafish liver, and the latter has analogous mechanisms for handling xenobiotic compounds as the former, including both phase 1 and phase 2 biotransformation (Chu and Sadler, 2009; Alderton *et al.*, 2010; Jones *et al.*, 2010). Morphologically, massive hepatocellular necrosis can result in smaller, dark livers owing to the loss of transparency and these have been suggested as a phenotypic endpoint for assessing hepatotoxicity (Hill *et al.*, 2012). When exposed to a hepatotoxicant, changes to liver morphology can be evaluated visually since larval zebrafish is virtually transparent (Hill *et al.*, 2012; He *et al.*, 2013). The unique nature of larval zebrafish presented above makes this animal an attractive model to study DIHT, being capable of mimicking the complexity of the whole liver system in humans.

Quetiapine fumarate (Seroquel®; AstraZeneca, London, UK) is a second-generation antipsychotic agent of the dibenzothiazepine class used for control of positive and negative symptoms in patients with refractory psychosis, mood disorders, and bipolar disorders (Lacy *et al.*, 2001). It was firstly approved by the FDA in 1997 for the treatment of schizophrenia, and recently approved in 2004 as a monotherapy and adjunct therapy with lithium or divalproex for the short-term treatment of acute manic episodes associated with bipolar I disorder. The molecular formula is $C_{21}H_{25}N_3O_8S_2$ with a molecular weight of 883.1. The chemical designation is 2[2-(4-dibenzo [b,f] thiazepin-11-yl-1-piperazinyl)ethoxy]-ethanol fumarate (2:1) salt (Lee, 2003; Bjornsson *et al.*, 2013), as shown in Fig. 1. Quetiapine fumarate (QF) exerts its effect through a combination of dopamine type 2 (D_2) and 5-hydroxytryptamine-2 (5HT₂) receptor antagonism. It also has

affinities to a range of other neurotransmitter receptors such as serotonin 5HT_{1a}, D1, histamine H1, and adrenergic α -1 and α -2, other than to cholinergic muscarinic and benzodiazepine receptors (McConville *et al.*, 2000). As proposed for the first-line and long-term treatment, QF provides superior efficacy with better patient compliance and fewer side effects, and has been particularly appropriate in patients at the beginning of their illness (Green, 1999). It could generally be well tolerated by the elders and has long been deemed safer than other antipsychotic agents (Hustey and Duffull, 1999). To date, QF has rarely been linked to DIHT by experimental reports. In this study, via the transparent model of larval zebrafish, QF was assessed for evaluating its *in vivo* acute hepatotoxicity for the first time.

MATERIALS AND METHODS

Drug preparation

Commercial tablet of QF (batch number: B141137) was purchased from Hunan Dongting Pharmaceutical Co., Ltd. (Changde, China) and stored in room temperature. One tablet with an additive (0.1 g) was powdered and dissolved with ultra-pure water into a 10 mg/mL stock solution for use.

Zebrafish handling

A wild-type AB strain of zebrafish (batch number: 20150319) was employed for this study. Four to five pairs of adult zebrafish were randomly set up for each mating in a temperature- and light- controlled aquaculture facility with a standard 14:10 hr day/night photoperiod and fed with live brine shrimp twice a day and dry flake once a day. About 200-300 embryos were then generated and maintained at 28°C in fish water (0.2% Instant Ocean Salt in deionized water, pH 6.9-7.2, conductivity 480-510 μ S/cm, and hardness 53.7-71.6 mg/L $CaCO_3$). The embryos were washed and staged at 6 and 24 hpf to obtain larval zebrafish. The zebrafish facility at Hunter Biotechnology, Inc is accredited by the Association for Assessment and

Fig. 1. Chemical structure of QF, $(C_{21}H_{25}N_3O_2S)_2C_4H_4O_4$.

Accreditation of Laboratory Animal Care (AAALAC) International.

Drug treatment

Larval zebrafish (72 hdf) were grouped ($n = 30$ per group) and distributed into 6-well plates (Nest Biotech, China) in 3 mL fresh fish water for a treatment period of 48 hr until 120 hpf. QF solution was dissolved in fish water to mimic oral administration. Untreated larvae were used as blank control. The dissolved oxygen concentration in each well was maintained above 80% throughout the experiments. After 48 hr treatment of QF, larval zebrafish were subject to visual observation and image acquisition under a dissecting stereomicroscope (Olympus, Japan).

LC₁₀, NOAEL, and LOAEL and estimation

Larval zebrafish was treated with QF from 72 to 120 hpf. Fish mortality was recorded every 24 hr, and dead zebrafish were counted as its heartbeat stopped under the dissecting stereomicroscope (Nikon, Japan). According to the preliminary experiments, ten concentrations (0, 20, 30, 35, 40, 45, 50, 55, 60, 65 µg/mL) were selected to produce a mortality curve via Origin 8.0 (OriginLab, USA). The LC₁₀ (10% lethal concentration) was determined with logistic regression. Meanwhile, toxic manifestations in every larval were observed in double-blind to estimate the NOAEL (no observed adverse effect level) and LOAEL (lowest observed adverse effect level).

Hepatotoxicity assessment

Four concentrations (1/10 LC₁₀, 1/3 LC₁₀, LC₁₀ and 4/3 LC₁₀) of QF were selected for hepatotoxicity assessment. With 48 hr treatment from 72 to 120 hpf, the larvae were visually observed for imaging acquisition of specific phenotypic endpoints under the dissecting stereomicroscope installed with a high-speed video camera. As described by He *et al.*, the larval liver is approximately globular in structure with a clearly recognizable periphery against the neighboring tissues and is perfused with circulating blood cells (He *et al.*, 2013). The normal liver is clear, whereas the hepatotoxicity attacked liver becomes darker with a brown or gray coloration and the texture of liver tissue becomes amorphous undergoing degeneration and/or necrosis (McGrath and Li, 2008; Hill *et al.*, 2012). Three hepatotoxic phenotypic endpoints (liver size changes, liver degeneration, and yolk absorption delay) were adopted for assessing hepatotoxicity and calculated based on the formulas below:

Liver size (%)

= liver area (treated)/liver area (control) × 100%

Liver degeneration (%)

= [liver optical density (treated) – liver optical density (control)] / liver optical density (control) × 100%

Yolk absorption delay (%)

= [yolk sac area (treated) – yolk sac area (control)] / yolk sac area (control) × 100%

Phenotypic quantitative analyses on liver size and opacity as well as yolk sac size were conducted by using image-based morphometric analysis (NIS-Elements D3.1, Japan).

Statistical analysis

All tests were replicated until the experimental condition was optimized. Sigmoidal regression for concentration-response curve was used for estimation of LC₁₀ in Origin 8.0. One-way ANOVA followed by Dunnett's test was used to compare differences between different groups. All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, USA) and $P < 0.05$ was considered statistically significant. For the quantitative hepatotoxicity analyses, all data were presented as mean ± standard error (S.E.).

RESULTS

LC₁₀, NOAEL, and LOAEL determination

A dosage-mortality curve of larval zebrafish was presented in Fig. 2. QF induced death was observed in a dose-dependent manner, and no survival could be seen with QF above 55.0 µg/mL and no death occurred with QF below 20 µg/mL. By means of sigmoidal regression, LC₁₀ of QF was estimated as 39.0 µg/mL. Through the preliminary replications and double blind observations, the NOAEL and LOAEL could be estimated as 3.9 µg/mL (1/10 LC₁₀) and 13.0 µg/mL (1/3 LC₁₀), respectively.

Qualitative assessment of hepatotoxicity

As shown in Fig. 3, hepatotoxicity-associated manifestations in larval zebrafish were observed. The untreated larvae exhibited clear liver tissue perfused with circulating blood cells. The larvae livers were found turned to non-transparent from brown to dark and liver blood flow was hardly observable with QF treatment at doses from 13.0 to 52.0 µg/mL in a dose-dependent manner. Neither hepatomegaly nor hepatoatrophy was observed in the QF treated larvae as compared with the control. A grossly swollen yolk sac was only seen in each larvae treated with QF at 13.0 µg/mL and above doses, indicating an observable delay of yolk absorption. The occurrence rate of QF-induced hepatotoxicity in zebrafish attained 100% (30/30).

Quantitative assessment of hepatotoxicity

The QF-induced changes of liver size, liver opacity, and yolk sac size in larval zebrafish relative to the normal control were further measured using an image-based

Fig. 2. QF-induced dose-dependent mortality of larval zebrafish between 72 and 120 hpf ($n = 30$).

morphometric analysis. Little change of liver size was seen between the control and each of the treated groups (Fig. 4), whereas significant increases of liver opacity and yolk sac size presented with QF treatment at doses from 13.0 to 52.0 µg/mL, indicating a severe liver degeneration and yolk absorption delay induced by QF (Figs. 5,6). Such hepatotoxicity was exerted in a dose-dependent manner. Furthermore, a dramatic change was found induced by QF at LC₁₀ (39 µg/mL), in which yolk sac size was 4.4-fold increased and continued to get 5.0-fold increased at QF dose above LC₁₀ (52 µg/mL).

DISCUSSION

As a first-line antipsychotics for management of schizophrenia, QF has good tolerability and compliance in humans with lowest incidence of adverse events among all antipsychotic agents (Hustey and Duffull, 1999; Cheer and Wagstaff, 2004). Unfortunately, QF abuse (overdose) has become more common in clinical cases, resulting in a variety of adverse reactions including cardiovascular disorders (e.g. palpitation, tachycardia, agitation, anxiety, hypotension), gastrointestinal disorders (e.g. abdomi-

Fig. 3. Observation of larval zebrafish at 120 hpf after treatment of QF for 48 hr. The circled L and Y were larvae liver and yolk sac.

Quetiapine fumarate induces acute hepatotoxicity

Fig. 4. Liver size changes of larval zebrafish induced by QF. A: liver area; B: liver area relative to control (%). All data were represented as mean $\pm$ standard error (S.E.).

Fig. 5. Liver opacity changes of larval zebrafish induced by QF. A: liver opacity; B: increased folds of liver opacity to control (%). All data were represented as mean $\pm$ standard error (S.E.). **P < 0.01 and ***P < 0.001 vs. control.

Fig. 6. Yolk sac size changes of larval zebrafish induced by QF. A: yolk sac area; B: increased folds of yolk sac area to control (%). All data were represented as mean $\pm$ standard error (S.E.). ** $P < 0.01$ and *** $P < 0.001$ vs. control.

nal pain, dyspepsia, vomiting), and behavioral disorders (e.g. tardive dyskinesia, myoclonus, dystonia, parkinsonism, akathisia, drowsiness, unconsciousness) (Rizos *et al.*, 2009; Strachan and Benoff, 2006; Desarkar and Sinha, 2006; Bharadwaj and Grover, 2008; Shah *et al.*, 2010; George *et al.*, 2013; Dev and Raniwalla, 2000). The toxicity of QF is thought to be mediated by blockade of adrenergic $\alpha 1$ - and $\alpha 2$ -receptors (hypotension and tachycardia) and blockade of histaminergic receptors (somnolence) (Pollak and Zbuk, 2000). Liver is the main site for QF metabolism and clearance, in which sulfoxidation took place by means of cytochrome P450 3A4 isoenzyme and QF extensively metabolized to over 20 metabolites and eliminated with urine (~73%) and faeces (~21%) (Green, 1999; Gunasekara and Spencer, 1998). Except to induce a mild and transient asymptomatic elevation of liver enzymes (AST, ALT, and bilirubin), QF was once considered to be a non-hepatotoxic agent by the market (Atasoy *et al.*, 2007.). Afterwards to date, three clinical cases reported hepatic injury secondary to use of QF, raising a concern whether QF is of hepatotoxicity (Shpaner *et al.*, 2008; Mutairi *et al.*, 2012; El Hajj *et al.*, 2004). However, it is insufficient to make a judgment on QF's hepa-

totoxicity according to such extremely rare occurrences of liver abnormalities, since the etiology of liver injury could not be 100 percent determined and other concurrent medications (e.g. metformin and ramipril for diabetes and hypertension, respectively) might obscure the results in those 'case-by-case' reports (El Hajj *et al.*, 2004; Ostapowicz *et al.*, 2002). Therefore, reliable evidence is strongly needed for verifying the suspected hepatotoxicity of QF.

In the present study, a qualitative and quantitative *in vivo* assay using larval zebrafish was performed to determine the hepatotoxicity of QF. The morphological observation found that QF at $1/3$ LC_{10} (13.0 µg/mL) induced typical toxic manifestations of liver (non-transparency) and yolk sac (edema), and the hepatotoxicity increased with increasing doses of QF (Fig. 3). The loss of liver transparency (being dark or brown) might be associated with reduction of liver blood flow, presenting a typical phenotype of liver degeneration that was also observed in other studies utilizing zebrafish to assess mammalian hepatotoxicants (Hill *et al.*, 2012; Hill, 2011; Zhang *et al.*, 2003). The edema of yolk sac was another hepatotoxic endpoint associated with an outcome of yolk

absorption delay in clinic. Correspondingly, the quantitative measurement showed a similar result—that is, both levels of liver opacity and yolk sac size were increased significantly with treatment of increasing doses of QF (Figs. 5, 6). The above hepatotoxic endpoints in larval zebrafish indicate a certain hepatotoxicity of QF, verifying the aforementioned deduction from the clinical ‘case-by-case’ reports. There was a significant positive correlation of lethality values and toxicity endpoints between zebrafish and rodents (mouse or rat), and a parallel comparison has shown that LC_{50} of cyclophosphamide in zebrafish (650 $\mu\text{g/mL}$) was about twice times higher than the LC_{50} (315 mg/kg) in mouse (p.o.) (Zhang *et al.*, 2003; Hill *et al.*, 2008). Accordingly, in this study, the LOAEL (13.0 $\mu\text{g/mL}$) and above doses (upon to 52.0 $\mu\text{g/mL}$) of QF can be converted as a potentially hepatotoxic dose range (~6.5 to 26.0 mg/kg) to mouse (p.o.). By converting the clinical daily dose of QF from human (5.0 to 10.0 mg/kg) to mouse (~45.5 to 91.0 mg/kg), it can be concluded that the QF’s hepatotoxic doses (6.5 to 26.0 mg/kg for mouse) determined by our assay are far more less than the minimal dose (45.5 mg/kg for mouse) applied in clinic, indicating a hepatotoxicity associated potential safety hazard to patients exposed to QF. Covering our findings, the overall prediction success rate of zebrafish for toxicity of drugs attained 100% and can thereby be ranked as excellent (> 85%) for identifying toxicity agents by the ECVAM (European Center for the Validation of Alternative Methods) criteria (Genschow *et al.*, 2002).

A question is, why the traditional assays using cell lines or rodent animals could not predict the hepatotoxicity of QF during its development process, but zebrafish can? It may be due to the advantages of larval zebrafish, such as transparent body and hypersensitive response to toxicity, so that the potential or chronic organ toxicity could not be easily ignored. The transparency can achieve rapid and specific observation on morphologies and functions of liver and yolk sac, and the sensitivity of larval zebrafish can maximize the potential toxic responses to drugs that may not be seen in rodents (McGrath and Li, 2008). Other studies have also verified the zebrafish as a highly predictive animal model for *in vivo* evaluation of drug hepatotoxicity (He *et al.*, 2013; Du *et al.*, 2009; Mesens *et al.*, 2015). Using zebrafish as an alternative animal model for drug screening can not only greatly accelerate the drug discovery process, decrease costs, and provide more specific results than traditional assays, but can also early predict the drug toxicity before drug development, and more importantly, determine the potential toxic hazard of commonly used drugs in clinic, such as QF. This study pro-

vides the first experimental evidence for QF’s hepatotoxicity, suggesting that a particular consideration should be given to the QF-dependent patients for their liver functions and physicians should be aware of the potential serious hepatotoxicity of QF prior to its use. There is clearly a need to further improve insights into the molecular mechanisms of QF’s hepatotoxicity *in vivo*.

ACKNOWLEDGMENTS

This work was supported by the Science and Technology Project Grant of Zhejiang Food and Drug Administration system (Grant no. 2014002), National Natural Science Foundation of China (Grant no. 81302989), Zhejiang Provincial Science and Technology Project of Traditional Chinese Medicine of China (Grant no. 2013ZQ007), Independent Project of Zhejiang Provincial Scientific and Technological Innovation Team of China (Grant no. 2011R50022-02), Research Fund for the Doctoral Program of Higher Education of China (Grant no. 20133322120006), Scientific research project of Zhejiang Provincial Department of Education (Grant no. Y201534534, Y201226186), and Ningbo Natural Science Foundation (Grant no. 2014A610277).

Conflict of interest---- The authors declare that there is no conflict of interest.

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