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Testing the protective effects of cyclodextrins vs. alternariol-induced acute toxicity in HeLa cells and in zebrafish embryos

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ABSTRACT

Alternariol (AOH) is a mycotoxin produced by *Alternaria* fungi, it appears as a contaminant in tomatoes, grains, and grapes. The chronic exposure to AOH may cause carcinogenic and xenoestrogenic effects. Cyclodextrins (CDs) are cyclic oligosaccharides, they form host-guest complexes with apolar molecules. In this study, the interactions of AOH with CD monomers and polymers were examined employing fluorescence spectroscopy. Thereafter, the protective effects of certain CDs vs. AOH-induced toxicity were investigated on HeLa cells and on zebrafish embryos. Our major observations are the following: (1) Sugammadex forms highly stable complex with AOH ($K = 4.8 \times 10^4$ L/mol). (2) Sugammadex abolished the AOH-induced toxicity in HeLa cells, while native β-CD did not show relevant protective effect. (3) Each CD tested decreased the AOH-induced mortality and sublethal adverse effects in zebrafish embryos: Interestingly, native β-CD showed the strongest protective impact in this model. (4) CD technology may be suitable to relieve AOH-induced toxicity.

1. Introduction

Alternariol (AOH) is a dibenzo-α-pyrone mycotoxin produced by *Alternaria* species. AOH is a frequent contaminant in several commodities, and it can spoil them during the transport, storage, and even refrigeration (Escrivá et al., 2017). AOH occurs in tomato (and the related products), grains, grapes, wine, and food supplements (Escrivá et al., 2017; Fraeyman et al., 2017). According to the report of the European Food Safety Authority (EFSA, 2011), the human dietary exposure to AOH may exceed the toxicological threshold of concern. However, there are still no regulation limits in regard to the acceptable amounts of AOH in foodstuffs and beverages (Fraeyman et al., 2017). AOH exerts low acute toxicity (EFSA, 2011), while the chronic exposure may result in genotoxic, carcinogenic, mutagenic, and endocrine disruptor effects (Solhaug et al., 2016). Previous studies demonstrated the in vitro cytotoxic action of AOH in human cervical cancer (HeLa) (Balázs et al., 2021), human colon adenocarcinoma (Caco-2) (Fernández-Blanco et al., 2014; Chiesi et al., 2015), and human colon carcinoma (HCT116) (Bensassi et al., 2012) cell lines.

Cyclodextrins (CDs) are cyclic oligosaccharides. The most commonly applied CDs are built up from six (α-CD or ACD), seven (β-CD or BCD), or eight (γ-CD or GCD) glucose subunits. The hydrophilic outer surface provides excellent aqueous solubility to CDs, while their hydrophobic internal cavity makes CDs suitable for the formation of host-guest type inclusion complexes (Galaverna et al., 2008). Previous studies demonstrated the interactions of native and chemically-modified CDs with mycotoxins, including AOH (Fliszár-Nyúl et al., 2019a), ochratoxin A (Verrone et al., 2007), and zearalenone derivatives (Poór et al., 2015; Faisal et al., 2019). In HepG2 cells, native BCD proved to be protective against zearalenone-induced toxicity (Poór et al., 2015). In addition, certain chemically-modified (sulfobutyl, randomly methylated, and succinyl-methyl) β-CDs relieved or even abolished the harmful impacts

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of zearalenone in HeLa cells and/or in zebrafish embryos (Faisal et al., 2020). Sulfobutylether-β-CD (SBECD) is a highly water-soluble CD derivative, it contains sulfobutyl-substituted primary hydroxyl groups (Das et al., 2019). SBECD is widely used in pharmaceutical industry as an excipient, due to its low nephrotoxicity and negligible hemolytic activity (Ueda et al., 1998). Sugammadex is a chemically-modified γ-CD, perfunctionalized with eight sulphanylpropanoic acids at its primary hydroxyl groups (Braga, 2019). As an antidote, sugammadex can rapidly reverse the effects of certain skeletal muscle relaxants (e.g., rocuronium and vecuronium), due to the formation of highly stable inclusion complexes (Keating, 2016). Molecular interactions of sugammadex with mycotoxins have not been tested yet.

In our earlier study, we demonstrated that AOH typically prefers γ-CDs vs. β-CD monomers (Fliszár-Nyúl et al., 2019a). Interestingly, both soluble and insoluble BCD polymers proved to be approximately tenfold stronger binders of AOH compared to the BCD monomer (Fliszár-Nyúl et al., 2019a). In addition, with β-CD bead polymer, we successfully extracted AOH from red wine and tomato juice samples (Fliszár-Nyúl et al., 2020). The binding constants of AOH-CD complexes (with native β- and γ-CDs, as well as with their methyl- and (2-hydroxy-3-N,N,N-trimethylamino)propyl-substituted derivatives) were relatively low ($\log K$ = 2.5–3.2; at pH 7.4) (Fliszár-Nyúl et al., 2019a). Therefore, we aimed to identify chemically-modified CDs which are able to form more stable complexes with this mycotoxin than the previously reported derivatives. Based on our previous observations with zearalenone, the formation of properly stable mycotoxin-CD complexes can inhibit the cellular uptake of the mycotoxin and consequently can alleviate its toxic effects (Faisal et al., 2020). Thus, our second goal was to test the outcome of AOH-CD co-treatments in regard to the toxic impacts of the mycotoxin.

In this study, the complex formation of AOH with CDs was examined employing fluorescence spectroscopy. Furthermore, the protective effects of CDs vs. AOH-induced acute toxicity were also evaluated employing in vitro (HeLa cell line) and in vivo (zebrafish embryo) models. Our results demonstrated that certain CDs can strongly relieve the toxic impacts of AOH.

2. Materials and methods

2.1. Reagents

Alternariol (AOH) was obtained by Cfm Oskar Tropitzsch GmbH (Marktredwitz, Germany). Beta-cyclodextrin (BCD), soluble BCD polymer (BCDSP), sulfobutylether-β-cyclodextrin (SBECD), soluble SBECD polymer (SBSP), and sugammadex were provided by CycloLab Cyclodextrin Research and Laboratory, Ltd. (Budapest, Hungary). Bioluminescent ATP Assay Kit CLSII was purchased from Roche (Basel, Switzerland). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Merck (Darmstadt, Germany). For spectroscopic studies and cell experiments, stock solutions of AOH (5 mM and 40 mM, respectively) were prepared in DMSO (spectroscopic grade; Fluka, Charlotte, NC, US), and were stored at − 20 °C.

2.2. Spectroscopic studies

Fluorescence spectroscopic measurements were performed using a Hitachi F-4500 spectrofluorimeter (Tokyo, Japan) at 25 °C, in the presence of air. Similar to our earlier study (Fliszár-Nyúl et al., 2019a), emission spectra of AOH (5 μM) were recorded in the presence of increasing concentrations of CD (0–5 mM; λ_{ex} = 345 nm). To mimic extracellular physiological conditions, experiments were carried out in phosphate-buffered saline (PBS, pH 7.4). The required amounts of SBSP were determined based on the monomer (SBECD) content of this polymer (60 m/m% according to the manufacturer's description). Binding constants (K; with L/mol unit) of AOH-CD complexes were determined employing the graphical application of the Benesi-Hildebrand equation (Verrone et al., 2007; Poór et al., 2015):

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

where I_0 and I are the fluorescence emission intensity of AOH without and with CDs, respectively. *[CD]* is the molar concentration of CDs, A is a constant, and n is the number of binding sites. Each experiment was performed in triplicates, data represent means ± SEM values.

2.3. Cell experiments

The protective effects of CD monomers and soluble polymers against the AOH-induced cytotoxicity were tested in HeLa cells (ATCC: CCL-2; adherent, human cervix carcinoma). Cells were cultured in DMEM (with 4.5 g/L glucose, 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin) at 37 °C and 5% CO_2. Considering the strong interaction of AOH with serum albumin (Fliszár-Nyúl et al., 2019b), the incubations were performed without fetal bovine serum in the medium. After 24 h treatment in 96-well sterile plates (Perkin-Elmer, Waltham, MA, US), changes in cell viability were evaluated based on intracellular ATP levels, determined by luciferin-luciferase reaction as described previously (Csepregi et al., 2018). Luminescence was measured employing a Perkin Elmer Enspire Multimode plate reader (Perkin Elmer, Waltham, MA, US). Means ± SEM values are demonstrated. The concentration of DMSO did not exceed 0.25 v/v% (the solvent of AOH), which had no effect on ATP levels (100.7 ± 2.0% compared to the solvent-free control). In AOH-CD co-treatment experiments, DMSO concentrations were uniformly 0.18 v/v% in each well.

To select the proper AOH concentration for mycotoxin-CD co-treatments, HeLa cells were incubated for 24 h with increasing concentrations of AOH (0, 10, 30, 50, 70, and 100 μM). The mycotoxin induced concentration-dependent decrease in ATP levels: Approximately 80% reduction was produced by 70 μM of AOH, while no further decrease was observed in the presence of its higher (100 μM) concentration (see later in Fig. 2A). To test the protective effects of CDs, we aimed to produce a strong AOH-induced toxicity. Therefore, in the co-treatment experiments, we applied 70 μM AOH with non-toxic concentrations of CDs (0.0, 0.1, 0.25, 0.5, and 1.5 mM).

Means ± SEM values are derived from three independent experiments (where eight data points were measured in each single experiment). Since the statistical comparison of many groups were performed, the evaluation was carried out employing one-way ANOVA (with Tukey's post hoc) test, employing the SPSS Statistics 26.0 (IBM, Armonk, NY, US) software. The level of significance was set to $p < 0.05$ and $p < 0.01$. ATP levels of cells treated with AOH alone were compared to the ATP concentrations of non-treated cells. Furthermore, ATP levels of AOH-CD co-treated cells were compared to the ATP concentrations of cells incubated with the same concentration of AOH alone (without CDs).

2.4. Experiments on zebrafish embryos

Laboratory-bred AB zebrafish line was held in breeding groups of 30 females and 30 males at the Department of Environmental Toxicology (Hungarian University of Agriculture and Life Sciences, Gödöllő, Hungary) in a Tecniplast ZebTEC recirculation system (Tecniplast S.p.a., Italy) at 25.5 ± 0.5 °C (system water: pH 7.0 ± 0.2, conductivity 550 ± 50 μS) and on a 14 h:10 h light:dark cycle. Fish were fed twice a day with dry granulate food (Zebrafeed 400–600 μm, Sparos Lda., Portugal) supplemented with freshly hatched live *Artemia* salina twice a week. The fish were placed in breeding tanks (Tecniplast S.p.a., Italy) in the afternoon before the day of the experiment, and allowed to spawn by removing the dividing walls next morning. The collected eggs were incubated in Petri dishes filled with system water (diameter: 10 cm). Four to eight cell stage normally developing embryos were collected. To

keep the experiment sterile, the embryos were disinfected by a bleaching method, as it has been described previously (Faisal et al., 2020). To assess the effect of the bleaching method, bleached embryo control was also used.

To test the toxic effects of AOH in the absence and presence of CDs, the Zebrafish Embryo Toxicity Assay (ZETA) was applied (Haq et al., 2016), which is a modified version of the standard OECD Fish Embryo Acute Toxicity (FET) Test (OECD, 2013). In order to find the final treatment concentration of AOH, a pre-test was carried out with five concentrations (2.5, 5.0, 10, 15, and 20 mg/L) in five replicates with five embryos each (n = 25). We observed mortality and sublethal effects daily. After 48 h, more than 25% of the embryos died in the 10 mg/L (38.7 μM) group, and each surviving embryo showed significant sublethal effects (see in Table S1). Based on the results obtained from this preliminary study, the 38.7 μM AOH concentration and 48 h exposure were selected for the co-treatment experiments with CDs.

Three concentrations (0.25 mM, 0.5 mM, and 1.0 mM) of CDs (BCD, SBECD, and sugammadex) were diluted in sterilized E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) (Brand et al., 2002) with and without AOH (38.7 μM). Mixtures were filtered through 0.2 mm syringe filters (VWR, Debrecen, Hungary) to gain bacteriologically sterile solutions. Bleached embryos were placed into 24-well tissue culture plates (JET Biofil; Guangzhou, China) in groups of five. Then E3 medium was removed and the wells were filled with CD solutions (0.25, 0.5, and 1.0 mM; 2 mL/well) in five replicates (n = 25). Another set of the same treatment was carried out with the addition of AOH (38.7 μM) to the CD solutions. Solvent controls were also tested, the final concentration of DMSO (solvent of AOH) did not exceed 0.25 v/v%. Plates were placed in an incubator at 25.5 ± 1 °C. After 48 h, treatment solutions were removed and the wells were filled with fresh E3 medium. Then the plates were incubated for another 72 h. All work phases were carried out under a sterile box in order to avoid contamination. After 120 h (48 h treatment + 72 h in E3 medium), mortalities and deformities of the zebrafish embryos were evaluated under a dissecting microscope (Leica Microsystems GmbH; Wetzlar, Germany).

The five-day old embryos were moved into Petri dishes (diameter: 6 cm; JET Biofil; Guangzhou, China) from each group. Overplus solutions were removed with a plastic transfer pipette and were filled with 2 mL of 0.02% tricaine-methanesulfonate (MS-222; Merck, Darmstadt, Germany) anesthetic solution. Specially designed petri dishes (with two cube-shaped tape, diameter: 10 cm) were filled with 4% methyl-cellulose solution (Csenki et al., 2020). Anaesthetized embryos were placed into the methyl-cellulose, oriented on their right side, and pushed to the bottom of the methyl-cellulose solution with a microloader pipette tip (Eppendorf; Hamburg, Germany). Bright field (exposure time: 6 msec, magnification: 30x) images of the embryos were taken under a stereomicroscope (Leica M205 FA stereomicroscope, Leica DFC 7000 T camera, Leica Application Suite X, Leica Microsystems GmbH; Wetzlar, Germany).

In zebrafish experiments, means ± SD values are demonstrated, where 25 embryos were used in each treatment group. Statistical analyses of data (both mortality and sublethal effects) were carried out with GraphPad Prism 8.4.3 software (GraphPad Software; San Diego, CA, US), employing two-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

3. Results

3.1. Fluorescence spectroscopic investigation of AOH-CD interactions

First, the effects of CDs on the fluorescence emission signal of AOH were tested in PBS (pH 7.4). Under the applied conditions, CDs did not exert fluorescence; nevertheless, the background corrections of spectra have been performed.

Both SBECD monomer and soluble polymer (SBSP) induced strong increase in the emission signal of AOH showing the wavelength maximum of the formed complexes at 460 nm (Fig. 1A and B). SBSP proved to be a stronger fluorescence enhancer than SBECD. The emission wavelength maximum of AOH-sugammadex complex was observed at 476 nm (Fig. 1C). Sugammadex caused a larger (approximately 25-fold) increase in the fluorescence signal of the mycotoxin than SBECD (sixfold) or SBSP (tenfold).

Binding constants of AOH-CD complexes were determined employing the graphical application of the Benesi-Hildebrand equation (Eq. (1)). Benesi-Hildebrand plots showed good fitting ($R^2 = 0.992–0.998$) with the 1:1 stoichiometry model (Fig. 1D, E, and F). Both SBECD ($\log K = 3.3$) and SBSP ($\log K = 3.7$) formed relatively stable complexes with the mycotoxin. However, as an unexpected result, sugammadex proved to be a superior binder of AOH (Fig. 1F). The binding constant of AOH-sugammadex ($\log K = 4.7$) was far higher than the $\log K$ values of other AOH-CD complexes examined.

3.2. Protective effects of CDs vs. AOH-induced toxicity on HeLa cells

The toxic impact of AOH was tested on HeLa cell line in the absence and presence of CDs. Cell viability was evaluated after 24 h incubation based on ATP level/well. The ATP content is an indicator of metabolically active cells; therefore, it is a suitable parameter to investigate the number of viable cells (Adan et al., 2016; Kocyigit et al., 2018). Under the applied conditions, even 1.5 mM concentration of CDs alone (without AOH) did not affect ATP levels (data not shown). However, AOH (10–100 μM) induced a concentration-dependent decrease in ATP levels (Fig. 2A): Even 10 μM caused a statistically significant ($p < 0.01$) reduction, while above 70 μM concentration no further changes were noticed. For the co-treatments, the 70 μM concentration of AOH was applied (caused approximately 80% decrease in ATP levels) with 0.00, 0.10, 0.25, 0.5, and 1.5 mM of CDs (soluble CD polymers were added based on their molar monomer contents).

Combined effects of AOH with CDs are demonstrated in Fig. 2B. Native BCD did not affect the AOH-induced toxicity even at 1.5 mM concentration, and BCDSP caused only a slight elevation without statistical significance ($p > 0.05$). SBECD and SBSP caused minor increase in ATP levels, but only the 1.5 mM concentration of SBSP caused statistically significant impact ($p < 0.05$). However, in a concentration-dependent fashion, sugammadex successfully relieved the AOH-induced acute toxicity. Even 0.5 mM concentration of sugammadex almost completely restored ATP levels (Fig. 2B).

3.3. Effects of AOH and/or CDs on zebrafish embryos

Zebrafish model is widely applied for the evaluation of toxicity and/or teratogenic effects of xenobiotics (Kim et al., 2020; de Esch et al., 2012; Rubinstein, 2006). Therefore, to further assess the protective effects of selected CDs (BCD, SBECD, and sugammadex), zebrafish experiments were also performed. Sterile conditions and the bleaching of embryos was reasonable to avoid the bacterial breakdown of CD rings to glucose subunits. Under the applied conditions, the bleaching method did not affect the viability of zebrafish embryos. Furthermore, in the solvent and E3 controls, no mortality was observed (Table 1).

First, the effects of BCD, SBECD, and sugammadex were examined to compare them later with the co-exposure experiments. CDs tested did not cause mortality (Table 1), while their mild sublethal effects were noticed (Fig. 3A). The detailed quantitative description in regard to the sublethal effects of CDs is demonstrated in Table 2. Two phenotypes, the uninflated swim bladder and the irregular edges of the dorsal fin occurred in all CD-treated embryos (at each concentration applied), while body axis curvature and edemas occurred only in certain groups. Uninflated swim bladder was typically more frequent in the BCD- and SBECD-treated groups, while it was slightly less common in the sugammadex-treated groups (Table 2). There was no clear difference in the appearance of the irregular edges of the dorsal fin between the three CDs examined (Table 2). Curvature of the body axis was induced by

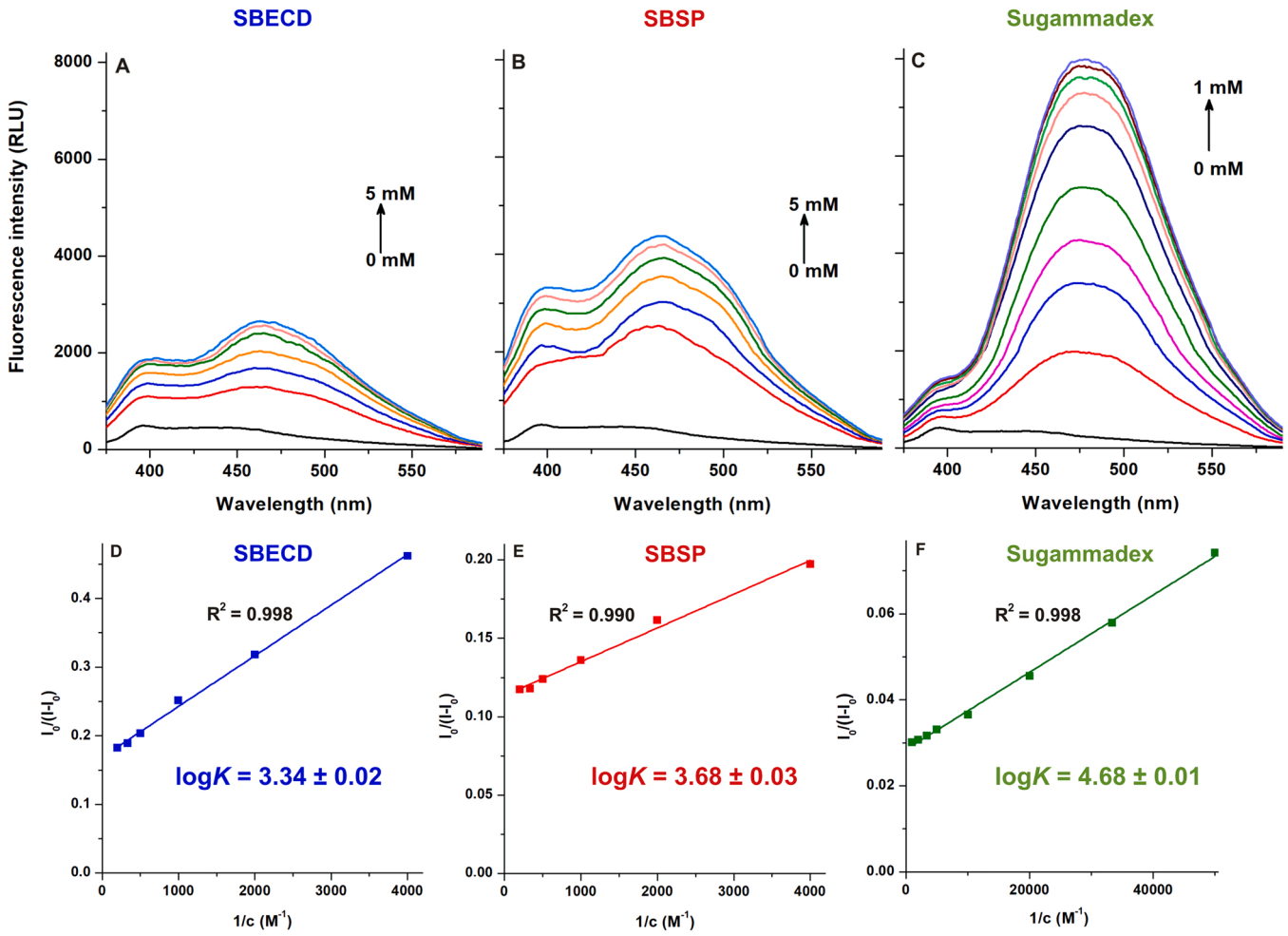

Fig. 1. Interaction of AOH with sulfobutylether-β-cyclodextrin (SBECD; A and D), soluble SBECD polymer (SBSP; B and E), and sugammadex (C and F). Representative fluorescence emission spectra of AOH (5 μM) in the presence of increasing SBECD (A; 0.0, 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0 mM), SBSP (B; 0.0, 0.25, 0.5, 1.0, 2.0, 3.0, and 5.0 mM), and sugammadex (C; 0.00, 0.01, 0.02, 0.03, 0.05, 0.10, 0.20, 0.30, 0.50, and 1.0 mM) concentrations in PBS (pH 7.4; λ_{ex} = 345 nm). Benesi-Hildebrand plots of AOH-SBECD (D), AOH-SBSP (E), and AOH-sugammadex (F) complexes (λ_{ex} = 345 nm; λ_{em} = 460 nm for SBECD and SBSP, and 476 nm for sugammadex). Decimal logarithmic values of the binding constants (log*K* ± SEM; the unit of *K* is L/mol) are also indicated (n = 3). At pH 7.4, the previously reported log*K* values of AOH-BCD and AOH-GCD complexes were 2.5 and 3.2, respectively (Fliszár-Nyúl et al., 2019a).

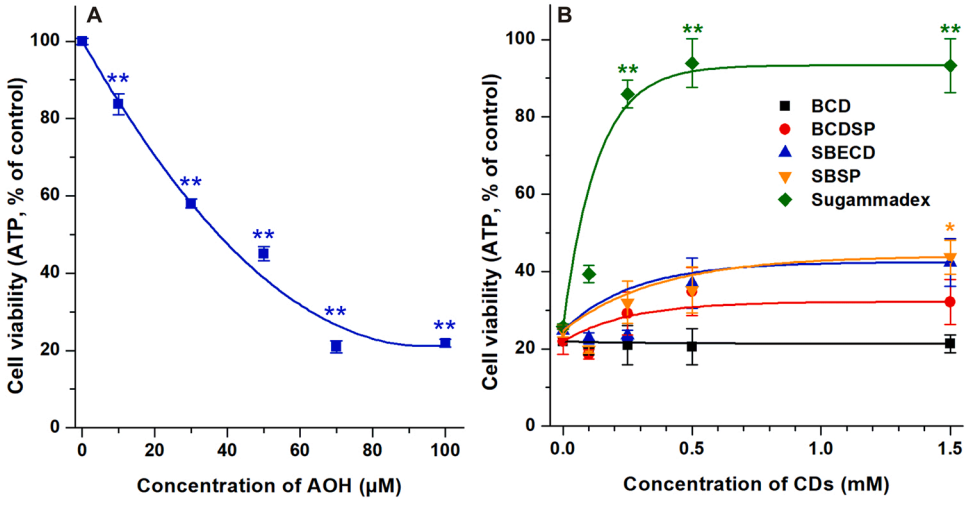

Fig. 2. Concentration-dependent impact of AOH (0–100 μM) on the viability of HeLa cells (ATP/well, % of control) after 24 h incubation (A). Effects of AOH (70 μM) on the viability of HeLa cells (ATP/well, % of control) after 24 h incubation in the presence of increasing concentrations of CDs (B). Data represent means ± SEM values from three independent experiments (*$p < 0.05$, **$p < 0.01$; BCD, beta-cyclodextrin; BCDSP, soluble BCD polymer; SBECD, sulfobutylether-β-cyclodextrin; SBSP, soluble SBECD polymer).

Table 1
Mortality (% ± SD) of zebrafish embryos at 120 hpf treated with AOH or CDs alone, and with their combinations (n = 25). Each treatment group was statistically compared to each other using two-way ANOVA with Tukey's multiple comparisons test. The different letters indicate statistically significant differences ($p < 0.0001$).

Treatment		Mortality (% ± SD)
E3 control		0.00 (± 0.00)[a]
E3 control with bleached embryos		0.00 (± 0.00)[a]
38.7 μM AOH (DMSO solvent) in E3 control		36.0 (± 2.00)[b]
38.7 μM AOH (DMSO solvent) in E3 control with bleached embryos		36.0 (± 2.00)[b]
Solvent (DMSO) control in E3		0.00 (± 0.00)[a]
BCD	0.25 mM	0.00 (± 0.00)[a]
	0.5 mM	0.00 (± 0.00)[a]
	1.0 mM	0.00 (± 0.00)[a]
BCD + 38.7 μM AOH	0.25 mM	4.00 (± 1.25)[a]
	0.5 mM	8.00 (± 1.25)[a]
	1.0 mM	0.00 (± 0.00)[a]
SBECD	0.25 mM	0.00 (± 0.00)[a]
	0.5 mM	0.00 (± 0.00)[a]
	1.0 mM	0.00 (± 0.00)[a]
SBECD + 38.7 μM AOH	0.25 mM	44.0 (± 2.00)[b]
	0.5 mM	4.00 (± 1.25)[a]
	1.0 mM	8.00 (± 1.25)[a]
Sugammadex	0.25 mM	0.00 (± 0.00)[a]
	0.5 mM	0.00 (± 0.00)[a]
	1.0 mM	0.00 (± 0.00)[a]
Sugammadex + 38.7 μM AOH	0.25 mM	28.0 (± 2.00)[b]
	0.5 mM	8.00 (± 1.25)[a]
	1.0 mM	4.00 (± 1.25)[a]

SBECD at each concentration applied, while among the other CDs, only 1.0 mM BCD caused the appearance of this symptom (Table 2). Formation of edemas occurred in the presence of 1.0 mM sugammadex and at each applied concentration of SBECD (but only less than 10% of the embryos were involved). Hatching rate was also investigated at 120 h post-fertilization (hpf): Each CD-treated embryo hatched prior to that time. Furthermore, CDs induced a specific phenotype of the caudal fin primordium which is characterized by the abnormal arrangement of melanophores in that region (Fig. 3E).

AOH (38.7 μM) caused 36.0 ± 8.9% mortality at 120 hpf (Table 1), and a wide variety of sublethal effects was observed in the surviving embryos. 100% of the survived embryos showed tail malformation, formation of edemas, uninflated swim bladder, and curvature of the body axis (Table 2). In addition, none of the embryos hatched up until the 120 hpf observation time. Fig. 3B depicts an AOH-treated embryo in the chorion, while Fig. 3C shows an embryo from which the chorion has been removed for the better visualization of malformations.

CDs significantly reduced the lethal effects of the mycotoxin (Table 1), suggesting their considerable protective impacts vs. AOH-induced toxicity. BCD exerted the strongest protective action and significantly reduced the mortality even at 0.25 mM concentration. Moreover, BCD completely prevented mortality at the highest concentration applied (1.0 mM). Surprisingly, SBECD and sugammadex showed weaker protective effects compared to BCD (Table 1). Their lowest concentration (0.25 mM) did not decrease the toxin-induced mortality, while the 0.5 mM and 1.0 mM concentrations of SBECD and sugammadex significantly reduced the toxin-induced lethal effects.

Sublethal impacts were also evaluated (Table 2). In general, CDs proved to be protective vs. certain AOH-induced sublethal effects (Fig. 4). Each applied concentration of BCD, SBECD, and sugammadex significantly reduced the appearance of tail malformation, edemas, and curvature of the body axis ($p < 0.0001$). The appearance of uninflated swim bladder has been significantly reduced in some CD co-treatments (Table 2). Nevertheless, we did not consider this phenotype as an index of recovery from AOH toxicity because this phenotype was abundant in single CD treatments as well. As we described above, in the absence of CDs, none of the AOH-treated embryos hatched up. In contrast, each CD co-treated zebrafish embryo hatched prior to 120 hpf (except the 0.25 mM sugammadex co-treated group).

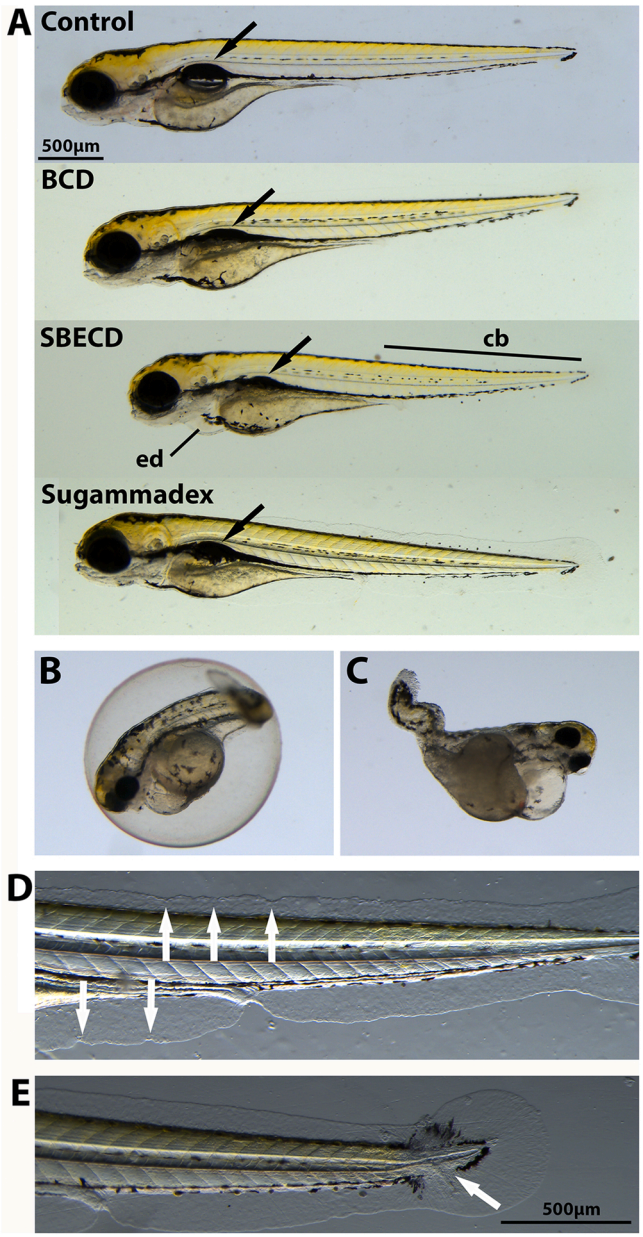

Fig. 3. Representative developmental defects in 120 hpf zebrafish embryos after 48 h treatment with CDs (A) or AOH (B and C) alone (bright field; exposure time: 6 msec; magnification: 30x (A, B, C), 50x (D, E); Leica M205 FA stereomicroscope; Leica DFC 7000 T camera; Leica Application Suite X): uninflated swim bladder (A, arrows), curvature of the body axis (cb), edema (ed), irregular edges of the dorsal fin (SBECD 1 mM, D, arrows) and abnormal arrangement of melanophores in the caudal fin primordium (BCD 1 mM, E, arrow).

In comparison to the in vitro HeLa cell assay, only sugammadex showed significant protective impacts in both tests (Fig. 2, Tables 1 and 2). BCD and SBECD did not alleviate the toxic effects of AOH in HeLa cells (Fig. 2). However, both BCD and SBECD relieved AOH-induced mortality and sublethal adverse effects in the in vivo zebrafish embryo model (Tables 1 and 2). The observed differences can be partly explained by the distinct in vitro (human adenocarcinoma cell line) and in vivo (zebrafish embryo) models applied, and also suggest that these tests can complement each other when they are used together.

Table 2
The mean appearance (% ± SD) of representative developmental defects after CD (0.0–1.0 mM), AOH (0.0 or 38.7 μM), or AOH+CD exposure (tm, tail malformation; nh, not hatched; ed, edema; cb, curvature of the body axis; usb, uninflated swim bladder; am, abnormal arrangement of melanophores in the caudal fin primordium; fin, irregular edges of dorsal fin). Recovery of developmental defects in AOH+CD co-treatments compared to the 38.7 μM AOH treatment has been analyzed using two-way ANOVA with Tukey's multiple comparisons test (significant differences are marked as ****$p < 0.0001$). In each group, 25 zebrafish embryos were treated. After 120 hpf, the developmental defects were evaluated in surviving animals.

		BCD			**SBECD**			**Sugammadex**			**E3 control**
		0.25 mM	**0.5 mM**	**1.0 mM**	**0.25 mM**	**0.5 mM**	**1.0 mM**	**0.25 mM**	**0.5 mM**	**1.0 mM**	
Freq. (%)	**tm**	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	nh	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	ed	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 5.5	8.0 ± 4.5	4.0 ± 5.5	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 5.5	0.0 ± 0.0
	cb	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 5.5	12.0 ± 4.5	20.0 ± 4.5	20.0 ± 4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	usb	56.0 ± 8.9	68.0 ± 11.0	88.0 ± 11.0	56.0 ± 8.9	56.0 ± 8.9	68.0 ± 11.0	56.0 ± 8.9	44.0 ± 8.9	44.0 ± 8.9	0.0 ± 0.0
	am	8.0 ± 4.5	10.0 ± 7.1	28.0 ± 11.0	12.0 ± 8.4	16.0 ± 8.9	24.0 ± 8.9	12.0 ± 8.4	24.0 ± 8.9	20.0 ± 7.1	0.0 ± 0.0
	fin	36.0 ± 8.9	52.0 ± 11.0	48.0 ± 11.0	24.0 ± 8.9	48.0 ± 11.0	56.0 ± 8.9	40.0 ± 0.0	32.0 ± 11.0	64.0 ± 8.9	0.0 ± 0.0
		BCD + 38.7 μM AOH			**SBECD + 38.7 μM AOH**			**Sugammadex + 38.7 μM AOH**			**38.7 μM**
		0.25 mM	**0.5 mM**	**1.0 mM**	**0.25 mM**	**0.5 mM**	**1.0 mM**	**0.25 mM**	**0.5 mM**	**1.0 mM**	**AOH**
Freq. (%)	**tm**	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	100.0 ± 0.0
	nh	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	12.0 ± 8.4****	0.0 ± 0.0****	0.0 ± 0.0****	100.0 ± 0.0
	ed	0.0 ± 0.0****	0.0 ± 0.0****	0.0 ± 0.0****	8.0 ± 4.5****	0.0 ± 0.0****	0.0 ± 0.0****	32.0 ± 11.0****	4.0 ± 5.5****	4.0 ± 5.5****	100.0 ± 0.0
	cb	0.0 ± 0.0****	4.0 ± 5.5****	0.0 ± 0.0****	28.0 ± 11.0****	12.0 ± 4.5****	0.0 ± 0.0****	40.0 ± 11.0****	4.0 ± 5.5****	0.0 ± 0.0****	100.0 ± 0.0
	usb	92.0 ± 11.0	96.0 ± 8.9	68.0 ± 11.0	72.0 ± 11.0	80.0 ± 11.0	80.0 ± 11.0	80.0 ± 11.0	76.0 ± 8.9	60.0 ± 8.9	100.0 ± 0.0
	am	12.0 ± 4.5	20.0 ± 11.0	8.0 ± 4.5	24.0 ± 8.9	40.0 ± 8.9	44.0 ± 8.9	8.0 ± 4.5	12.0 ± 4.5	16.0 ± 5.5	0.0 ± 0.0
	fin	24.0 ± 8.9	28.0 ± 11.0	40.0 ± 0.0	20.0 ± 8.9	40.0 ± 8.9	56.0 ± 8.9	40.0 ± 0.0	80.0 ± 11.0	80.0 ± 11.0	0.0 ± 0.0

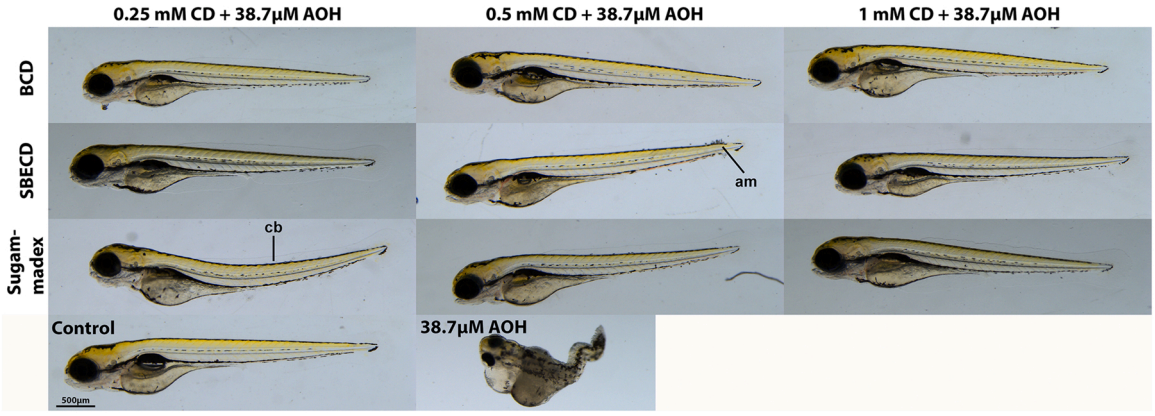

Fig. 4. Representative developmental defects in 120 hpf zebrafish embryos after 48 h co-treatment with AOH (38.7 μM) and CDs (0.0–1.0 mM) (bright field; exposure time: 6 msec; magnification: 30x; Leica M205 FA stereomicroscope; Leica DFC 7000 T camera; Leica Application Suite X): curvature of the body axis (cb) and abnormal arrangement of melanofores in the caudal fin primordium (am). Control and AOH-treated (38.7 μM, without CDs) animals are demonstrated in the bottom panels.

4. Discussion

Our previous studies demonstrated the host-guest type complex formation of AOH with certain CDs (Fliszár-Nyúl et al., 2019a, 2020). In order to find more effective AOH binders, in the current study, further chemically-modified CDs were examined, including SBECD, SBSP, and sugammadex. Typically, in an aqueous solution, the water molecules in the hydration shell can partly quench the fluorescence of an aromatic fluorophore (Faisal et al., 2019; Fliszár-Nyúl al, 2019a). The accommodation of an aromatic fluorophore in the CD cavity results in the partial decomposition of its hydration shell, leading to the decreased quenching effects of water molecules and consequently the elevated emission signal of the guest molecule (Faisal et al., 2019; Fliszár-Nyúl al, 2019a). Due to the CD-induced enhancement in the fluorescence emission signal of AOH, fluorescence spectroscopy is a suitable technique to examine the complex formation of the mycotoxin with CDs (Fliszár-Nyúl et al., 2019a). SBSP caused a higher increase in the fluorescence of AOH compared to the SBECD monomer (Fig. 1A and B), which is in agreement with our previous observation where BCD polymer (BCDSP) also induced larger enhancement than BCD monomer (Fliszár-Nyúl et al., 2019a). Nevertheless, sugammadex proved to be more effective fluorescence enhancer of AOH (Fig. 1C) than any other CDs tested in the current or in previous studies. As it has been reported, native GCD induced 14-fold increase in the fluorescence of AOH at pH 7.4 (Fliszár-Nyúl et al., 2019a); while sugammadex produced approximately 25-fold enhancement in the current study (Fig. 1). GCD forms aggregates in aqueous solution due to the hydrogen bonds between the exterior hydroxyl groups (Szente et al., 1998), and the self-assembling

may affect the fluorescence signal of its complexes. However, self-aggregation is not typical in regard to sugammadex because its negatively charged substituents induce repulsion between the CD molecules (Kurkov et al., 2011). Therefore, the greater increase in the fluorescence of the guest molecule can be observed in the presence of sugammadex.

At pH 7.4, SBECD formed stable complexes with AOH (log$K$ = 3.3; Fig. 1). In our previous study, 3- to 7-fold lower binding constants were determined for native BCD, randomly methylated β-CD, and quaternary ammonium β-CD (Fliszár-Nyúl et al., 2019a). Furthermore, based on the molar SBECD content of SBSP, the polymer was a stronger binder of AOH than the monomer (Fig. 1). This observation suggests the slight cooperative effect of CD rings in the soluble polymer (Zhang and Ma, 2010).

At physiological pH, sugammadex formed highly stable complex with AOH (log$K$ = 4.7) (Fig. 1C). It bound the mycotoxin with 30-fold higher affinity compared to the native GCD, based on the previously reported data for AOH-GCD complex (log$K$ = 3.2; pH 7.4) (Fliszár-Nyúl et al., 2019a). The outstanding stability of AOH-sugammadex complex can likely be explained by structural features: the sulphonylpropanoic acid side chains of sugammadex elongate the internal cavity to 1.3-fold vs. the native GCD (Booij, 2009). Furthermore, the repelling electrostatic force between the anionic functional groups keeps the cavity wide open and may promote the interaction (Kovac, 2009).

Previous studies demonstrated that the formation of highly stable ligand-CD complexes can limit the cellular uptake and consequently the cytotoxicity of certain guest molecules (Poór et al., 2015; Weiss-Errico et al., 2017; Nam et al., 2017; da Silva et al., 2019; Faisal et al., 2020). Based on the binding constants of AOH-CD complexes determined in this study (Fig. 1), we successfully identified SBECD and sugammadex as promising AOH binders.

Therefore, we examined the effects of CD co-treatment on the AOH-induced cytotoxicity in HeLa cells. The protective effects of CDs showed excellent correlation with the binding constants of AOH-CD complexes. The affinity of sugammadex toward AOH is at least one magnitude higher compared to the other CDs tested, which explains why sugammadex was highly the most successful CD against the AOH-induced toxicity in HeLa cells (Fig. 2B). The formation of stable AOH-sugammadex complexes leads to the entrapment of the mycotoxin in the CD cavity, and consequently decreases the harmful effects of AOH. These observations are in agreement with our previous results with zearalenone, where the formation of highly stable complexes (log$K$ = 4.1–4.7) with chemically-modified (sulfobutyl, methyl, and succinyl-methyl) β-CDs completely abolished the mycotoxin-induced viability loss in HeLa cells (Faisal et al., 2020).

Under the applied conditions, CDs did not cause mortality or strong malformations in zebrafish embryos (Table 1); however, their mild sublethal effects have been observed (Table 2). The uninflated swim bladder phenotype was noticed both in the current (Fig. 3) and in our previous study (Faisal et al., 2020), suggesting that this phenotype is typically caused by CDs. Furthermore, the same concentrations of BCD and SBECD (tested on *tg(vtg1:mcherry)* transgenic zebrafish embryos in a different exposure window) caused mostly the same phenotypic changes (Faisal et al., 2020). In another study, sugammadex induced neither visible malformations, nor lethality in WT zebrafish embryos in the 0–200 μg/mL (0.00–0.09 mM) concentration range (Kim et al., 2020). Nevertheless, these concentrations were markedly lower compared to our experiment. CDs caused a specific phenotype of the caudal fin primordium (abnormal arrangement of melanophores in that region; see in Fig. 3E) suggesting their effects on the Sonic hedgehog (Shh) signaling pathway. Shh signaling is required for the normal patterning of the caudal fin primordium, while the earliest development of this region occurs at 1.5 days post-fertilization (dpf) in zebrafish embryos, marked by the appearance of medial fin precursor cells (Hadzhiev et al., 2007).

Only limited data are available in regard to the effects of native and chemically-modified CDs on zebrafish embryos, therefore, our results help the deeper understanding of this issue. On the other hand, these observations do not confute the safety of certain CDs (e.g., SBECD and sugammadex) which are used even in the human pharmacotherapy. Most of the CDs can barely pass through the cell membrane (except methyl derivatives), thus CDs typically have low oral bioavailability and consequently they do not cause adverse effects after *per os* administration (Irie and Uekama, 1997; Crini, 2014; Jansook et al., 2018). Hydroxypropyl-β-CD, SBECD, and sugammadex can be administered parenterally with good tolerability, while the intravenous administration of BCD can cause nephrotoxicity (Irie and Uekama, 1997; Gould and Scott, 2005; Stella and He, 2008; Jansook et al., 2018). Sugammadex itself is applied as a medication, it can rapidly reverse the rocuronium- or vecuronium-induced skeletal muscle relaxation (Keating, 2016). In addition, hydroxypropyl-β-CD is an investigational drug in the US, it may be suitable for the treatment of Niemann-Pick C disease (Erickson et al., 2018). Importantly, SBECD or sugammadex have some adverse effects; however, considering the human clinical data available, these CDs seem to be safe enough even for the human parenteral application.

In the current study, the 38.7 μM concentration of AOH caused 36% mortality in zebrafish embryos (Table 1). In a previous report, the ichtyotoxic (toxic or lethal towards fish) effect of AOH in zebrafish embryos has also been demonstrated, where the half-lethal concentration of the toxin was determined as 15.9 μg/mL (61.6 μM), inducing coagulation of egg (24 hpf) and embryonic death (72 hpf) (Bai et al., 2016). Embryotoxicity of AOH was tested previously on chicken embryos by microinjection, it caused no mortality or teratogenic effects at the highest dose applied (1000 μg/egg) (Griffin and Chu, 1983). In another study, AOH was administered to brine shrimp (*Artemia salina* L.) larvae, where 387.3 μM was calculated as the 50% lethal concentration (Panigrahi and Dallin, 1994). These findings suggest that the embryotoxic/fetotoxic effects of AOH show large species-dependent differences and they are milder compared to tenuazonic acid (another *Alternaria* mycotoxin) (Griffin and Chu, 1983).

Interestingly, BCD did not show protective effect in in vitro cell experiments (Fig. 2B), while it strongly decreased the AOH-induced mortality and alleviated the subtoxic effects of the mycotoxin in the in vivo zebrafish model (Tables 1 and 2). In our previous study, BCD also showed strong protective impacts in zebrafish experiments against zearalenone-induced toxicity, while it was not effective in cell experiments (Faisal et al., 2020). These results highlight that the zebrafish model is more sensitive. On the other hand, we have no clear explanation why BCD showed better results in zebrafish experiments compared to SBECD or sugammadex, since the latter two CDs bind AOH with much higher affinity (Fig. 1) and demonstrated stronger protective effects in cell experiments than BCD (Fig. 2). This phenomenon may be resulted from other interactions of AOH and/or CDs in zebrafish. CDs can interact with several endogenous molecules in vivo, which cannot be properly modeled in in vitro cell experiments. Some other studies, performed on zebrafish, also demonstrated that CDs can alleviate the acute toxicity of certain chemicals: BCD decreased the toxic impacts of estradiol (da Silva et al., 2019), perfluorooctanoic acid (Weiss-Errico et al., 2017), and 20 (S)-protopanaxadiol 20-O-D-glucopyranoside (Nam et al., 2017). However, CDs increased the caffeine-induced toxicity and the frequency of embryonic malformations in zebrafish, likely due to their synergistic effects (Szmeja et al., 2021). Considering the previously reported results and our new observations, the stability of the ligand-CD complexes can help to predict the in vivo effects of CDs; however, these expectations are sometimes overwritten by other factors. Since the number of available studies are limited, further extensive investigations are reasonable for the better understanding of the in vivo application of CDs as toxin binders.

5. Conclusions

In summary, the interactions of AOH with CDs were examined, including the stability of the formed complexes as well as the protective

effects of CDs vs. AOH-induced acute toxicity. Based on fluorescence spectroscopic studies, sugammadex bound AOH with much higher affinity (log$K$ = 4.7) than the other CDs tested in the current or previous studies. In agreement with this observation, sugammadex showed concentration-dependent, strong protective effect in cell experiments. In zebrafish studies, each CD examined (BCD, SBECD, and sugammadex) decreased the AOH-induced mortality and sublethal adverse effects. Unexpectedly, native BCD did not affect the viability of AOH-treated HeLa cells, while it showed stronger protective effects in zebrafish experiments than SBECD or sugammadex. Our results demonstrate that CDs may be suitable as in vivo AOH binders. Nevertheless, besides the stability of AOH-CD complexes, other details may also be important. Thus, further in vivo investigations are required for the deeper understanding of the protective effects of CDs vs. AOH and other xenobiotics. Importantly, in animal studies with rodents or other mammals, we should focus on the less toxic CD derivatives (e.g., SBECD, sugammadex, or hydroxypropyl-β-CD).

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CRediT authorship contribution statement

Eszter Fliszár-Nyúl: Formal analysis, Investigation, Methodology, Writing – original draft. **Illés Bock:** Formal analysis, Investigation. **Rita Csepregi:** Formal analysis, Investigation. **Lajos Szente:** Resources, Methodology. **István Szabó:** Resources, Methodology. **Zsolt Csenki:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft. **Miklós Poór:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2022.103965.

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