Protective effects of beta-cyclodextrins vs. zearalenone-induced toxicity in HeLa cells and *Tg(vtg1:mCherry)* zebrafish embryos

Zelma Faisal [a,b,#], Edina Garai [c,#], Rita Csepregi [b,d], Katalin Bakos [c], Eszter Fliszár-Nyúl [a,b], Lajos Szente [e], Adrienn Balázs [f], Mátyás Cserháti [f], Tamás Kőszegi [b,d], Béla Urbányi [c], Zsolt Csenki [c,*], Miklós Poór [a,b,*]

[a]Department of Pharmacology, Faculty of Pharmacy, University of Pécs, Szigeti út 12, H-7624 Pécs, Hungary

[b]János Szentágothai Research Center, University of Pécs, Ifjúság útja 20, H-7624 Pécs, Hungary

[c]Department of Aquaculture, Institute of Aquaculture and Environmental Safety, Faculty of Agricultural and Environmental Sciences, Szent István University, Páter Károly u. 1, H-2100 Gödöllő, Hungary

[d]Department of Laboratory Medicine, Medical School, University of Pécs, Ifjúság út 13, H-7624 Pécs, Hungary

[e]CycloLab Cyclodextrin Research & Development Laboratory, Ltd., Illatos út 7, H-1097 Budapest, Hungary

[f]Department of Environmental Safety and Ecotoxicology, Institute of Aquaculture and Environmental Safety, Faculty of Agricultural and Environmental Sciences, Szent István University, Páter Károly u. 1, H-2100 Gödöllő, Hungary

[#]These authors contributed equally to this work.

*Corresponding authors:

Miklós Poór, PharmD, PhD; Department of Pharmacology, Faculty of Pharmacy, University of Pécs, Szigeti út 12, H-7624 Pécs, Hungary; Phone: +36-72-536-000 ext: 35052; E-mail: poor.miklos@pte.hu

Zsolt Csenki, PhD; Department of Aquaculture, Institute of Aquaculture and Environmental Safety, Faculty of Agricultural and Environmental Sciences, Szent István University, Páter Károly u. 1, H-2100 Gödöllő, Hungary; Phone: +36-28-522-000 ext: 2316; E-mail: csenki.zsolt@mkk.szie.hu

E-mail addresses: faisal.zelma@gytk.pte.hu (Z.F.), edina.garai@phd.uni-szie.hu (E.G.), ritacsepregi93@gmail.com (R.C.), bakos.katalin@mkk.szie.hu (K.B.), eszter.nyul@aok.pte.hu (E. F-N.), szente@cyclolab.hu (L.S.), balazs.adrienn@mkk.szie.hu (A.B.), cserhati.matyas@mkk.szie.hu (M.C.), koszegi.tamas@pte.hu (T.K.), urbanyi.bela@mkk.szie.hu (B.U.)

Abstract

Zearalenone is a xenoestrogenic mycotoxin produced by *Fusarium* species. High exposure with zearalenone induces reproductive disorders worldwide. Cyclodextrins are ring-shaped host molecules built up from glucose units. The apolar cavity of cyclodextrins can entrap so-called guest molecules. The formation of highly stable host-guest type complexes with cyclodextrins can decrease the biological effect of the guest molecule. Therefore, cyclodextrins may be suitable to decrease the toxicity of some xenobiotics even after the exposure. In this study, the protective effect of beta-cyclodextrins against zearalenone-induced toxicity was investigated in HeLa cells and zebrafish embryos. Fluorescence spectroscopic studies demonstrated the formation of stable complexes of zearalenone with sulfobutyl-, methyl-, and succinyl-methyl-substituted beta-cyclodextrins at pH 7.4 (K = 1.4-4.7 × 10^4 L/mol). These chemically modified cyclodextrins considerably decreased or even abolished the zearalenone-induced loss of cell viability in HeLa cells and mortality in zebrafish embryos. Furthermore, the sublethal effects of zearalenone were also significantly alleviated by the co-treatment with beta-cyclodextrins. To test the estrogenic effect of the mycotoxin, a transgenic bioindicator zebrafish model (*Tg(vtg1:mCherry)*) was also applied. Our results suggest that the zearalenone-induced vitellogenin production is partly suppressed by the hepatotoxicity of zearalenone in zebrafish. This study demonstrates that the formation of stable zearalenone-cyclodextrin complexes can strongly decrease or even abolish the zearalenone-induced toxicity, both *in vitro* and *in vivo*. Therefore, cyclodextrins appear as promising new mycotoxin binders.

Keywords: zearalenone; beta-cyclodextrins; mycotoxin binders; transgenic; bioindicator; vitellogenin

1. Introduction

Zearalenone (ZEN; Fig. 1) is a xenoestrogenic mycotoxin produced by *Fusarium* species, which is a contaminant in cereals (e.g., maize and wheat), spices, and in different beverages, e.g., milk and beer (Maragos, 2010; EFSA, 2017). Because of the high thermal stability and wide occurrence of ZEN, its removal from the food chain is difficult (Ryu et al., 1999). Based on cell and animal experiments, several adverse effects are attributed to ZEN, e.g., hepatotoxicity and genotoxicity (Zinedine et al., 2007; Cheraghi et al., 2015). Furthermore, ZEN can activate estrogen receptors in humans and animals, therefore, ZEN is an endocrine disruptor molecule which induces reproductive disorders (EFSA, 2017; Shier et al., 2001). ZEN is extensively metabolized in the body, during which reduced derivatives (zearalenols, zearalanone, and zearalanols) and glucuronic acid conjugates of ZEN and its reduced metabolites are produced (EFSA, 2017). Some of these metabolites (e.g., α-zearalenol and α-zearalanol) bind with significantly higher affinity to the estrogen receptors (and consequently exert higher toxicity) than ZEN (Shier et al., 2001; Filannino et al., 2011).

Cyclodextrins (CDs) are ring-shaped host molecules with a hydrophilic external part, which ensures excellent aqueous solubility, and an apolar internal cavity, which can accommodate lipophilic guest molecules (Szente and Szejtli, 1999; Szente et al., 2018). Therefore, they are frequently utilized by food, cosmetic, and pharmaceutical industries. The pharmaceutical application of beta-CDs is most common, due to their favorable cavity size for drugs (Challa et al., 2005). The native beta-CD (BCD) is often contained by orally administered drugs, however, its parenteral use is limited due to its nephrotoxicity and relatively low aqueous solubility of BCD (Jambhekar and Breen, 2016a). Methylated beta-CDs are absorbed from the gastrointestinal tract and cause nephrotoxic effects, therefore, they are not used neither orally nor parenterally (Jambhekar and Breen, 2016a). The sulfobutylated beta-CD is an excellent solubilizer without nephrotoxic adverse effect, thus, it is even suitable for parenteral

application (Jambhekar and Breen, 2016b). Generally, the pharmaceutical industry applies drug-CD complexes with low binding constants to increase the aqueous solubility, gastrointestinal absorption, and/or cellular uptake of drugs (Jambhekar and Breen, 2016a). However, formation of highly stable CD complexes can strongly decrease the pharmacological effect and tissue uptake of drugs and other xenobiotics (Schaller and Lewald, 2016; Weiss-Errico et al., 2017).

Native and chemically modified beta-CDs can form stable complexes with mycotoxins, including aflatoxins (Dall'asta et al., 2003), citrinin (Poór et al., 2016), ochratoxin A (Poór et al., 2015a), and ZEN/zearalenols (Poór et al., 2017). The interaction of ZEN with beta-CDs has been reported in previous studies, demonstrating that native and chemically modified beta-CDs form highly stable complexes with ZEN (K is in the 10^4-10^5 L/mol range) (Dall'Asta et al., 2008; Dall'Asta et al., 2009; Poór et al., 2015b). Among beta-CDs tested, ZEN formed the most stable complexes with methyl and sulfobutyl derivatives (Poór et al., 2015b).

A beta-CD bead polymer has been shown recently to effectively remove ZEN and zearalenols added to aqueous solutions and corn beer samples (Poór et al., 2018). Furthermore, BCD strongly alleviated the toxic effect of ZEN in HepG2 cells, probably by limiting toxin uptake by the cells, as a result of the formation of highly stable mycotoxin-CD complexes (Poór et al., 2015b). Based on these observations, we hypothesize that CDs may also be effective as *in vivo* binders of ZEN.

There are numerous of endocrine disruptors in the environment, especially estrogenic xenobiotics. Sensitive biomonitor/bioindicator organisms are commonly applied to test xenoestrogenic effects. Among these biomonitoring organisms, several fish models, including zebrafish, exist (Chen et al., 2010; Fetter et al., 2014; Bakos et al., 2019). The main advantage of zebrafish as a biosensor is the transparent body of embryos and larvae; therefore, the

fluorescence signal of a reporter protein can be easily studied *in vivo* in the living animal (Strähle et al., 2012). Zebrafish embryo is widely used as a model in developmental toxicology tests (Braunbeck et al., 2005; Scholz et al., 2008) because the developing and transparent zebrafish can be assessed conveniently for lethality and developmental abnormalities from fertilization through larval stages. Furthermore, the development of zebrafish embryos is very similar to the embryogenesis in higher vertebrates (including humans); therefore, this species is highly suitable for the investigation of the fundamental processes underlying embryonic development (Nagel, 2002; Weight et al., 2011). In addition to animal protection, it is also favorable that the same individual fish can be studied throughout the treatment (Segner, 2009). In our experiments, we used a vitellogenin reporter transgenic zebrafish line, the *Tg(vtg1:mCherry)* (Bakos et al., 2019).

In this study, we examined the hypothesis that beta-CDs can limit the toxic effects of ZEN, employing BCD and its chemically modified derivatives, namely sulfobutylated beta-cyclodextrin (SbBCD), randomly methylated beta-cyclodextrin (RAMEB), succinyl-beta-cyclodextrin (SucBCD), and succinyl-methyl-beta-cyclodextrin (SuRAMEB) (Fig. 1). The stability of ZEN-CD complexes was tested in a physiological buffer by fluorescence spectroscopy. In our previous study, the cytotoxic effects of ZEN in the absence and presence of CDs were examined on HepG2 cell line (Poór et al., 2015b). Because HepG2 liver cells may significantly biotransform ZEN. Therefore, in this study, the toxic actions of ZEN were examined in HeLa (cervical cancer) cell line, in the absence and presence of CDs. The cytotoxicity of ZEN and CDs were evaluated based on ATP levels/well. Furthermore, the acute toxicity of ZEN was also examined on zebrafish embryos, in the absence and presence of CDs. Our results demonstrate that CDs can strongly alleviate the ZEN-induced toxicity both *in vitro* and *in vivo*.

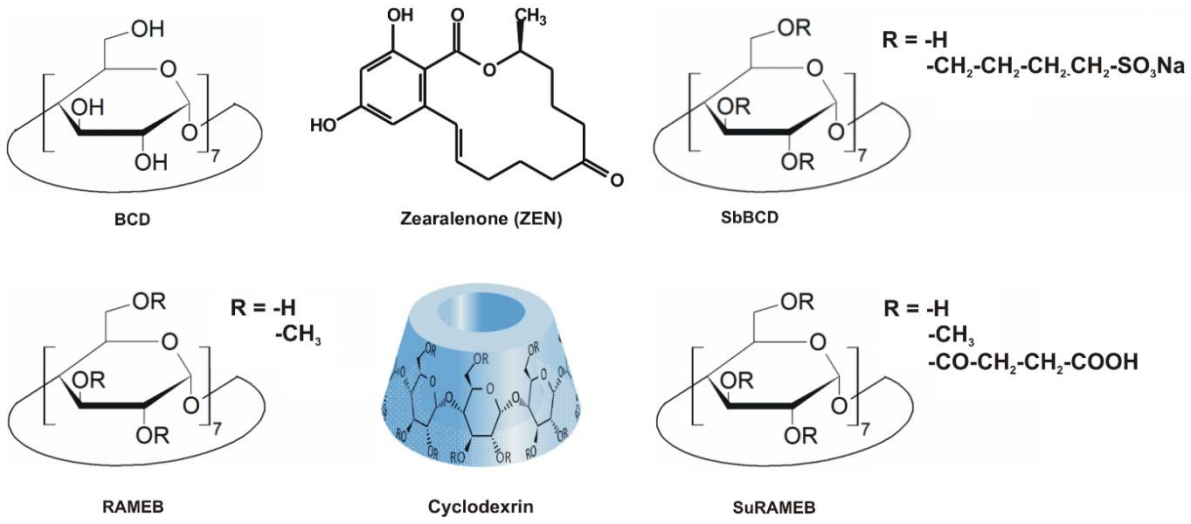


Fig. 1: Chemical structures of zearalenone and beta-cyclodextrins tested.

2. Materials and Methods

2.1. Reagents

Zearalenone (ZEN), Dulbecco's Modified Eagle Medium (DMEM), and fluorescamine (Fluram) were purchased from Sigma-Aldrich (St. Louis, MO, US). Cyclodextrins, including beta-cyclodextrin (BCD), sulfobutylated beta-cyclodextrin (SbBCD), randomly methylated beta-cyclodextrin (RAMEB), succinyl-beta-cyclodextrin (SucBCD), and succinyl-methyl-beta-cyclodextrin (SuRAMEB) were provided by CycloLab Cyclodextrin Research and Development Laboratory, Ltd (Budapest, Hungary). Bioluminescent ATP Assay Kit CLSII (Roche; Basel, Switzerland), fetal bovine serum (Pan-Biotech; Aidenbach, Germany), and bovine serum albumin (Biosera; Nuaille, France) were used as received.

2.2. Steady-state fluorescence spectroscopic studies

Fluorescence spectroscopic measurements were performed using a Hitachi F-4500 fluorimeter (Tokyo, Japan). Increasing amounts of CDs (final concentrations: 0, 25, 50, 100, 250, and 500 μM) were added to ZEN (2 μM), after which fluorescence emission spectra of ZEN and ZEN-CD complexes were recorded (λ_{ex} = 315 nm). To approximate extracellular physiological

conditions, experiments were carried out in phosphate-buffered saline (PBS, pH 7.4; containing 8.00 g/L NaCl, 0.20 g/L KCl, 1.81 g/L $Na_2HPO_4 \times 2H_2O$, and 0.24 g/L KH_2PO_4). Stock solution of ZEN (5000 μM) was prepared in 96 v/v(%) ethanol (Reanal; Budapest, Hungary). In fluorescence spectroscopic studies, the concentration of ethanol did not exceed 0.04 v/v (%). Binding constants (*K*, unit: L/mol) of ZEN-CD complexes were determined employing the graphical application of the Benesi-Hildebrand equation, assuming 1:1 stoichiometry of complex formation (Poór et al., 2015b):

$$\frac{I_0}{(I-I_0)} = \frac{1}{A} + \frac{1}{A \times K \times [CD]^n} \qquad (1)$$

where I_0 and I are the fluorescence emission intensity of ZEN without and with CDs, respectively (λ_{ex} = 315 nm, λ_{em} = 455 nm). *[CD]* denotes the molar concentration of CDs (unit: mol/L), *A* is a constant, and *n* is the number of binding sites.

2.3. Cell experiments

2.3.1. Cell culturing and treatment

Cell experiments were performed on HeLa cervical cancer cell line (ATCC: CCL-2). The adherent cells were cultured in DMEM with high glucose (4500 mg/L) containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) in 75 cm^2 sterile cell culture flasks in humidified atmosphere with 5% CO_2 and at 37 °C. Cells were trypsinized and plated onto 96-well sterile plastic plates. Stock solution of ZEN (5000 μM) were prepared in 96 v/v(%) ethanol. In cell experiments, solvent controls were also applied; however, the final concentrations of ethanol did not exceed 1 v/v(%), which did not influence significantly the viability of HeLa cells. During the treatments, the culture medium was replaced with fresh one, containing the appropriate concentrations of ZEN (50 μM) and/or CDs (0.0-1.0 mM). Then the cells were incubated for 48 h before analysis.

183 2.3.2. Measurements of cellular ATP and total protein levels

184 To test the effects of ZEN and CDs alone and in combinations on the viability of HeLa cells,
185 intracellular ATP and total protein levels were quantified (based on luciferin-luciferase ATP
186 and fluram protein assays, respectively) as described previously (Csepregi et al., 2018).

187

188 2.3.3. Statistical analyses in cell experiments

189 Means and standard error (± SEM) values were derived from at least three independent
190 experiments. The data showed normal distribution based on the Shapiro-Wilk normality test
191 (IBM SPSS Statistics, V21). Statistical evaluation was performed using one-way ANOVA
192 test (IBM SPSS Statistics, V21). The level of significance was set at $p < 0.05$ and $p < 0.01$.

193

194 *2.4. Experiments on zebrafish embryos*

195 2.4.1. Characterization of the *Tg(vtg1:mCherry)* biomarker zebrafish line

196 The zebrafish line used in these experiments is a vitellogenin reporter transgenic zebrafish
197 line. Vitellogenin is a glycoprotein that is inducible by environmental estrogens. The
198 transgene construct used for the development of *Tg(vtg1:mCherry)* carried a long (3.4 kbp)
199 natural vitellogenin-1 promoter sequence with a high number of ERE (estrogen responsive
200 element) sites. The *mCherry* reporter is only produced in the liver, similarly to endogenous
201 vitellogenin. The sensitivity and usability of the embryos of the line have been tested on
202 several estrogenic compounds (including ZEN) as well as on environmental samples (Bakos
203 et al., 2019).

204

205 2.4.2. Zebrafish maintenance and egg collection

206 Laboratory-bred *Tg(vtg1:mCherry)* zebrafish strain was held in breeding groups of 30 females
207 and 30 males at the Department of Aquaculture (Szent István University, Hungary) in a

208 Tecniplast ZebTEC recirculation system (Tecniplast S.p.a., Italy) at 25.5 ± 0.5 °C (system
209 water: pH 7.0 ± 0.2, conductivity 550 ± 50 μS) and on a 14h:10 h light:dark cycle. The fish
210 were fed twice a day with dry granulate food (Zebrafeed 400-600 μm, Sparos Lda., Portugal)
211 supplemented with freshly hatched live *Artemia salina* once a day. The fish were placed in
212 breeding tanks (Tecniplast S.p.a.) late in the afternoon before the day of the experiment and
213 allowed to spawn by removing the dividing walls next morning. The collected eggs were
214 incubated in system water with methylene blue (2 mL 0.1% methylene blue in 1 L system
215 water) (25 ± 2 °C) in Petri dishes (diameter: 10 cm). After 24 h, coagulated and/or non-
216 fertilized eggs were assorted, and a part of the embryos were disinfected with bleaching
217 method to keep the experiment sterile.
218
219 2.4.3. Embryo bleaching
220 Bleaching of embryos was necessary because some microorganisms can break down the CD
221 ring to glucose units during long-term experiments in aqueous solution. System water was
222 removed with a plastic pipette and embryos were bathed in a bleach solution (0.0035%
223 sodium hypochlorite) for 5 min. Then, the bleach solution was removed, and Petri dishes were
224 filled with sterilized E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33
225 mM $MgSO_4$ in 1 L sterilized deionized water) for 5 min. E3 medium was removed and dishes
226 were filled with new E3 solutions under a sterile box.
227
228 2.4.4. Determination of lethal concentration (LC) values of ZEN
229 96 hpf (hours post-fertilization) *Tg(vtg1:mCherry)* embryos were placed in groups of five in
230 24-well plates (JET Biofil; Guangzhou, China). E3 medium were removed then zebrafish
231 embryos were treated (2 mL/well) with 0, 1, 2, 3, 4, 5, 6, and 7 mg/L (equal to 0-22 μM)
232 ZEN, each treatment was performed in four replicates. ZEN was dissolved in methanol, the

final concentrations of the solvent did not exceed 0.4 v/v (%) during the treatments. Solvent controls were also tested: At the applied concentrations, methanol alone did not affect the viability of zebrafish embryos. The mortality was evaluated after 24 h exposure.

2.4.5. Testing the effects of CDs on zebrafish embryos in the absence and presence of ZEN

Three concentrations (0.25 mM, 0.5 mM, and 1 mM) of beta-CDs (BCD, SbBCD, RAMEB, and SuRAMEB) with and without ZEN (final concentration: 4.0 mg/L or 12.6 μM) were diluted in sterilized E3 medium. Mixtures were filtered with 0.2 μm syringe filters (VWR International Ltd., Hungary) to gain bacteriologically sterile solutions. ZEN control (with methanol solvent) was diluted in E3 medium to 4.0 mg/L (12.6 μM) final concentration. Each treatment were prepared with bleached and non-bleached larvae to test disinfection procedure. 96 hpf transgenic larvae were transferred in groups of ten in sterile 6-well plates (JET Biofil, China), the experiment was performed in three replicates. Thereafter, E3 medium was removed, each well were filled with 10 mL of treatment solution and larvae were incubated at 26°C (± 1°C) on 14 h:10 h light:dark cycle for 24 h in each treatment.

2.4.6. Imaging and analysis

Five-day old embryos were placed to petri dishes (diameter: 6 cm; JET Biofil; Guangzhou, China) from each group. Overplus solutions were removed with a plastic pipette and were filled with 2 mL of 0.02% MS-222 (Tricane-methane-sulfonate; from Sigma-Aldrich; St. Louis, MO, US) anesthetic solution. Special designed petri dishes (with two cube-shaped tape, diameter: 10 cm) were filled with 4% methyl-cellulose solution. Anaesthetized embryos were placed to methyl-cellulose, oriented to the left side, and pushed to the bottom of the cellulose solution with a cut ended Microloader pipette tip (Eppendorf; Hamburg, Germany). Bright field (exposure time: 6 msec, magnification: 30x and 60x), and fluorescent (*mCherry*

filter, exposure time: 2 sec, magnification: 60x) images of larvae were taken under a fluorescent stereomicroscope (Leica M205 FA fluorescent stereomicroscope, Leica DFC 7000T camera, Leica Application Suite X, Leica Microsystems GmbH; Wetzlar, Germany). Signals in the red range of the RGB (Red, Green, Blue) color range was evaluated by ImageJ software (Schneider et al., 2012) based on the prepared fluorescent images. An elliptical area of the same size was selected on each image and moved to the area of the liver, then the signal strength and the size of the affected area were determined. The integrated density values were determined for each treatment. The results of ZEN treatments (ZEN and ZEN+CDs) were corrected with the integrated density values of test solutions without the mycotoxin.

2.4.7. Statistical analyses in zebrafish experiments

The concentration-lethality curve was fitted and LC values were calculated by non-linear regression. Integrated density data were checked for normality with Shapiro-Wilk normality test and non-compliance with the requirements of parametric methods was established. Statistical significance was evaluated employing Kruskal-Wallis analysis with Dunn's multiple comparisons test. Results were analyzed and plotted by GraphPad Prism 6.01 (GraphPad Software; San Diego, CA, US).

3. Results and Discussion

3.1. Interaction of ZEN with beta-CDs in physiological buffer

Whereas the complex formation of ZEN with some beta-CDs has been reported, the interaction of SucBCD and SuRAMEB with ZEN has not been tested. Furthermore, previous experiments did not try to approximate extracellular physiological conditions; therefore, our spectroscopic experiments were performed in PBS buffer (pH 7.4). Each tested CD induced a strong increase in the fluorescence of ZEN (which is the sign of complex formation), showing

283 the following order in the fluorescence enhancement: SuRAMEB > RAMEB > SbBCD >
284 SucBCD > BCD (Fig. 2A). Our results are in agreement with the previously published
285 studies, which also suggest that the chemical modifications of BCD strongly increase the
286 fluorescence signal of ZEN (Dall'Asta et al., 2009; Poór et al., 2015b). Then, the binding
287 constants of ZEN-CD complexes were determined employing the Benesi-Hildebrand equation
288 (Eq. 1). As it is demonstrated in Fig. 2B, Benesi-Hildebrand plots showed excellent linearity
289 with the 1:1 stoichiometry model, and suggesting the formation of stable mycotoxin-CD
290 complexes. ZEN forms similarly stable complexes with SucBCD ($K = 5.5 \times 10^3$ L/mol) than
291 with BCD ($K = 6.5 \times 10^3$ L/mol), while other chemically modified beta-CDs bound to ZEN
292 with higher affinity. The most stable mycotoxin-CD complexes were formed with SuRAMEB
293 ($K = 4.7 \times 10^4$ L/mol) followed by RAMEB ($K = 2.0 \times 10^4$ L/mol) and SbBCD ($K = 1.4 \times 10^4$
294 L/mol). Since succinyl substitution of BCD resulted in slightly less stable ZEN-CD
295 complexes than BCD, we did not use SucBCD in the following experiments.
296 Our results demonstrate that each beta-CDs tested form stable complexes with ZEN in PBS
297 (pH 7.4). The binding constants of BCD, SbBCD, and RAMEB complexes were similar but
298 slightly lower than those previously found in ammonium acetate buffer (0.05 M) at pH 5.0
299 (Poór et al., 2015b). These findings indicate that methyl and sulfobutyl substitutions of BCD
300 strongly increase the stability of ZEN-CD complexes (Dall'Asta et al., 2009; Poór et al.,
301 2015b). Despite succinyl derivative of BCD slightly decreased the stability of the complexes
302 formed, the simultaneous presence of succinyl and methyl substituents in SuRAMEB resulted
303 in higher binding constants compared to both BCD and RAMEB. Succinyl-methyl, methyl,
304 and sulfobutyl substitutions of BCD led to the approximately 7.2-, 3.1-, and 2.2-fold increase
305 in binding constants of ZEN-CD complexes, respectively.
306

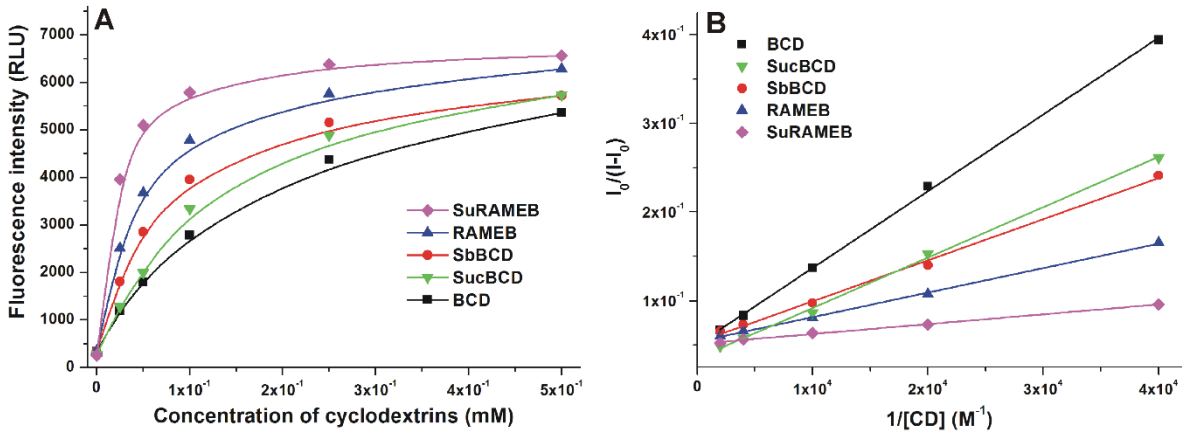


Fig. 2: (A) Fluorescence emission intensity of ZEN (2 μM) in the absence and presence of increasing concentrations of CDs (0-500 μM) in PBS (pH 7.4). (B) Benesi-Hildebrand plots of ZEN-CD complexes (λ_{ex} = 315 nm, λ_{em} = 455 nm).

3.2. Effects of ZEN on HeLa cells in the absence and presence of beta-CDs

To test the effects of CDs on the ZEN-induced cytotoxicity, HeLa cells were treated with ZEN and/or CDs. After 48 h incubation, cell viability was mainly evaluated based on the cellular ATP content/well. Quantitation of cellular ATP levels is a widely accepted method to determine cell viability. However, previous studies indicated that the ATP level alone may be a misleading parameter (Sali et al., 2016; Kőszegi et al., 2007; Hochachka and Mcclelland, 1997; Andreoli and Mallett, 1997). Therefore, to confirm the results from ATP assay, total protein levels were also quantified. Changes of cellular ATP and total protein levels showed good correlation (Fig. 3). To produce a strong decrease in cell viability, ZEN was applied at 50 μM concentration in these experiments. Our data are in good agreement with a previous study on HeLa cells which reported that the IC_{50} value of ZEN is approximately 60 μM (Ayed et al., 2011). However, a wide cytotoxic concentration range for ZEN has been found in other cell lines: 5-40 μM in Caco-2 (colorectal adenocarcinoma) cells and 31-157 μM in HL-60 (human leukemia) cells (Rai et al., 2019). As Fig. 3 demonstrates, BCD failed to significantly alleviate the ZEN-induced toxicity (it caused only slight increases in ATP and total protein

levels); however, other CDs considerably decreased or even abolished the toxic effects of ZEN. In a concentration-dependent fashion, the co-treatment of ZEN-exposed cells with SbBCD, RAMEB, or SuRAMEB increased both ATP and total protein levels compared to the cells exposed to ZEN alone. Low CD concentrations (0.25 mM) were minimally effective (only the total protein level was increased significantly by SuRAMEB), while 0.5 mM concentrations of CDs induced spectacular elevation of cell viability. In addition, SbBCD, RAMEB, and SuRAMEB completely abolished the ZEN-induced loss of cell viability at the highest concentration (1 mM).

Considering the high stability of ZEN-CD complexes as well as the previously reported protective effect of BCD against ZEN in HepG2 cells (Poór et al., 2015b), it was reasonable to hypothesize that some of these CDs may also effectively alleviate the ZEN-induced cytotoxicity *in vivo*. BCD failed to significantly affect cell viability even at 1 mM concentration in HeLa cells, although it strongly alleviated the toxic effects of ZEN in HepG2 cells in a previous study (Poór et al., 2015b). However, chemically modified beta-CDs (SuRAMEB, RAMEB, and SbBCD) caused the significant decrease of ZEN-induced loss of cell viability. This can be explained by the higher binding affinity of the mycotoxin towards these CDs. In the cell medium, ZEN can form stable complexes with bovine serum albumin contained by the fetal bovine serum. In previous fluorescence spectroscopic studies, similar K values (6.0×10^4 and 2.6×10^4 L/mol) of ZEN-BSA complex have been reported (Faisal et al., 2018; Ma et al., 2018). Therefore, ZEN is likely present in the cell medium mainly in albumin-bound form. Because CDs can form similarly stable complexes with ZEN than with albumin (see in 3.1), CDs can further decrease the free fraction of ZEN in the cell medium, thus further decreasing the cellular uptake of the mycotoxin.

Under the applied conditions, even the highest concentration (1 mM) of CDs (BCD, SbBCD, RAMEB, and SuRAMEB) did not affect significantly ATP and total protein levels (Fig. S1).

Based on previous studies, SbBCD is a less toxic while the methylated derivatives are less tolerable compared to the native BCD (Kiss et al., 2010; Jambhekar and Breen, 2016b). Furthermore, *in vitro* studies suggests that SuRAMEB is a less toxic derivative compared to RAMEB (Kiss et al., 2010). In previous cell experiments, alpha-, beta-, and gamma-CDs as well as their hydroxypropyl, methyl, and carboxymethyl derivatives did not induce significant toxicity at 1 mM or lower concentrations in HEK293T (human embryonic kidney), HeLa, and TZM-bl (endocervical adenocarcinoma) cells (Szente et al., 2018).

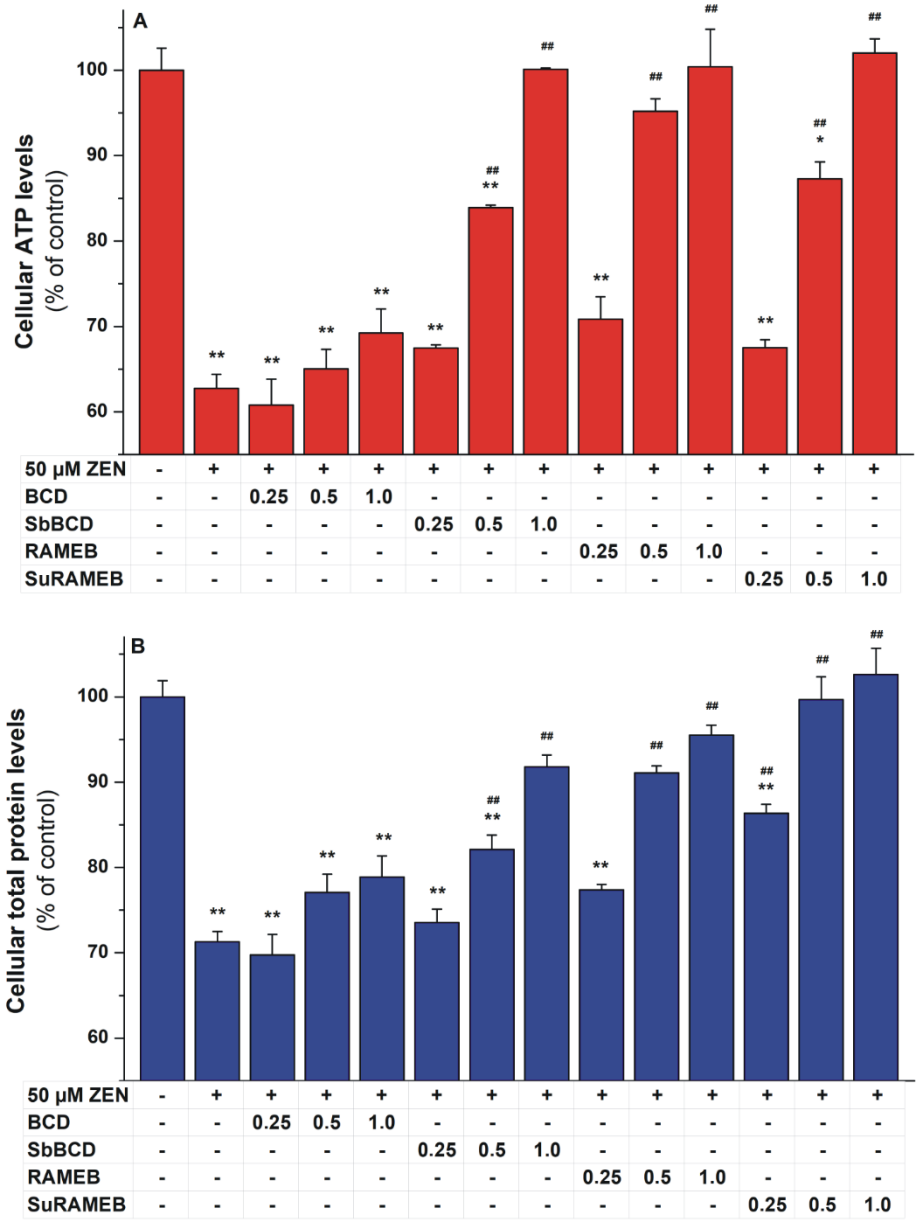


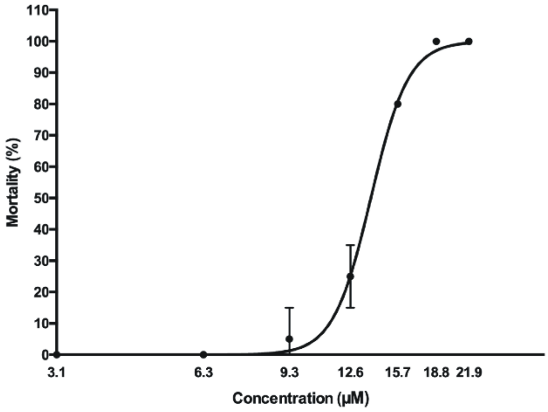
Fig. 3: Effects of ZEN (50 μM) on the cellular ATP (A) and total protein (B) levels in HeLa cells, in the absence and presence of CDs (0-1 mM) after 48 h incubation (compared to the control: *$p < 0.05$, **$p < 0.01$; compared to ZEN alone: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$).

3.3. Effects of ZEN on zebrafish embryos in the absence and presence of beta-CDs

To confirm our results indicating the protective effects of CDs in HeLa cell *in vitro*, their influence on the ZEN-induced toxicity was further examined in zebrafish embryos. As the first step, the toxicity indicators in the selected exposure window were determined. Therefore, the effect of ZEN on *Tg(vtg1:mCherry)* embryos was determined between 96-120 hpf. Fig. 4A demonstrates the concentration-mortality curve of ZEN. LC values (Fig. 4B) were higher than in an earlier study using the same strain, in which 0.893 mg/L (or 2.81 μM; in this study: 4.405 mg/L or 13.84 μM) and 0.335 mg/L (or 1.05 μM; in this study: 3.625 mg/L or 11.39 μM) LC_{50} and LC_{10} values of ZEN were reported, respectively (Bakos et al., 2013). Since earlier studies suggest that the survival of fish embryos decreases with their age (Gellert and Heinrichsdorff, 2001), these differences likely resulted from the different length of exposure (96-120 vs. 1-120 h period). The LC_{25} concentration of ZEN (4.0 mg/L or 12.6 μM) was selected for the following experiments (Fig. 4A and B) because it did not induce marked mortality while its sublethal effects were significant. Mortality data observed in the presence of ZEN and/or CDs are listed in Fig. 4C. ZEN-induced mortality (26.67%) was consistent with the previous treatments (see in Fig. 4A and B), and the bleaching method did not affect the viability of larvae. The OECD guideline criteria for fish embryo test accepts a maximum of 10% lethality in the control groups (OECD236, 2013). Since the mortality of the control groups was 0%, it obviously fulfills this criteria. Under the applied circumstances, CDs (0.25-1.0 mM) alone did not increase the mortality. Furthermore, the co-exposure of ZEN with beta-CDs completely abolished the lethal effects of ZEN (except 0.25 mM BCD) (Fig. 4C),

386 suggesting the considerable protective effects of CDs vs. ZEN-induced toxicity. The weaker
387 protective effect of BCD on ZEN-induced mortality is in agreement with the previous
388 observation that BCD forms less stable complexes with ZEN compared to SuRAMEB,
389 RAMEB, and SbBCD (see in 3.1), as well as it is also in accordance with the results of cell
390 experiments (Fig. 3). Some previous studies also pointed out that CDs can decrease the toxic
391 actions of several compounds, due to the formation of stable host-guest type complexes. BCD
392 strongly decreased the LC_{50} values of 1-dodecyl-3-methylimidazolium tetrafluoroborate
393 (Hodyna et al., 2016), 20(S)-Protopanaxadiol-20-O-D-glucopyranoside (Nam et al., 2017),
394 and perfluorooctanoic acid (Weiss-Errico et al., 2017) in zebrafish.
395

A



B

ZEN	Mean µM(mg/L)	Standard Deviation (SD) µM(mg/L)
LC_{50}	13.8 (4.405)	± 3.2 (1.01)
LC_{25}	12.6 (3.996)	± 0.9 (0.29)
LC_{10}	11.4 (3.625)	± 0.3 (0.09)
LC_{1}	9.2 (2.931)	± 0.03 (0.01)

C

Treatment	Mortality (%)(±SD)
E3 control	0 (± 0.00)
E3 control with bleached larvae	0 (± 0.00)
12.6 µM ZEN (methanol solvents) in E3 control	26.67 (± 5.77)
12.6 µM ZEN (methanol solvents) in E3 control with bleached larvae	26.67 (± 5.77)
Solvent (methanol) control in E3	0 (± 0.00)
BCD (0.25 or 0.5 or 1 mM)	0 (± 0.00)
BCD (0.25 mM) with 12.6 µM ZEN	6.67 (± 5.77)
BCD (0.5 or 1 mM) with 12.6 µM ZEN	0 (± 0.00)
SbBCD (0.25 or 0.5 or 1 mM)	0 (± 0.00)
SbBCD (0.25 or 0.5 or 1 mM) with 12.6 µM ZEN	0 (± 0.00)
RAMEB (0.25 or 0.5 or 1 mM)	0 (± 0.00)
RAMEB (0.25 or 0.5 or 1 mM) with 12.6 µM ZEN	0 (± 0.00)
SuRAMEB (0.25 or 0.5 or 1 mM)	0 (± 0.00)
SuRAMEB (0.25 or 0.5 or 1 mM) with 12.6 µM ZEN	0 (± 0.00)

Fig. 4: LC values and mortality data of *Tg(vtg1:mCherry)* zebrafish embryos (120 hpf). Concentration-mortality curve of ZEN (A); lethal concentration and the corresponding standard deviation (SD) values of ZEN with (B); and mortality data of ZEN and CDs alone as well as in combination (C). All experiments were performed in 96-120 hpf exposure window.

Besides the lethal outcome, sublethal effects of beta-CDs were also studied on 120 hpf zebrafish embryos. In general, beta-CDs caused mild phenotypic lesions on the treated embryos, such as uninflated swim bladder and mild pericardial edema (Fig. 5). Furthermore, a slight upward curvature of the body axis can be observed as a result of BCD and SuRAMEB treatments (Fig. 5A), whereas moderately irregular edges of the dorsal and ventral fins were noticed only on BCD-treated embryos (Fig. 5B). Quantitative values of sublethal effects are demonstrated in Fig. 6C. In previous studies, the presence of 1% or lower concentrations of hydroxypropyl-beta-CD did not affect the development in zebrafish embryos and larvae (Maes et al., 2012), and even 3 mM concentration of methyl-beta-CD did not induce abnormal cytokinesis of zebrafish embryos (Feng et al., 2002). Our results also suggest that BCD, SbBCD, RAMEB, and SuRAMEB do not cause strong malformations up to 1 mM concentrations.

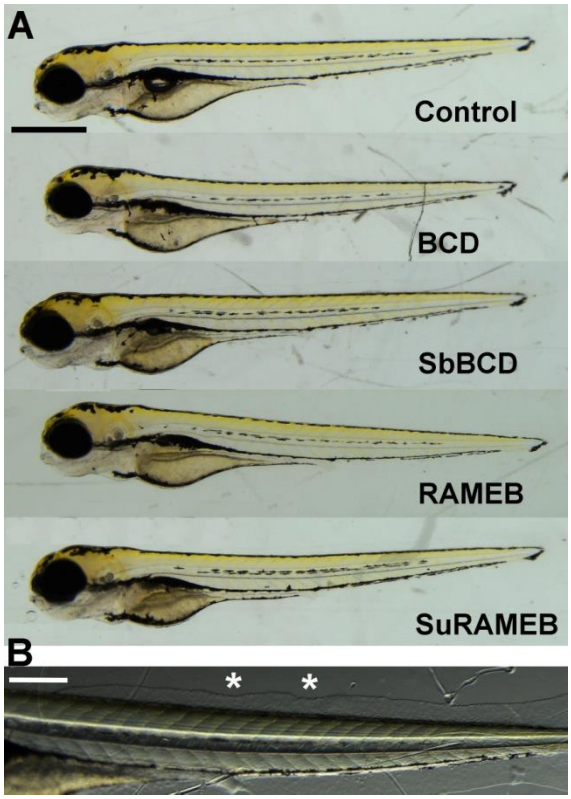
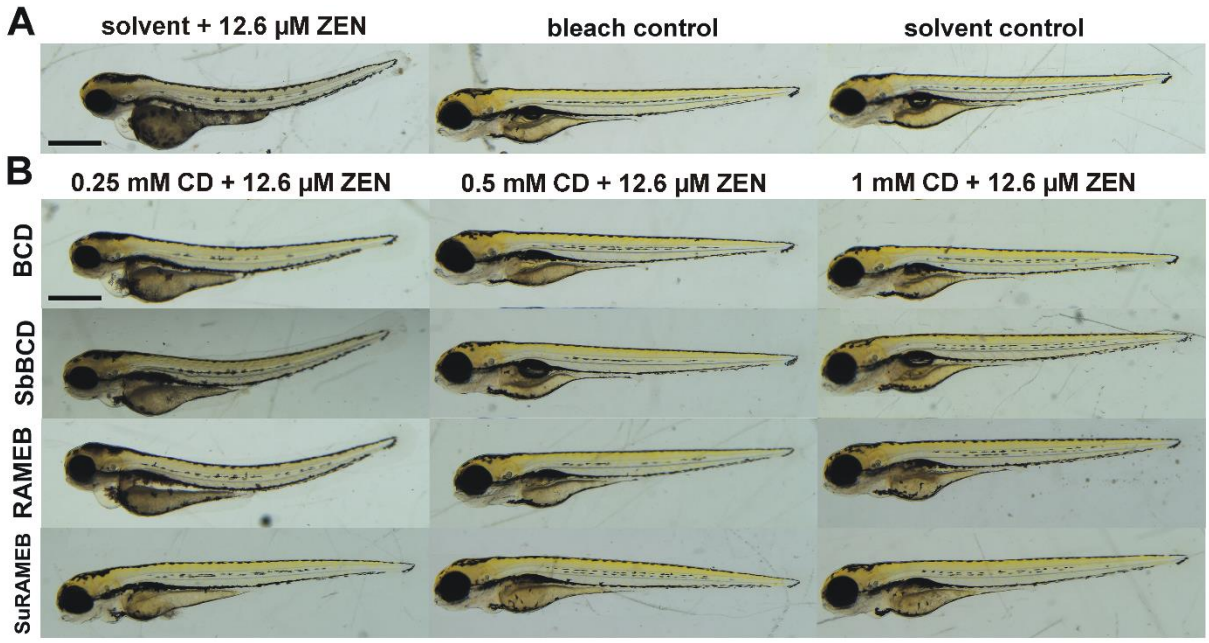


Fig. 5: Representative developmental defects in 120 hpf zebrafish embryos after 24 h treatment with beta-CDs. (A) An untreated control and embryos treated with 1 mM of BCD, SbBCD, RAMEB, and SuRAMEB. Pericardial edema and uninflated swim bladder appeared as a result of beta-CD treatments. Slight upward curvature of the body axis can be observed after BCD and SuRAMEB treatments. (B) Moderately irregular edges (marked with asterisks) of the dorsal fin are apparent in the BCD-treated embryo. Scale bar: 500 μm.

Sublethal effects were also studied on 120 hpf embryos treated with ZEN in the absence and presence of beta-CDs (Fig. 6). The effects of ZEN on the development of zebrafish embryos have been reported. During the 72-h treatment of the embryos, ZEN caused pericardial edema, eye deformity, and concentration-dependent dorsal curvature of the body axis (heart and soul (*has*) phenotype), which is also characterized by other estrogenic substances (Bakos et al., 2013). The *has* phenotype can be observed in ZEN-treated (12.6 μM) embryos as well as after the co-exposure of ZEN with 0.25 mM BCD, SbBCD, or RAMEB (Fig. 6). However, this morphological alternation was no longer observed during the co-treatment of ZEN-exposed embryos with higher concentrations (0.5 and 1.0 mM) of beta-CDs (Fig. 6B). Interestingly, the ZEN-induced formation of *has* phenotype was also eliminated by the co-treatment of 0.25 mM SuRAMEB, which is in good agreement with our previous observations: (1) SuRAMEB forms the most stable complex with ZEN among the beta-CDs tested (see in section 3.1) and (2) SuRAMEB was the only CD which significantly increased the total protein levels in ZEN-treated HeLa cells even at 0.25 mM concentration (Fig. 3B). Another developmental effect of ZEN is the lack of the gap in the melanophore streak along the ventral side at the base of the caudal fin (Bakos et al., 2013). This phenotype is also typical for endocrine disruptors in zebrafish embryos treated between 0-72 hpf (Yang et al., 2010; Georgescu et al., 2011). Less pigmentation disorder was observed only after ZEN

441 treatment (without CDs), and there was no complete closure of melanophores streak. This
442 may be explained by the fact that experiments were started with 96 hpf embryos when the
443 process of pigmentation was slowed down compared to the previous developmental stages,
444 and the duration of the treatment was too short for complete closure.
445 The co-treatment of ZEN with 0.5 and 1 mM BCD typically resulted in uninflated swim-
446 bladder, and some individuals exhibited mild pericardial edema. Fin disorder, which was
447 specific to BCD-treated embryos, was not observed. The co-exposure of ZEN with 0.5 and 1
448 mM SbBCD caused inflated swim bladder in most of the individuals (which appeared
449 regularly only with this CD) and a slight pericardial edema was also noticed. Typical
450 sublethal symptoms, as a result of the simultaneous treatment with ZEN and RAMEB (0.5
451 and 1 mM), were uninflated swim bladder and pericardial edema. There was no similar
452 abnormality with the *has* phenotype regarding ZEN-SuRAMEB co-treatments; however, a
453 slight pericardial edema was observed in all treated embryos. Furthermore, the 0.25 and 0.5
454 mM concentrations of SuRAMEB (in the presence of ZEN) led to a slight downward
455 curvature of the body axis. During the ZEN-SuRAMEB co-exposures, the swim bladders of
456 the embryos were not inflated until the end of the experiment. Based on the above-listed
457 observations, beta-CDs reduced the sublethal effects of ZEN (Fig. 6C).
458



C

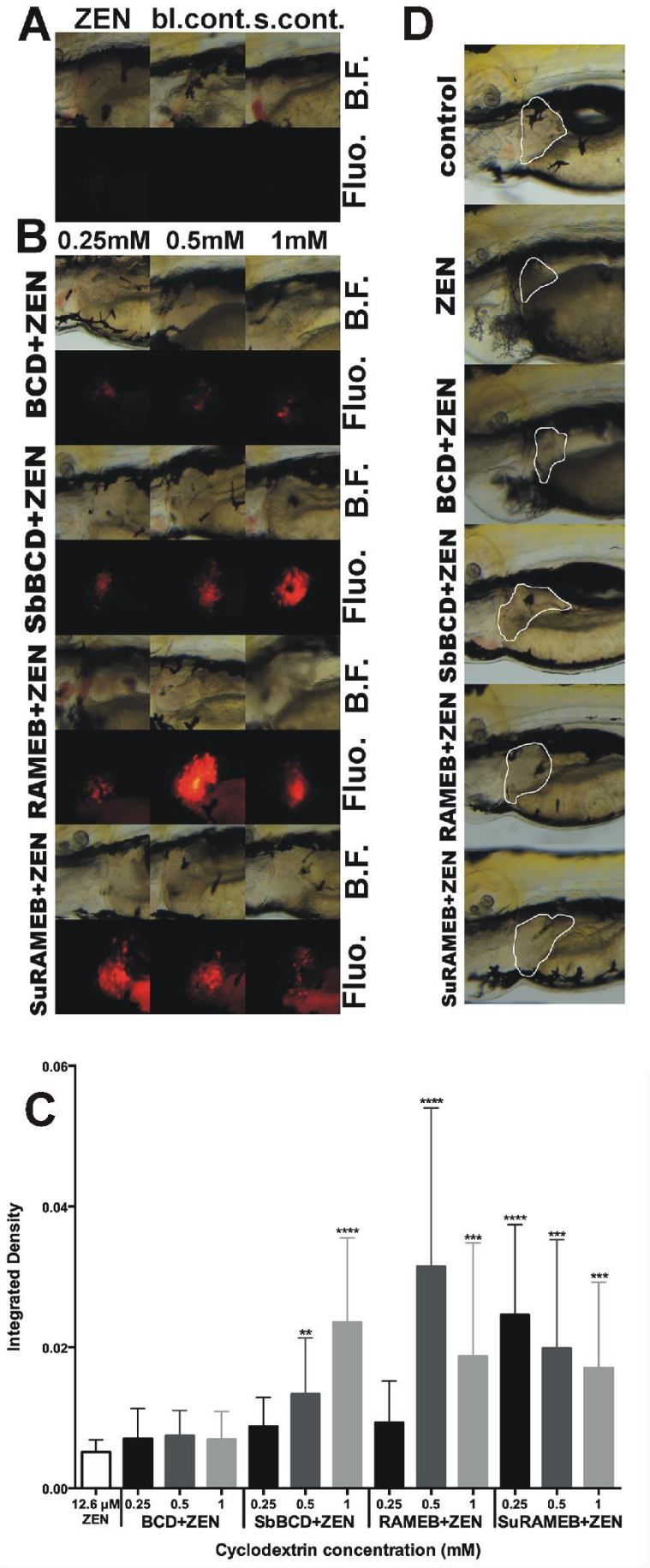
		BCD			SbBCD			RAMEB			SuRAMEB		
		0.25 mM	0.5 mM	1 mM	0.25 mM	0.5 mM	1 mM	0.25 mM	0.5 mM	1 mM	0.25 mM	0.5 mM	1 mM
Freq (%)	pe	66.7 ± 5.8	53.3 ± 5.8	83.3 ± 15.3	56.7 ± 11.6	56.7 ± 11.6	66.7 ± 5.8	70.0 ± 10.0	66.7 ± 11.6	76.7 ± 5.8	63.3 ± 11.6	93.3 ± 5.8	90.0 ± 10.0
	usb	93.3 ± 5.8	90.0 ± 10.0	96.7 ± 5.8	96.7 ± 5.8	86.7 ± 15.3	90.0 ± 10.0	76.6 ± 5.8	93.3 ± 5.8	100.0 ± 0.0	90.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
	cb	40.0 ± 10.0	46.7 ± 5.8	70.0 ± 0.0	–	–	–	–	–	–	53.3 ± 11.6	60.0 ± 17.3	76.7 ± 5.8
	fin	83.3 ± 11.6	96.7 ± 5.8	100.0 ± 0.0	–	–	–	–	–	–	–	–	–
		BCD + 12.6 µM ZEN			SbBCD + 12.6 µM ZEN			RAMEB + 12.6 µM ZEN			SuRAMEB + 12.6 µM ZEN		
		0.25 mM	0.5 mM	1 mM	0.25 mM	0.5 mM	1 mM	0.25 mM	0.5 mM	1 mM	0.25 mM	0.5 mM	1 mM
	has	82.3 ± 5.9	–	–	70.0 ± 10.0	–	–	96.7 ± 5.8	–	–	–	–	–
Freq (%)	pe	–	60.0 ± 0.0	66.7 ± 11.6	–	56.7 ± 5.8	60.0 ± 10.0	–	56.7 ± 11.6	76.7 ± 5.8	73.3 ± 5.8	63.3 ± 11.6	93.3 ± 11.6
	usb	–	100.0 ± 0.0	96.7 ± 5.8	–	–	–	–	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	96.7 ± 5.8	100.0 ± 0.0
	sb	–	–	–	–	83.3 ± 5.8	90.0 ± 10.0	–	–	–	–	–	–
	cb	–	–	–	–	–	–	–	–	–	46.7 ± 5.8	53.3 ± 5.8	73.3 ± 5.8

Fig. 6: Representative developmental defects in 120 hpf zebrafish embryos after 24 h treatment with ZEN (12.6 µM) in the absence and presence of beta-CDs. ZEN-exposed embryos as well as bleach and solvent controls are demonstrated in panel A, while embryos co-treated with ZEN and CDs are represented in panel B. CDs reduced the sublethal effects of ZEN, as it can be observed on the bright field images. Scale bar: 500 µm. (C) The mean appearance of representative developmental defects after ZEN and ZEN+CD exposure (%) (has: *heart and soul* phenotype; pe: pericardial edema; sb: inflected swim bladder; usb: uninflected swim bladder; cb: curvature of the body axis; fin: irregular edges of dorsal fin). As a result of ZEN treatment (without CDs), the *has* phenotype appeared in each zebrafish embryo (100%).

Transgenic bioindicator models for estrogenic effects are increasingly used in toxicological studies. *In vivo* models allow the investigation of complex processes in the organism. Several transgenic zebrafish lines are suitable for the investigation of estrogenic effects of test compounds, of which *Tg(vtg1:mCherry)* was used in our studies (Bakos et al., 2019). In these experiments, the effects of ZEN in the absence and presence of beta-CDs were examined on 96 hpf-120 hpf zebrafish embryos. We investigated the potential appearance of fluorescence signal in the liver of fish (at the end of the exposure time), indicating the xenoestrogenic effect of ZEN. ZEN treatment induced the transgene to function, which is indicated by fluorescence signal in the liver (Fig. 7A and B). The weakest fluorescence signal was observed in the liver of ZEN-treated embryos (in the absence of CDs; Fig. 7A). In ZEN-BCD co-treated fish, the intensity of the fluorescence signal was almost the same than in the presence of ZEN alone (without CDs). Surprisingly, ZEN-SbBCD co-exposure caused a concentration-dependent increase in the fluorescent signal; while SuRAMEB induced the strongest elevation at its lowest concentration (0.25 mM), above which the fluorescence signal gradually decreased (Fig. 7B). Simultaneous treatment of ZEN-exposed cells with RAMEB led to the strong increase in the fluorescence (0.5 mM RAMEB produced the highest effect in this whole experiment), however, no clear concentration-dependence can be observed. In untreated control embryos, no fluorescent signal was visible (Fig. 7A). The integrated density values were in agreement with the differences in the fluorescence intensities (Fig. 7C). When the integrated density values of ZEN-CD co-treatments were compared to ZEN, no significant differences were observed in the presence of BCD, however, statistically significant changes were noticed in the presence of 0.5 and 1.0 mM concentrations of SbBCD and RAMEB, and each concentration (0.25, 0.5, and 1.0 mM) of SuRAMEB.

The germ layers from which the liver of the zebrafish is formed start to develop 4 to 6 h after fertilization, hepatic budding starts at 24 h after fertilization, and the liver starts working after 50 h (Villenueve et al., 2014). First, the left lobe of the liver is formed, where the endogenous vitellogenin (and the fluorescent reporter) is produced; then, after 96 h (96 hpf), the right lobe of the liver also appears (Ober et al., 2003; Tao and Peng, 2009). The final shape of the liver appears around day 5 (120 hpf), and becomes well-defined in a relatively large area, where the fluorescence signal can be easily detected with a stereomicroscope (Bakos et al., 2019). Therefore, the liver works during the 96-120 h exposure window for short-term treatments when *Tg(vtg1:mCherry)* embryo model is used to test estrogen effects (as it is also confirmed in the current study). There are large variations in the fluorescence intensities and the integrated density values within the treatments. Interestingly, ZEN-CD co-treatments induced stronger fluorescence signals compared to ZEN alone. The reason is likely the high individual sensitivity of the embryos to the treatment. The cells of the embryos (including their liver cells) can be damaged by higher concentrations of toxic substances (Bakos et al., 2013, 2019). In that case, the induction of vitellogenin production can be strongly decreased, thus lowering the fluorescence signal in ZEN-treated fish as compared to ZEN-CD co-treatment. This hypothesis is also supported by our observations that the stronger fluorescence signal of the reporter and the higher integrated density values (Fig. 7) are accompanied with the considerably lower mortality (Fig. 4) and the substantially weaker sublethal symptoms (Fig. 6). Furthermore, Fig. 7D shows that both ZEN and ZEN+BCD treatments significantly altered the size, shape and color of the liver compared to the liver of untreated control embryos, suggesting the significant hepatotoxic effect of ZEN. In contrast, simultaneous treatment of embryos with ZEN and other CDs (SbBCD, RAMEB, SuRAMEB) resulted in much smaller hepatic lesions, confirming their hepatoprotective effects against ZEN. These observations are

519 in agreement with the integrated density values, where stronger fluorescent signal and higher
520 *mCherry* protein affected area were observed in the less damaged liver.
521


A
ZEN
bl.cont.
s.cont.
B.F.
Fluo.
B
0.25mM
0.5mM
1mM
BCD+ZEN
SbBCD+ZEN
RAMEB+ZEN
SuRAMEB+ZEN
D
control
ZEN
BCD+ZEN
SbBCD+ZEN
RAMEB+ZEN
SuRAMEB+ZEN
C
Integrated Density
0.06
0.04
0.02
0.00
12.6 µM ZEN
0.25
0.5
1
BCD+ZEN
SbBCD+ZEN
RAMEB+ZEN
SuRAMEB+ZEN
Cyclodextrin concentration (mM)

522

Fig. 7: Fluorescence signals of the vitellogenin reporter and integrated density values in *Tg(vtg1: mCherry)* embryos (n = 30/treatment) as well as the changes in liver size and shape as a result of ZEN and ZEN+CD treatments. ZEN-treated (12.6 μM) embryos as well as bleach (bl.cont.) and solvent controls (s.cont.) are demonstrated in panel A, while embryos co-treated with ZEN and CDs are represented in panel B. Livers of the treated embryos are demonstrated in Bright Field (B.F.) and in fluorescent (Fluo.) images. (C) Integrated density values of ZEN-CD co-treatments were compared to ZEN (**p < 0.01, *** p < 0.001, **** p < 0.0001). Data represent that co-treatments of embryos with ZEN and beta-CDs cause higher fluorescence signal than ZEN alone. (D) The changes in the liver size and shape (marked with white line) are shown as the results of ZEN and ZEN+CD treatments (ZEN: 12.6 μM; CDs: 1 mM). Integrated density values which were equal to or less than the untreated controls were excluded from the evaluation.

In addition to the solubilizing effect of CDs, the low stability of CD complexes ($K \approx 10^2$-10^3 L/mol) may support the cellular uptake of guest molecules, whereas CD complexes with significantly higher stability may impair uptake (Redenti et al., 2001; Irie and Uekama, 1999; Poór et al., 2015b). Therefore, the stability of CD complexes strongly affect the field of their application. Furthermore, some CD derivatives have proved to be suitable in the treatment of endotoxin shock in animal studies, likely due to their interactions with lipopolysaccharides (Arima et al., 2005). Moreover, CDs are also applied in the human therapy: hydroxypropyl-beta-CD is applied for the treatment of Niemann-Pick disease (Davidson et al., 2019) and Sugammadex (a chemically-modified gamma-CD derivative) terminates the muscle relaxant effect of rocuronium (Cada et al., 2016). These effects result from formation of highly stable complexes of hydroxypropyl-beta-CD and Sugammadex with cholesterol and rocuronium, respectively. In addition to these pharmaceutical applications, it is reasonable to hypothesize

548 that CD technology may suitable for development mycotoxin binders, which may counteract
549 the toxic effects of mycotoxins even after exposure. Our results demonstrate that some beta-
550 CDs are promising as binders of ZEN.
551
552 **4. Conclusions**
553 In summary, the protective effects of native and chemically modified beta-CDs on ZEN-
554 induced toxicity were investigated in HeLa cells and in zebrafish embryos. The chemically
555 modified beta-CDs that formed more stable complexes with ZEN had considerably stronger
556 protective effect on HeLa cells and zebrafish embryos against the toxic consequences of ZEN-
557 exposure. Since beta-CDs strongly decreased or even abolished the ZEN-induced toxicity
558 both in our *in vitro* and *in vivo* models, it is reasonable to hypothesize that CD technology
559 may be suitable for the development of new ZEN binders. However, further *in vivo* studies are
560 needed to confirm the suitability of CDs as protective agents against ZEN exposure.
561
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