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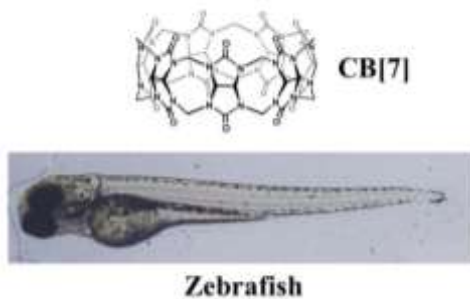


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The macrocyclic Cucurbit[7]uril was evaluated for its *in vivo* toxicity profile, including developmental toxicity and organ-specific toxicities using zebrafish models.

Developmental and organ-specific toxicity of cucurbit[7]uril: *in vivo* study on zebrafish models

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Abstract

The macrocyclic molecular container cucurbit[7]uril (CB[7]), the most water-soluble homologue in the cucurbit[*n*]uril family (*n* = 5-8, 10, 14), has been evaluated for its *in vivo* toxicity profile, including its developmental toxicity such as its effect on hatching, growth and survival, as well as its potential organ-specific toxicities such as cardiotoxicity, hepatotoxicity, and locomotion and behavioral toxicity, using zebrafish models. The results revealed that CB[7] has measureable cardiotoxicity and locomotion and behavioral toxicity at concentrations of ~500 μM or higher, and negligible developmental and hepatotoxicity at concentrations up to 750 μM, although extended exposure to CB[7] at the 500-750 μM concentration range induced the mortality of tested fish. These results demonstrate for the first time with live *in vivo* animal models that

CB[7] has relatively low developmental and organic specific toxicity, and support further exploration of the use of CB[7] in biomedical research at sub-toxic concentrations.

1. Introduction

Macrocyclic host molecules such as the cyclodextrins and calixarenes have received considerable attention as vehicles for drug delivery, due to their ability to improve the water solubility, bioavailability, physical and chemical stability, and release profiles of drugs.^{[1] [2] [3] [4]} During recent years a new family of macrocyclic host molecules, the cucurbit[*n*]urils (CB[*n*], *n* = 5-8, 10, 14) have received increasingly intensive attention and have been the subject of a number of reviews due to their potential application in drug formulation and delivery.^{[5] [6]} CB[*n*] consists of *n* glycoluril units that are bridged by 2*n* methylene groups, forming a hydrophobic cavity and two identical carbonyl-lined portals with negative dipole potentials. The reaction of glycoluril and excess formaldehyde in the presence of concentrated sulfuric acid or hydrochloric acid at a temperature of ~110 °C produces the macrocyclic CB[*n*] compounds through an acid-catalyzed condensation reaction, initially only CB[6] was isolated and characterized.^[7] In the years of 2000-2001, the research groups of Kim and Day independently isolated the homologues CB[5], CB[7] and CB[8], using a lower temperature of 75-90 °C for the synthesis of these macrocycles.^{[8] [9]} This development dramatically expanded the versatility of cucurbit[*n*]uril chemistry and has attracted more scientists to this field. During the past fifteen years, CB[*n*]s have demonstrated outstanding molecular recognition properties and superior interactions with a wide range of neutral and positively charged molecules, especially those possessing imine or amine groups, many of which are bioactive and medically relevant compounds.^{[5] [6] [10]}

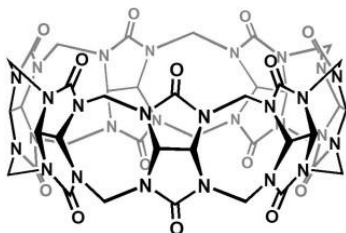


Fig. 1. The molecular structure of CB[7].

Among the CB[*n*] family, CB[7] (shown in Fig. 1) has received perhaps the greatest attention as a potential drug delivery vehicle due to its superior water-solubility and compatible size with various organic and organometallic drug molecules.^[5-6] Examples of drugs which have been studied with CB[7] include: atenolol (a beta-blocker),^[11] pyrazinamide (a tuberculosis drug),^[12] platinum-based anti-cancer drugs such as cisplatin,^[13] prilocaine (a local anaesthetic),^[14] coumarin (an anticoagulant),^[15] ranitidine (used to treat ulcers),^[16] and vitamin B₁₂ as well as coenzyme B₁₂.^[17] For instance, we have reported the encapsulation of ranitidine by CB[7], and this encapsulation protected the drug from thermal degradation and could potentially extend the shelf-life of this drug.^[16] Similarly, we studied the encapsulation of vitamin B₁₂ and coenzyme B₁₂ by CB[7], through binding to the 5,6-dimethylbenzimidazole α -nucleotide base, and observed stabilization of the base-off forms of these compounds.^[17] Wheate and coworkers reported that various platinum-based anticancer complexes have increased thermal stability and reduced cytotoxicity upon complexation with CB[7].^[18] ^[19] As demonstrated by these and other examples, the number of pharmaceutically relevant applications of CB[7] is rapidly expanding. Therefore, it has become critical to clearly understand the intrinsic toxicity profile of CB[7] before its application in real-world drug formulations.

Several *in vitro* studies on cell cultures have shown that CB[7] exhibits very low toxicity at sub-mM concentrations or even up to 1 mM concentrations.^[20] ^[21] When CB[7] incubated with Chinese Hamster Ovary cells (CHKO-K1) for 2 days, the IC₅₀ value was found to be 0.53 mM, and up to 1 mM of CB[7] was found to be tolerable at shorter incubation times (e.g. 3 h).^[21] When assays were used to evaluate their metabolic activity and cytotoxicity (AK assay and MTS assay), CB[7] macrocycles have shown high cell tolerance at concentrations of up to 1 mM in cell lines originating from the human kidney, liver or blood tissue.^[20] The effects observed for an intravenous single dose *in vivo* injection with a mice model demonstrated that CB[7] has a very low acute toxicity of 250 mg/kg, based on a body weight change of less than 10% within 5 days of the injections. In addition, all of the mice injected via slow infusion into the vein began to recover after 5-8 days of the injections, thus the maximum tolerated dose (MTD) was

established at approximately 250 mg/kg dose level.^[21] A limitation of these *in vitro* and *in vivo* studies is that little information can be collected on the toxicity of CB[7] to specific organs. Very recently, the tissue specific toxicity including neuro-, myo- and cardiotoxicity of CB[7] has been examined with the use of *ex vivo* electrophysiological models.^[22] The study reported that 1 mM of CB[7] did not exhibit statistically measurable neurotoxicity as examined using mouse sciatic nerve compound action potential. However, myotoxic and cardiotoxic activities were observed in the presence of CB[7] concentrations of 0.3 mM, which were determined by nerve-muscle force of contraction through chemical and electrical stimulation and changes in the rate and force of right and left atria contraction.^[22] Even with these very recent results, *in vivo* organ-specific toxicity profiles of CB[7] in live animal models are still not available. In addition, there has been no evaluation of CB[7]'s toxicity profile with regard to the development of any *in vivo* models.

Zebrafish has become a powerful and cost-efficient model for the evaluation of drug-induced toxicity, and it has been widely used for the assessment and prediction of developmental toxicity and organ-specific toxicities targeting the heart, central nervous system (CNS), the liver as well as the growth and development of living samples.^{[23] [24] [25] [26] [27] [28]} One of the significant advantages of the zebrafish model is its rapid and synchronous growth. In addition, the transparency of the chorion and body enables non-invasive and direct observations during embryogenesis. More importantly, several reports have shown that the biological responses of zebrafish to small molecules and drugs are similar to that of mammals.^[29] In addition, the gene networks of zebrafish regulating embryonic development, normal physiology and pathogenesis also showed high conservation in comparison with humans. These features suggest that zebrafish shall provide an useful model for the evaluation of CB[7] toxicity that exhibits a safety profile that may be similar to that of humans.^[30] With regard to studies of organ-specific toxicity, zebrafish has been identified as an ideal model for investigations involving cardiac function and human heart diseases.^[31] Functional and behavioral studies focusing on parameters such as hearing, aggression, learning and locomotion have also been developed that are useful for identifying neurotoxins without obvious phenotypic

changes.^[32] The clear chorion and embryo of the zebrafish allow for continuous visualization of the anatomical changes associated with the development of this species. This visual clarity, along with short maturation times and the capability of complex behavior, makes this model particularly useful for measuring changes to the development phases.^[33] Meanwhile, zebrafish models have also been employed for hepatotoxicity studies to provide first-hand *in vivo* liver-specific toxicity data, although their liver anatomy differed from that of rodents and humans.^[34]

Herein we will report our efforts to utilize zebrafish models to study the developmental and organ-specific toxicity profile of CB[7]. The results obtained through these efforts will provide valuable insight into the toxicity of CB[7], which will be of immense importance with respect to the future use of CB[7] in the biomedical field.

2. Materials & Methods

2.1 Zebrafish husbandry and breeding

Wild type zebrafish were used for developmental toxicity, hepatotoxicity and locomotion and behavioral assays, while Tg (*cmcl2*:GFP) zebrafish with GFP (green fluorescent protein) specifically expressed in the myocardial cells were used for cardiotoxicity assays.^[35] The procedures for zebrafish culture, breeding, embryo collection, embryonic and larval culture, fluorescent observation were performed according to the standard procedures.^[36] Briefly, adult fish were raised in an aquaculture system with 12 h alternating light/dark cycles, and fed twice a day with newly hatched brine shrimp. Mature male and female zebrafish (at a ratio of 1 male to 2 females) were transferred into a breeding tank and separated by a mesh screen the night before breeding. All fertilized embryos were examined under a microscope and those that developed normally were selected for subsequent experiments. The collection of embryos, and exposure experiments were all performed in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄ at pH 7.2-7.3). Zebrafish and embryos were maintained at 27 ± 1 °C. All experiments involving animals were conducted according to the ethical guidelines of the Institute of Chinese Medical Sciences, University of Macau.

2.2 Developmental toxicity assay

In order to determine the developmental toxicity of CB[7], the hatchability, morphology, survival rate and body length of the zebrafish were measured. During the hatchability study, at 1 day post fertilization (dpf), zebrafish embryos at the same developmental stage were randomly distributed into a 24-well microplate (14 to 17 fish per well) and exposed to 1 mL solutions of CB[7] at various concentrations (0, 125, 250, 500, and 750 μM , respectively). After 2 days of incubation, the hatched larvae were counted in each group, and the hatchability was analyzed by the ratio of hatched numbers/total treated numbers $\times 100\%$. In order to perform the morphology, survival rate and body length measurements, 1 dpf zebrafish embryos were dechorionated under microscope by the manual removal of the chorion with fine forceps, and then randomly distributed into a 24-well microplate (12 to 14 fish per well), before they were incubated with 1 mL solutions of CB[7] (at 0, 125, 250, 500, 750 μM , respectively) for 2 days. Survival rates were calculated, while the morphologies and body lengths of the zebrafish were measured using a Olympus DSU (Disk Scanning Unit) Confocal Imaging System.

2.3 Cardiotoxicity assay

Tg(*cmlc2*:GFP) zebrafish embryos were used as cardiotoxicity assay. All embryos were cultivated in embryo medium containing 0.003% (wt%) of 1-phenyl-2-thiourea (PTU) to block pigmentation since 1 dpf. Meanwhile, 2 dpf zebrafish embryos were dechorionated as mentioned above, and then randomly distributed into a 24-well microplate (12 to 16 fish per well), treated with 1 mL solutions of CB[7] (at various concentrations including 0, 125, 250, 500, 750 μM) and with a 1 mL solution of doxorubicin (40 μM , an anticancer drug with well-known cardiotoxicity) as a positive control. After 2 days of incubation, zebrafish were immersed into 1% (wt%) low-melting point agarose matrix (Gibco) to fix them in a dorsal orientation and restrict their movement. The heart morphology, heart rates (HR) and quantitative assessment of other cardiac functions were obtained from 15 s video segments recorded of individual fish using an Olympus Cell[^]R imaging system comprising IX71 microscope at room temperature. Ventricular functions were evaluated with various parameters, which were

measured as described previously.^[37] Images from the video were used to measure the longitudinal axis length (a) and lateral axis length (b) between the myocardial borders of ventricles at end-diastole and end-systole, respectively. In order to measure the HR, the number of heartbeats in a 15 s interval was counted. The ventricular volume at end-diastole (EDV) and end-systole (ESV) in the larvae was calculated from the heart dimensions using the formula for a prolate spheroid: $V = 4/3\pi ab^2$. The stroke volume (SV), cardiac output (CO) and percent fractional shortening (% FS) were calculated as follows: $SV = (EDV - ESV)$, $CO = SV \times HR$, $\%FS = (Diastolic\ diameter - Systolic\ diameter) / (Systolic\ diameter) \times 100\%$.

2.4 Assessment of liver toxicity

Zebrafish (4 dpf) were treated with 0, 125, 250, 500, 750 μ M of CB[7] in fish medium for 1 day. A fish medium sample that did not contain CB[7] was used as the blank control. Meanwhile, a fish medium sample containing 25 μ M amiodarone (ADR, with known liver toxicity) was used as the positive control. At the end of the treatment, 8-10 zebrafish from each group were randomly selected to be photographed under a microscope. The fish were anesthetized with 0.004% tricaine before they were placed dorso-laterally under the imaging system. The entire liver and yolk sac of each fish was measured, and analysis of the liver and yolk sac volumes were performed using the ImageJ software package.

2.5 Assessment of locomotion and behavioral toxicity

Zebrafish larvae (6 dpf) were placed into the wells of a 96-well plate (1 larvae/well). The fish was acclimatized for 30 min and their basal swimming behavior was monitored for 10 min using a Viewpoint Zebrabox system. Various concentrations of CB[7] (0, 125, 250, 500, 750 μ M) or 10 mM pentylenetetrazol (PTZ, a chemical known to induce seizure) as a positive control were added to each well (8-10 fish in each group). The swimming behavior of the larvae was then recorded for 10 min starting at time point of 0 min, 15 min, 30 min, 60 min, 120 min and 240 min, respectively. The

swimming distance reached by each zebrafish was then analyzed using the Zebrabox system.

3. Results and Discussion

3.1 Developmental toxicity

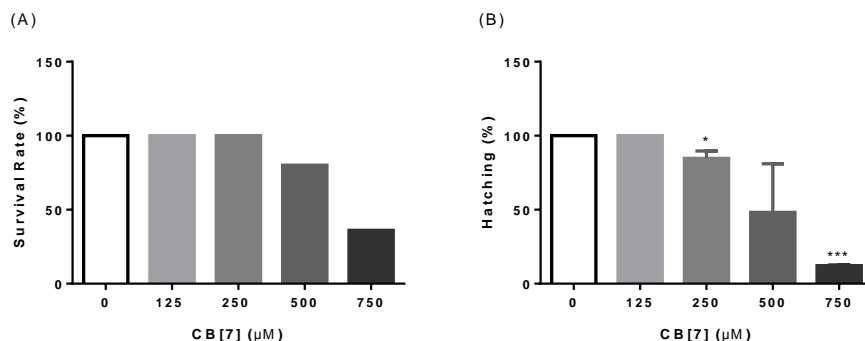


Fig. 2 Survival rates of zebrafish (1 dpf) exposed with various concentrations of CB[7] for 2 days (A), and hatchability of zebrafish embryos within 2 days of exposure to various concentrations of CB[7] (B). Data are presented as mean \pm S.D. (n = 14-17). * $P < 0.05$, *** $P < 0.001$ significantly different compared with the control group.

As shown in Fig. 2A, the mortality of the zebrafish increased when they were exposed to elevated concentrations of CB[7] for 2 days. No significant effect on the survival rate was observed for the 125 and 250 μM treatment groups. However, one treatment of groups of zebrafish larvae (n=13-14 in each group) with 500 and 750 μM of CB[7] for 2 days resulted in 77% and 38% survival rates, respectively. None of the zebrafish survived when they were exposed to CB[7] at concentrations of 1 mM or higher for 2 days. Thus the lethal dose LD_{50} is likely around 750 μM for these 1dpf zebrafish larvae. With shorter incubation times such as within 24 h, no mortality was observed even among the 750 μM CB[7] group, which indicates that extended exposure to CB[7] causes fish death. Similarly, the hatchability of zebrafish embryos also decreased in a

dosage-dependent manner after the embryos had been exposed to CB[7] for 2 days. While exposure to 250 μM CB[7] showed moderate impact on the hatchability, higher concentrations of CB[7] (>500 μM) significantly inhibited the hatchability of the zebrafish embryos when compared with that of the control group (Fig. 2B).

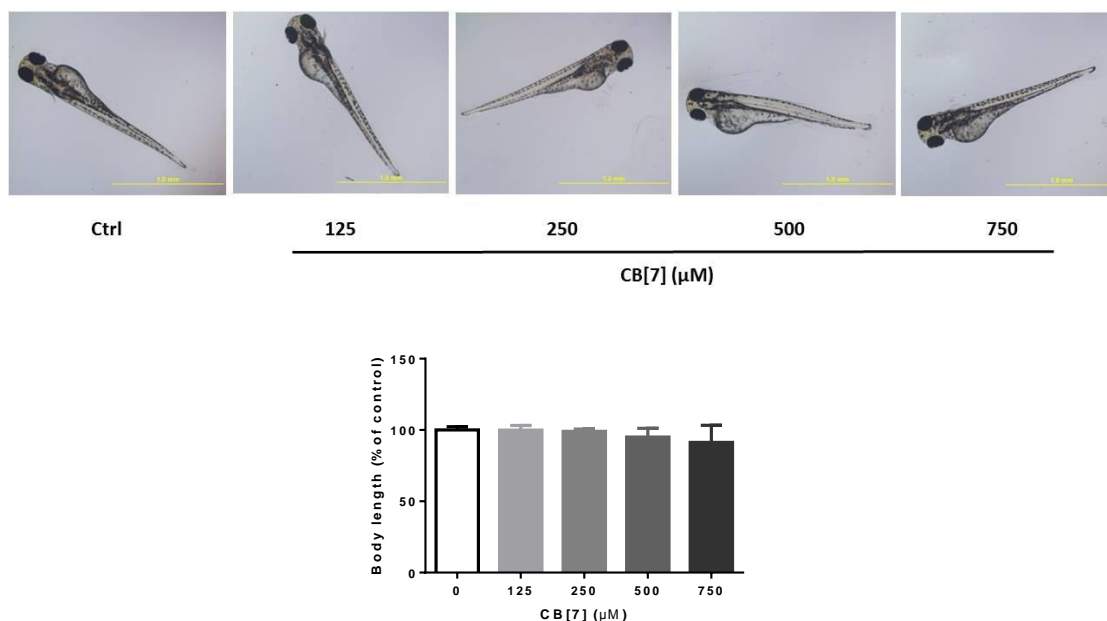


Fig. 3 Morphology of representative zebrafish larvae (3 dpf) after exposure to various concentrations of CB[7] for 2 days (top, scale bar: 1.0 mm), and their body lengths of each group, relative to the control group, by the end of incubation (bottom). Data are presented as mean \pm S.D. (n = 12). No statistical difference was observed across these groups.

When the morphologies of these CB[7] treated fish were examined, no obvious morphological abnormalities, such as crooked bodies (CB), spinal deformities (SD), yolk sac edemas (YSE) or pericardial edema (PE), were observed (Fig. 3 top). CB[7] treatment did not exhibit any significant effects on the growth of the body length of zebrafish after 2 full days of incubation of 1 dpf zebrafish. It is noteworthy that, even for the treatment group with 750 μM CB[7] that has shown high mortality, the fish that did survive still had similar body lengths with those of the control group, indicating that the

physical growth of zebrafish larvae had a high tolerance to CB[7] (Fig. 3 bottom). Overall, these results suggested that CB[7] exhibited certain toxic effects on the development (e.g. hatchability and mortality) of the zebrafish. At the same time, since no obvious morphological abnormalities or changes in the body length were observed for those fish that survived these mortality studies, we postulated that the developmental toxicity may be a non-lethal factor, and likely CB[7] toxicity on other organs or tissues may be the cause of zebrafish death.

3.2 Cardiotoxicity

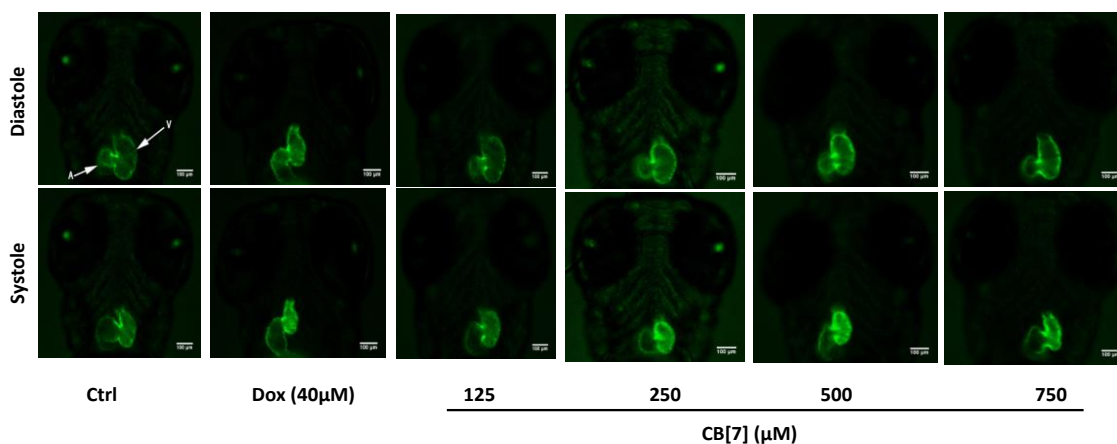


Fig. 4 Representative fluorescent microscopic images of Tg (*cmcl2::GFP*) zebrafish (4 dpf) with GFP specifically expressed in the myocardial cells, exposed to various concentrations of CB[7] or 40 μ M doxorubicin (Dox) as a positive control, for 2 days. “A” denotes atrium, and “V” denotes ventricle (Scale bar, 100 micron).

To evaluate the cardiotoxicity of CB[7], cardiac morphology was evaluated first when the zebrafish were treated with various concentrations of CB[7]. As shown in Fig. 4, representative images of zebrafish hearts displayed the systolic and diastolic functions after doxorubicin (Dox) or CB[7] treatment. Dox-induced cardiotoxicity has been widely reported both in humans and among laboratory animals, including mice, rats and zebrafish.^[38] Therefore, Dox was used as a positive control in our study. Exposure to Dox caused the hearts of the zebrafish to become distorted into an elongated shape with a

thin atrium, a compact ventricle, and a massive pericardial edema. These morphological defects are related to cardiac dysfunction that has been well known.^[38] Exposure to the 125, 250 and 500 μM CB[7] solutions did not exhibit an obvious influence on the heart morphology. However, it seems that exposure to 750 μM CB[7] resulted in similar but less severe responses to those induced by Dox, but no pericardial edema was found. As expected, no signs of heart failure or edema were found among the zebrafish hearts in the control group.

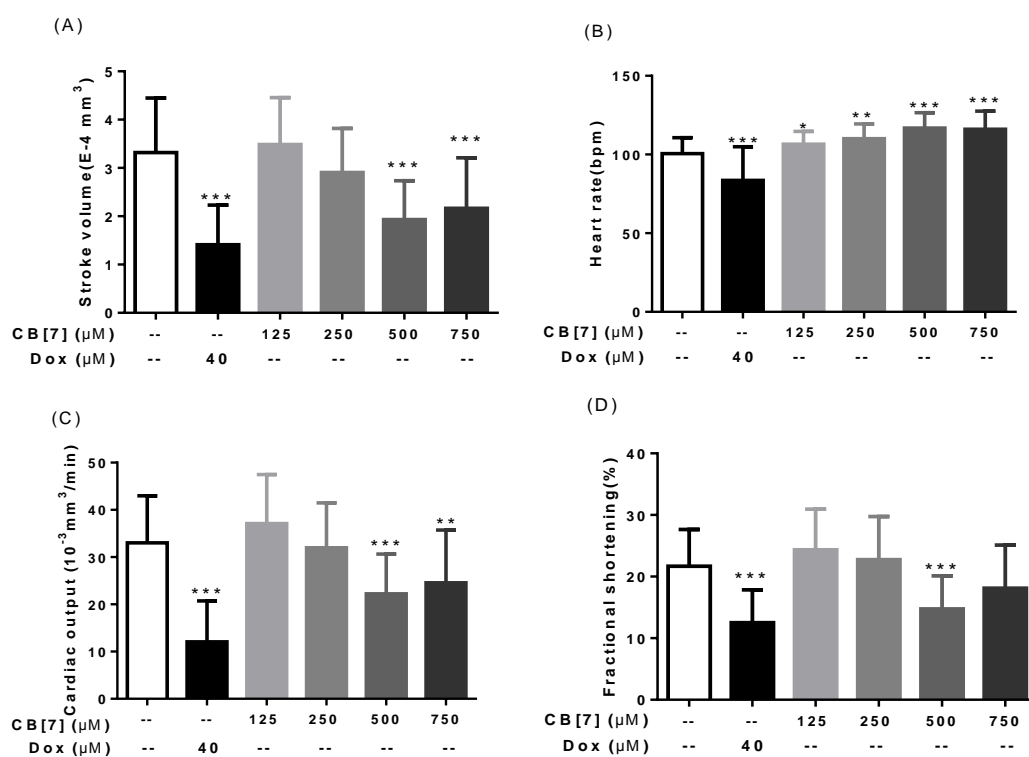


Fig. 5 Cardiac functions of zebrafish (2dpf) exposed to various concentrations of CB[7] for 2 days. Stroke volume (A), heart rate (B), cardiac output (C) and % FS (D) of zebrafish were shown. Data are presented as mean \pm S.D. ($n = 16$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different compared with the control group.

Additionally, cardiac function was evaluated according to several parameters, including the stroke volume (SV), heart rate (HR), cardiac output (CO), and fractional

shortening (FS). As demonstrated by Fig. 5, exposure to 500 μM or higher concentrations of CB[7] dramatically decreased the SV, CO, and FS, in comparison with the control group. Similar cardiotoxic effects on the SV, CO, and FS were observed upon exposure to Dox. Meanwhile, exposure to 125 and 250 μM CB[7] did not cause significant effects on these parameters. On the other hand, it is evident that Dox caused a moderate reduction of the HR. In contrast, CB[7] treatment slightly elevated the HR in a dose-dependent manner in the concentration range of 125-750 μM (Fig. 5B). The cardiotoxicity profile of CB[7] seems to be consistent with a observations reported in a recent *ex vivo* study.^[22]

With these results in hand, we speculate that CB[7]-induced heart failure may be a result of tachyarrhythmia or structural heart damage. It is generally accepted that tachycardia begets cardiac dysfunction and cardiac dysfunction begets tachycardia.^[39] As shown in Fig. 5B, tachycardia was observed in all treatment groups. However, significant reduction in the SV, CO and FS only appeared upon exposure to higher concentrations (≥ 500 μM) of CB[7]. This may be attributed to the initial compensatory mechanism, resulting in near normal stroke volume, cardiac output and fractional shortening. In more severe forms, greater increases in the HR can cause these parameters to deteriorate further and cause already reduced systolic function to worsen.^[40]

3.3 Locomotion and behavioral toxicity

The locomotion and behavior, namely the total swimming distance (including short route and long route), was measured at several time points within a period of 4 h after the fish had been initially treated with various concentrations of CB[7] and 10 mM PTZ (positive control). Exposure to PTZ, which is a convulsant and was thus used as the positive control here, caused a significant increase in the swimming distance at 15 min post treatment (Fig. 6B), but the fish recovered to normal swimming behaviors with longer time exposure. In contrast, it can be seen that CB[7] treatment significantly reduced the total swimming distance (in other words, inhibited the locomotion) of zebrafish after 2 h and 4 h of incubation in a dose-dependent manner at the concentration range of 250-750 μM (Fig. 6E & F). In contrast, shorter incubation times with CB[7] did

not result in statistically meaningful changes in the locomotion (see supporting information for the recorded swimming patterns and paths).

In many studies, changes in locomotion have been regarded as a behavioral endpoint.^{[41] [42]} The concept is simple but meaningful based on the idea that ‘all behaviors are predicated on the ability to move’.^[42] Among investigations involving fish, locomotory activity endpoints include swimming speed, distance covered and turning rate. Associations between changes in zebrafish locomotions and neurotoxicity have also been noted reports investigating the mechanisms of addiction to amphetamines,^[43] cocaine,^[44] and ethanol.^[45] Admittedly, non-neurological changes can also affect behaviour. For example, musculoskeletal injury and developmental abnormalities may be associated with behaviors that deviate from those exhibited by healthy individuals. However, in our study, the changes in the swimming behavior appeared within a short time of exposure (2-4 h) and no physical injuries or abnormalities were observed during subsequent physical examinations of the fish. Thus, we believe that the effect of CB[7] is likely on the neurological system, rather than the musculoskeletal system. In addition, the locomotion and behavior toxicity could be induced by other organ toxicity such as the already-seen cardiotoxicity. It is noteworthy that the previously reported *ex vivo* study did not find evidence of any measurable neurotoxicity arising from exposure to 1 mM concentration CB[7].^[22]

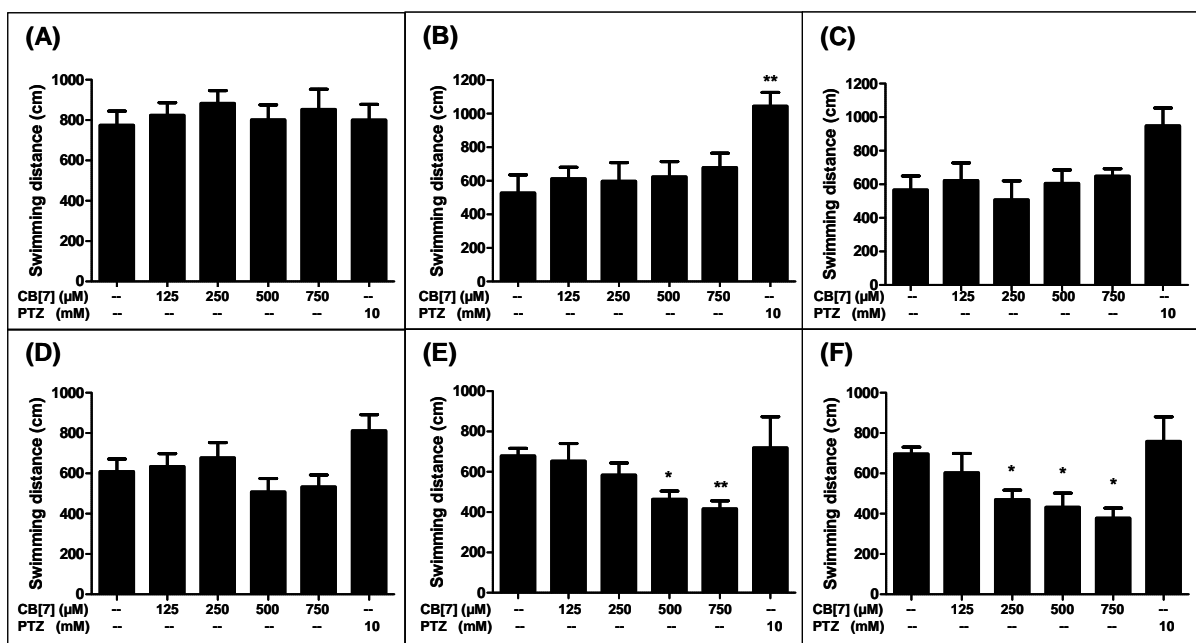


Fig. 6 Measurements of the total swimming distance of each fish in 10 min (wild type zebrafish at 6 dpf, the number of fish per group is 16) with CB[7] exposure durations of 0 (A), 15 (B), 30 (C), 60 (D), 120 (E), and 240 min (F). Data are presented as mean \pm S.D.% (n = 8). * P <0.05, ** P <0.01 significantly different compared with the control group.

3.4 Hepatotoxicity

Due to the similarity of the general structure, organization and functions of zebrafish liver to that of mammals, zebrafish has been used as a good vertebral model for drug metabolism studies.^[46] Another advantage of this model is that the liver of zebrafish is fully formed and functioning by 3 dpf. In our study, hepatotoxicity of CB[7] was determined by examining liver degeneration and yolk sac retention. As seen in Fig. 7, there were no obvious changes in either the liver size or the yolk sac size when the treated groups were compared with the control group. In contrast, exposure to ADR (25 μ M, as a positive control) significantly decreased the liver size (liver degeneration) and induced yolk sac retention. Thus, the results demonstrated the non-toxic property of CB[7] itself and likely its metabolites as well. However, a limitation of this assay is that only phenotypic changes were observed. The change of hepatic enzyme activity, which is

an important indicator of liver functions, could not be detected due to the limited size of the liver in zebrafish larvae. Nonetheless, the zebrafish liver's high tolerance to CB[7] at this concentration level seems to be supported by findings from previous investigations that utilized *in vitro* assays to evaluate the cytotoxicity of CB[7] (at concentrations of up to 1 mM) to cell lines originating from human liver tissue.^[20]

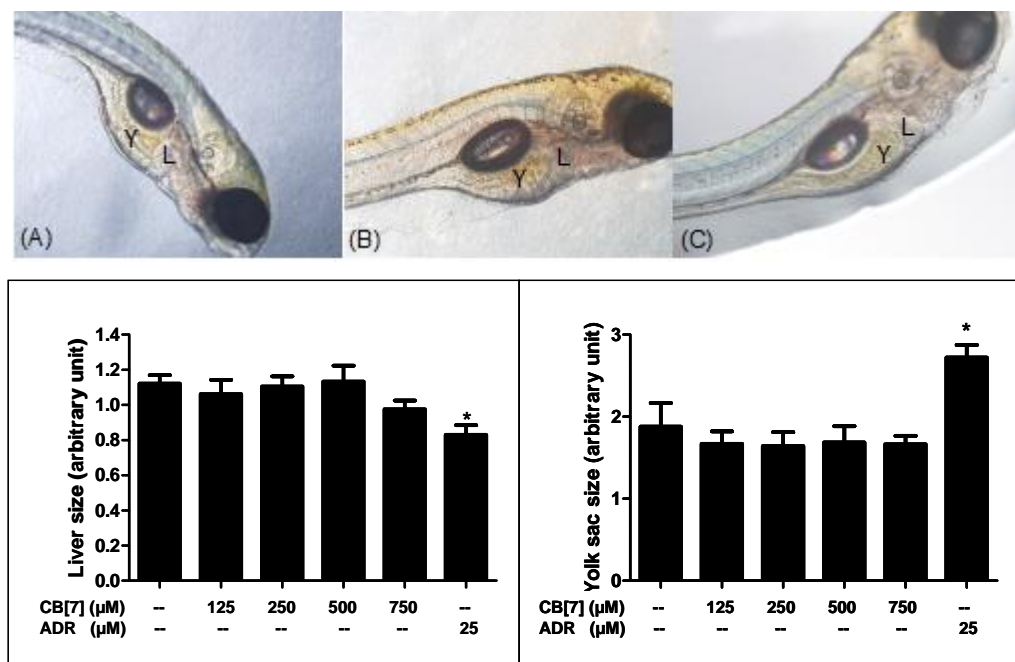


Fig. 7 Top panel: representative pictures showing the liver (L) and yolk sac (Y) of zebrafish larvae. (A) Negative control: zebrafish without treatment; (B) Positive control: zebrafish treated with ADR (25 μM) for 24 h; (C) Zebrafish treated with 750 μM of CB[7] for 24 h. **Bottom panel:** liver and yolk sac volume measurements of zebrafish treated with various concentrations of CB[7] or ADR (25 μM) for 24 h. Data are presented as mean ± S.D.% (n = 8). * $P < 0.05$ significantly different compared with the control group.

4. Conclusion

In this study, the CB[7] was examined for its potential developmental and organ-specific toxicities in live *in vivo* zebrafish models for the first time. The results show that CB[7] exhibits measurable cardiotoxicity and locomotion and behavioral toxicity (likely a result of neurotoxicity) at concentrations of 0.50 mM or higher, without significantly observable change on hepatotoxicity phenotype at concentrations up to 0.75 mM. In addition, CB[7] presented a LD₅₀ likely around 0.75 mM for 1 dpf zebrafish larvae when treated with CB[7] for 2 days, although interestingly at this concentration range CB[7] did not seem to affect the physical growth and morphology of the surviving fish. The functional concentration of CB[7] employed in drug complexation and delivery applications is often at 0.25 mM or lower, and thus the overall *in vivo* toxicity profile is still safe at this concentration range. Consequently, these results still support future exploration of the use of CB[7] in pharmaceutical sciences and medicinal chemistry at sub-toxic concentrations, and these results warrant further investigation of the biosafety of CB[7] in large animal models and human tissue samples.

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