Interaction of mycotoxins zearalenone, α-zearalenol, and β-zearalenol with cytochrome P450 (CYP1A2, 2C9, 2C19, 2D6, and 3A4) enzymes and organic anion transporting polypeptides (OATP1A2, OATP1B1, OATP1B3, and OATP2B1)

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ABSTRACT

Zearalenone (ZEN) is a mycoestrogen produced by *Fusarium* fungi. ZEN is a frequent contaminant in cereal-based products, representing significant health threat. The major reduced metabolites of ZEN are α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL). Since the toxicokinetic interactions of ZEN/ZELs with cytochrome P450 enzymes (CYPs) and organic anion transporting polypeptides (OATPs) have been barely characterized, we examined these interactions applying *in vitro* models. ZEN and ZELs were relatively strong inhibitors of CYP3A4 and moderate inhibitors of CYP1A2 and CYP2C9. Both CYP1A2 and CYP3A4 decreased ZEN and β-ZEL concentrations in depletion assays, while only CYP1A2 reduced α-ZEL levels. OATPs tested were strongly or moderately inhibited by ZEN and ZELs; however, these mycotoxins did not show higher cytotoxicity in OATP-overexpressing cells. Our results help the deeper understanding of the toxicokinetic/pharmacokinetic interactions of ZEN, α-ZEL, and β-ZEL.

1. Introduction

Mycotoxin zearalenone (ZEN; Fig. 1) is typically produced by *Fusarium* molds *(e.g., F. graminearum)*. ZEN is a common contaminant in maize, in other cereals, and in cereal-based food products (European Food Safety Authority (EFSA), 2017). Furthermore, ZEN is an endocrine disruptor; due to its interaction with estrogen receptors, it is classified as a xenoestrogen (Rai et al., 2020). The ZEN-induced vulvovaginitis in swine was described nearly a century ago (McNutt et al., 1928). In mammals, 3α- and 3β-hydroxysteroid dehydrogenases are the dominant enzymes in the phase I metabolism of ZEN, resulting in the formation of reduced metabolites, including α-zearalenol (α-ZEL; Fig. 1), β-zearalenol (β-ZEL; Fig. 1), zearalanone, α-zearalanol, and β-zearalanol (Rogowska et al., 2019). Importantly, α-ZEL and α-zearalanol have remarkably higher xenoestrogenic potency compared to the parent mycotoxin (European Food Safety Authority (EFSA), 2017). Glucuronic acid conjugation is the most relevant phase II biotransformation reaction of ZEN (and its reduced metabolites); glucuronides are significantly weaker xenoestrogens than ZEN and they are subjected to enterohepatic cycling (European Food Safety Authority (EFSA), 2017; Rogowska et al., 2019).

The members of the cytochrome P450 (CYP) protein family are microsomal enzymes: CYP1A2, 2C9, 2C19, 2D6, and 3A4/5 are the most frequently involved CYP enzymes in the oxidative biotransformation of drugs and other xenobiotics (Zhao et al., 2021). ZEN can activate pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR), resulting in the increased
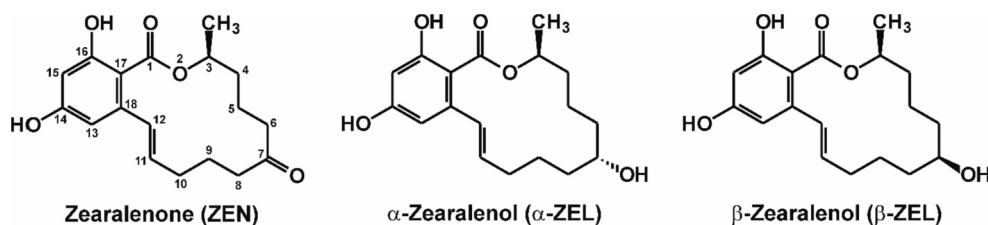
Fig. 1. Chemical structures of zearalenone (ZEN), α-zearalenol (α-ZEL), and β-zearalenol (β-ZEL).

transcription of several genes and the elevated expression of CYP1A, CYP2C, CYP2E, and CYP3A enzymes (Ayed-Boussema et al., 2011; Duca et al., 2012; European Food Safety Authority (EFSA), 2017). In addition, CYP enzymes also take part in the biotransformation of ZEN, the CYP-catalyzed production of 6-hydroxy-, 8-hydroxy-, 13-hydroxy-, and 15-hydroxy-ZEN has been reported (European Food Safety Authority (EFSA), 2017). In rat and human liver microsomes, the formation of 13-hydroxy- and 15-hydroxy-catechol derivatives is the most typical (Fleck et al., 2012). The xenoestrogenic impacts of hydroxylated ZEN metabolites are limited compared to the parent mycotoxin (Bravin et al., 2009); however, the catechol derivatives can produce DNA-reactive quinones, which are likely involved in ZEN-induced genotoxicity (Fleck et al., 2012). Based on *in vitro* experiments with human CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 enzymes, mainly CYP1A2 and CYP3A4 took part in the oxidative biotransformation of ZEN and α-ZEL (Pfeiffer et al., 2009): CYP1A2 produced exclusively catechol derivatives, while CYP3A4 catalyzed both aliphatic and aromatic hydroxylation. In another *in vitro* study, where the impacts of human CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, and 3A5 enzymes were tested, the authors described the involvement of CYP2C8 and CYP3A4/5 in the hydroxylation of ZEN (Bravin et al., 2009). Typically, mycotoxin levels are low in the human body; therefore, it is unlikely that they can influence the pharmacokinetics of drugs (Lootens et al., 2022). However, the inhibitory effect of ZEN on CYP3A4 has been demonstrated both *in vitro* and *in vivo* (Schelstraete et al., 2019): ZEN inhibited CYP3A4-catalyzed midazolam hydroxylation in porcine hepatic microsomes, and higher C_{max} and AUC values of midazolam (*per os*, single dose) were observed in pigs after the two-week exposure to ZEN. These observations underline the potential importance of ZEN-CYP interactions. In addition, only limited data are available regarding ZEN metabolites (*e.g.*, α-ZEL and β-ZEL).

Organic anion transporting polypeptides (OATPs) are transporters expressed in the plasma membrane of human endothelial or epithelial cells (Svoboda et al., 2011; Oswald, 2019). OATPs facilitate the uptake of a variety of *endo-* and xenobiotics, including clinically used drugs (Stieger and Hagenbuch, 2014). Of those, OATP1A2 and OATP2B1 are ubiquitously expressed in the human body, particularly in the intestinal epithelium and brain capillary endothelial cells; they are implicated in the intestinal absorption and blood-brain barrier penetration of various organic compounds (Shitara et al., 2013; Tamai and Nakanishi, 2013; Gao et al., 2015). OATP1B1 and OATP1B3 are predominantly expressed on the basolateral membrane of hepatocytes, where they contribute to hepatic uptake than detoxification (Kalliokoski and Niemi, 2009; Shitara et al., 2013). The multispecific members of the OATP family are key modulators of the absorption, distribution, metabolism, and excretion (ADME) of many endogenous compounds (*e.g.*, steroid hormones and bile acids), drugs (*e.g.*, statins, fexofenadine, and docetaxel), polyphenols (*e.g.*, quercetin), and toxins (*e.g.*, microcystin) (Kovacsics et al., 2017). In addition, OATPs can be victims of food-drug interactions, resulting in adverse health effects (Ali et al., 2020). Therefore, we aimed to elucidate the extent to which ZEN and ZELs interact with multispecific OATPs, as we found no data regarding these interactions in the scientific literature.

To get a deeper insight into the toxicokinetic and pharmacokinetic interactions of ZEN and ZELs, in the present *in vitro* study, the interactions of ZEN, α-ZEL, and β-ZEL were examined with CYP1A2, 2C9, 2C19, 2D6, and 3A4 enzymes, as well as with OATPs, 1A2, 1B1, 1B3, and 2B1. The inhibitory effects of the mycotoxins on CYP activity were tested on human recombinant enzymes, after which mycotoxin depletion assays were performed to examine the potential involvement of CYPs in the biotransformation of ZEN and ZELs. The inhibitory actions of mycotoxins on OATP activity were investigated on OATP-overexpressing cells. Thereafter, the cytotoxicity of ZEN and ZELs on OATP-expressing cells *vs.* their mock-transfected controls was also tested.

2. Materials and methods

2.1. Reagents

Zearalenone (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), CypExpress Cytochrome P450 (CYP1A2, 2C9, 2C19, 2D6, and 3A4) human kits, α-naphthoflavone, ticlopidine hydrochloride, testosterone, 6β-hydroxytestosterone, ketoconazole, and quinidine were purchased from Merck (Darmstadt, Germany). Diclofenac, 4′-hydroxydiclofenac, sulfaphenazole, (*S*)-mephenytoin, 4-hydroxymephenytoin, dextromethorphan, and dextrorphan were obtained from Carbosynth (Berkshire, UK). Nicotinamide adenine dinucleotide phosphate sodium salt (NADP$^+$) and glucose-6-phosphate barium salt (G6P) were from Reanal (Budapest, Hungary). Pyranine, sulforhodamine 101 (SR101), and further chemicals, if not stated otherwise, were purchased from Merck (Darmstadt, Germany).

2.2. Testing the inhibitory effects of ZEN and ZELs on CYP enzymes

For CYP inhibition and mycotoxin depletion assays, stock solutions of mycotoxins (each 10 mM) were prepared in acetonitrile (HPLC grade; Molar Chemicals, Halásztelek, Hungary) and stored at −20 °C.

The impacts of ZEN and ZELs were examined on CYP2C9-catalyzed diclofenac hydroxylation (Mohos et al., 2020a), CYP2C19-catalyzed (*S*)-mephenytoin hydroxylation (Fliszár-Nyúl et al., 2019), CYP2D6-catalyzed dextromethorphan demethylation (Mohos et al., 2020b), and CYP3A4-catalyzed testosterone hydroxylation (Mohos et al., 2018) employing the previously reported assays with CypExpress Cytochrome P450 human kits (containing both CYP enzyme and NADPH generating system) without modifications. Quantitative analyses of both substrates and products were carried out by the HPLC-UV methods described earlier (Faisal et al., 2021).

To examine the inhibitory effects of ZEN and ZELs on CYP1A2 enzyme (Fliszár-Nyúl et al., 2023), the Fluorometric CYP1A2 Inhibitor Assay Kit (ab211075; Abcam, Cambridge, UK) was used exactly following the manufacturer's description.

2.3. Mycotoxin depletion assays

In mycotoxin depletion assays, the time-dependent decrease in ZEN and ZEL concentrations was examined in the presence of CYP1A2, 2C9, 2C19, 2D6, and 3A4 enzymes. Samples contained ZEN or ZEL (5 μM), G6P (500 μM), NADP$^+$ (200 μM), and CypExpress Cytochrome P450 reagent (15 mg/mL) in 200 μL final volume of potassium phosphate
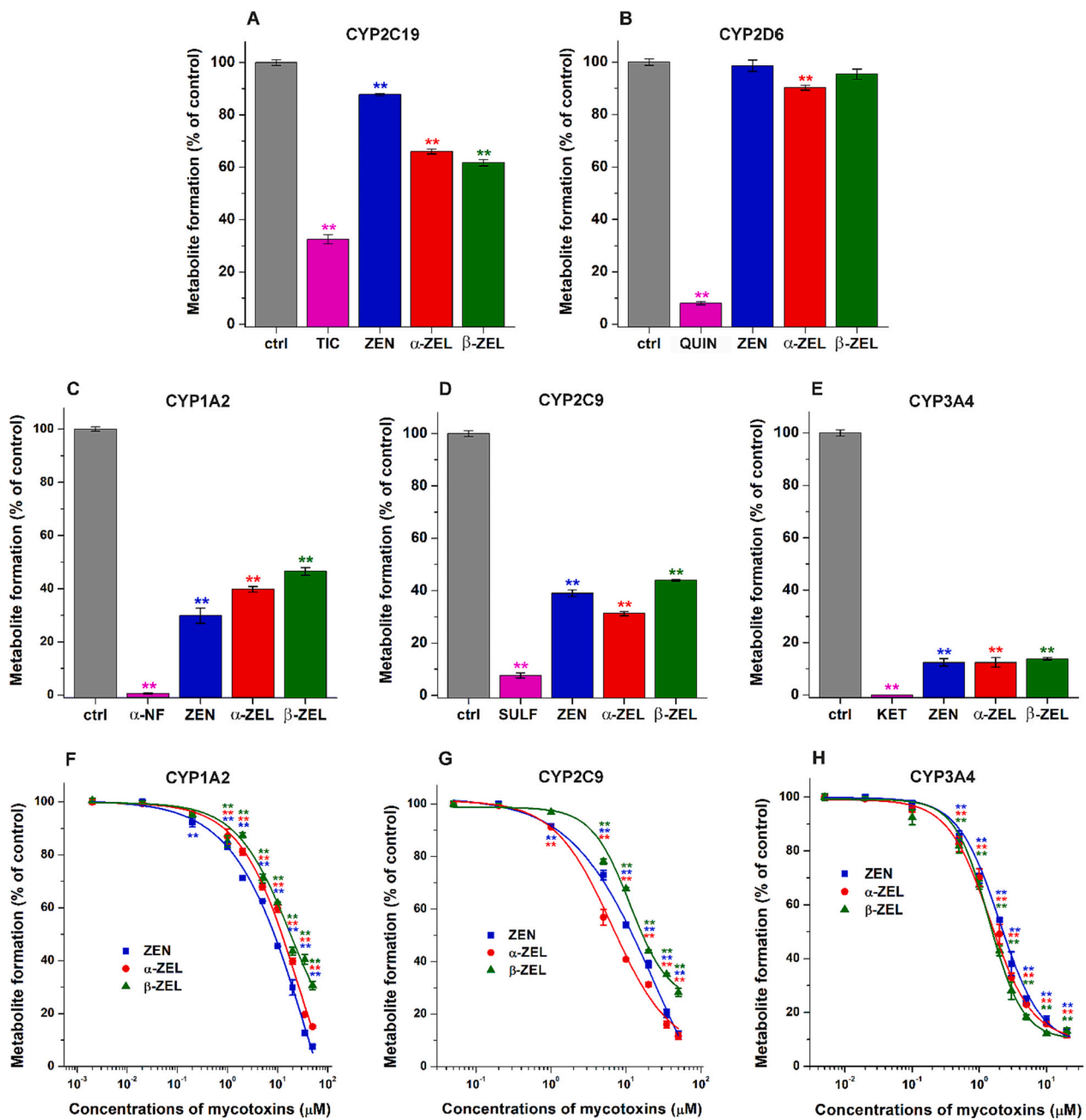
Fig. 2. Effects of ZEN (blue), α-ZEL (red), β-ZEL (green), and positive control inhibitors (magenta) (each 20 μM) on CYP2C19 (**A**), CYP2D6 (**B**), CYP1A2 (**C**), CYP2C9 (**D**), and CYP3A4 (**E**) (TIC, ticlopidine; QUIN, quinidine; α-NF, α-naphthoflavone; SULF, sulfaphenazole; KET, ketoconazole). Concentration-dependent impacts of ZEN and ZELs (0–50 μM) on CYP1A2 (**F**), CYP2C9 (**G**), and CYP3A4 (**H**) enzymes (data represent means ± SEM; *n* = 3; ** *p* < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Inhibitory effects (IC$_{50}$ ± SEM) of ZEN, α-ZEL, and β-ZEL on CYP1A2, CYP2C9, and CYP3A4 enzymes.

Mycotoxin	CYP1A2 IC$_{50}$ (μM)	CYP2C9 IC$_{50}$ (μM)	CYP3A4 IC$_{50}$ (μM)
ZEN	7.84 ± 1.09	9.51 ± 1.21	2.01 ± 0.10
α-ZEL	9.09 ± 1.14	6.07 ± 0.49	1.44 ± 0.10
β-ZEL	> 10.0	> 10.0	1.38 ± 0.08

buffer (0.05 M, pH 7.5), and were incubated for 0, 60, and 120 min in a thermomixer at 700 rpm and 30 °C (Fliszár-Nyúl et al., 2023). After the reaction was stopped with 100 μL of ice-cold methanol, the incubates

were centrifuged for 10 min at 14,000 g and room temperature. Myco-toxin levels in the supernatants were quantified with the previously reported HPLC-FLD method (Poór et al., 2018). Statistical differences were evaluated compared to the incubates that contained CypExpress Control reagent (CYPnull; 15 mg/mL), which was produced by the manufacturer using an empty vector and processed identically to the CypExpress Cytochrome P450 products.

2.4. OATP inhibition assays

A431 cells overexpressing OATP1A2, OATP1B1/3, or OATP2B1 and their mock transfected controls were generated, as has been previously reported (Patik et al., 2018; Bakos et al., 2020). Cells were cultured in
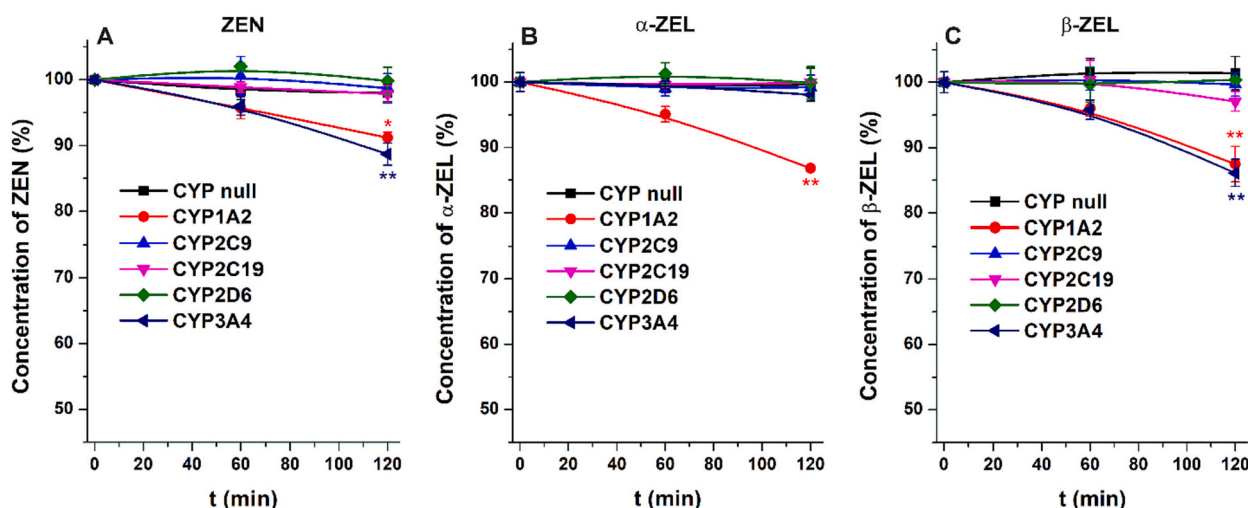
Fig. 3. Mycotoxin depletion assays. The concentrations (% of control ± SEM) of ZEN and ZELs in the solution after 0, 60, and 120 min incubation with CYP enzymes. Effects of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 enzymes on ZEN (**A**), α-ZEL (**B**), and β-ZEL (**C**) in the presence of the different CYP isoenzymes (n = 3; *$p < 0.05$, **$p < 0.01$; initial concentrations of mycotoxins: 5 μM). Statistical differences were evaluated compared to the incubation with CYPnull under the same conditions.

Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermofisher Scientific, Waltham, MA, US), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO_2.

For OATP inhibition experiments, stock solutions of mycotoxins (each 10 mM) were prepared in dimethyl sulfoxide (spectroscopic grade; Fluka, Charlotte, NC, US) and stored at −20 °C. The inhibitory actions of ZEN, α-ZEL, and β-ZEL on OATP1A2, OATP1B1/3, and OATP2B1 were tested in uptake assays using sulforhodamine 101 (SR101; OATP1A2) or pyranine (OATP1B1/3 and OATP2B1) as fluorescent substrates (Patik et al., 2018; Bakos et al., 2020). A431 cells overexpressing OATP1A2, OATP1B1/3, or OATP2B1 and their mock transfected controls were seeded on 96-well plates at a density of 8×10^4 cells/well in 200 μL of cell culture medium one day prior to the transport measurements. The following day, the medium was removed, and the cells were washed three times with 200 μL phosphate-buffered saline (PBS, pH 7.4) and preincubated for 5 min at 37 °C with 50 μL uptake buffer (pH 5.5; 125 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 12 mM $MgSO_4$, 25 mM MES, and 5.6 mM glucose) with or without increasing concentrations of the mycotoxins tested. The uptake reaction was initiated with 50 μL of uptake buffer containing SR101 (0.5 μM in OATP1A2 assay) or pyranine (10 μM in OATP1B1 assay; 20 μM in OATP1B3 and OATP2B1 assays). After incubating the cells for 10 min (OATP1A2), 15 min (OATP1B1 and OATP2B1), or 30 min (OATP1B3) at 37 °C, the reaction was stopped by aspirating the supernatant and washing the cells three times with 200 μL of ice-cold PBS. The fluorescence of the cells was determined in 200 μL of PBS using an Enspire plate reader (PerkinElmer, Waltham, MA, US) at excitation/emission wavelengths of 586/605 nm (SR101) or 460/510 nm (pyranine). OATP-dependent transport was calculated by subtracting the fluorescence measured in mock control cells from that measured in OATP-expressing cells. The percent inhibition of each compound was determined by comparing the fluorescence of cells treated with mycotoxins to that of cells treated with the dye alone. All experiments were performed at least three times with three technical replicates.

2.5. Cytotoxicity assay

A431 cells overexpressing OATP1A2, OATP1B1/3, or OATP2B1 and their mock controls were seeded in 96-well plates in a density of 5000 cells/well. After 24 h, each well was supplemented with 100 μL of DMEM with or without 20 μM of ZEN, α-ZEL, or β-ZEL, after which the

cells were cultured at 37 °C and 5% CO_2 for 120 h. On the third day, the supernatant was discarded, and Presto Blue reagent solution (5%) was added to the cells. Following 60 min incubation at 37 °C, the fluorescence of the cells was measured at 555/585 nm excitation/emission using an Enspire plate reader (PerkinElmer, Waltham, MA, US). Cytotoxicity was quantified by comparing the fluorescence of non-treated control and mycotoxin-treated cells. Data represent the mean of three biological replicates.

2.6. Data analyses

Means ± standard error of the mean (SEM) values presented are at least from three independent experiments. Statistically significant differences ($p < 0.05$ and $p < 0.01$) were established based on the one-way ANOVA and Tukey's post-hoc tests employing SPSS Statistics software (IBM, Armonk, NY, US).

The IC_{50} values were determined with sigmoidal fitting (Hill1) using the Origin software (OriginLab Corporation, Northampton, MA, US).

3. Results

3.1. Inhibition of CYP enzymes by ZEN and ZELs

First, the impacts of high ZEN/ZEL concentrations (20 μM) were examined on CYP activity. Under these conditions, ZEN was a weak inhibitor of CYP2C19, and ZELs induced only 35–40% decrease in metabolite formation (Fig. 2A). ZEN and β-ZEL did not affect CYP2D6-catalyzed metabolite production, and α-ZEL caused statistically significant ($p < 0.01$) but slight (10%) inhibition (Fig. 2B). ZEN, α-ZEL, and β-ZEL induced approximately 70%, 60%, and 55% inhibition of CYP1A2 activity, respectively (Fig. 2C). Furthermore, the moderate inhibitory effects of the mycotoxins (55–70%) were observed on CYP2C9 enzyme (Fig. 2D), where α-ZEL showed the strongest impact. Moreover, ZEN and ZELs caused almost 90% inhibition of CYP3A4 activity (Fig. 2E).

Since ZEN and ZELs showed no or only weak effects on CYP2C19 and CYP2D6, the concentration-dependent (0–50 μM) inhibitory actions of these mycotoxins were tested on CYP1A2, CYP2C9, and CYP3A4 enzymes. Mycotoxins typically caused statistically significant ($p < 0.01$) decrease in metabolite formation at nanomolar or low micromolar concentrations (Fig. 2F, G, and H), except β-ZEL, which produced a significant impact on CYP2C9 activity only at 5 μM. ZEN and α-ZEL induced approximately 90% decreases in CYP1A2- and CYP2C9-
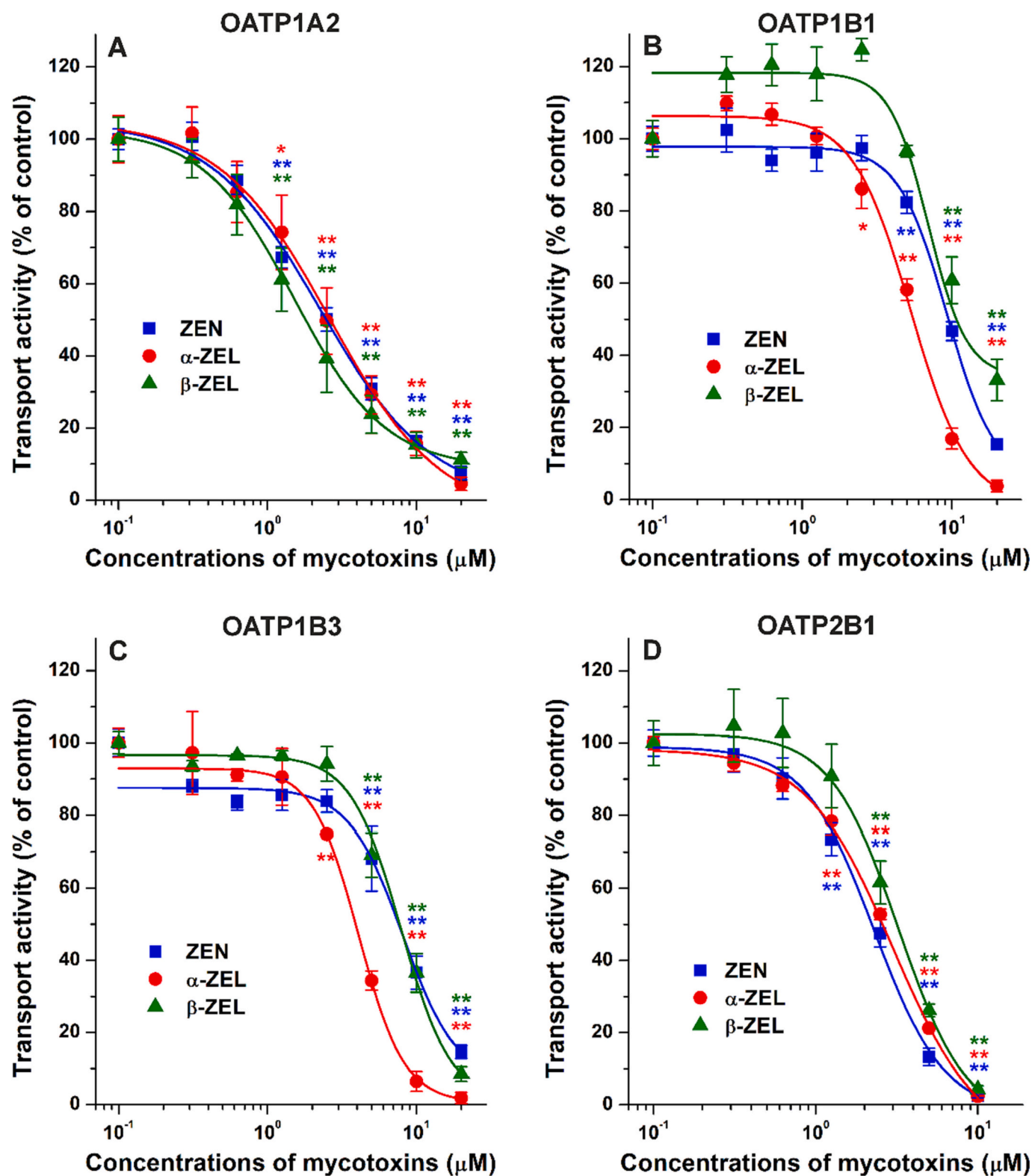
Fig. 4. Concentration-dependent inhibitory effects of ZEN and ZELs (0–20 μM) on the transport activity of OATP1A2 (**A**), OATP1B1 (**B**), OATP1B3 (**C**), and OATP2B1 (**D**) in A431 cells. OATP activity was measured using SR101 (OATP1A2) or pyranine (OATP1B1/3 and OATP2B1) as test substrates (see further experimental details in Section 2.4). The mean (% of control ± SEM) values are presented, where the fluorescence with the dyes alone was set as 100% ($n = 3$; *$p < 0.05$, **$p < 0.01$).

catalyzed metabolite formation at 50 μM (Fig. 2F and G). Nevertheless, even in the presence of 50 μM concentration of β-ZEL, approximately 70% inhibition of CYP1A2 and CYP2C9 was noticed. ZEN, α-ZEL, and β-ZEL caused close to complete inhibition of CYP3A4 even at 20 μM (Fig. 2H).

The IC$_{50}$ values determined are listed in Table 1. The lowest IC$_{50}$ values were presented by ZEN, α-ZEL, and β-ZEL on CYP3A4 enzyme (IC$_{50}$ = 1–2 μM), followed by ZEN and α-ZEL on CYP1A2 and CYP2C9 (IC$_{50}$ = 6–10 μM), and then β-ZEL on CYP1A2 and CYP2C9 (IC$_{50}$ > 10 μM).

Table 2
Inhibitory effects (IC$_{50}$ ± SEM) of ZEN, α-ZEL, and β-ZEL on OATPs (1A2, 1B1, 1B3, and 2B1).

Mycotoxin	OATP1A2 IC$_{50}$ (μM)	OATP1B1 IC$_{50}$ (μM)	OATP1B3 IC$_{50}$ (μM)	OATP2B1 IC$_{50}$ (μM)
ZEN	2.28 ± 0.26	> 10.0	8.10 ± 2.26	2.30 ± 0.13
α-ZEL	2.63 ± 0.29	5.30 ± 0.48	4.10 ± 0.16	3.00 ± 0.17
β-ZEL	1.51 ± 0.05	6.94 ± 1.77	7.89 ± 1.22	3.24 ± 0.09

3.2. Mycotoxin depletion assays

In the following experiments, we examined the involvement of CYP enzymes in the biotransformation of ZEN and ZELs. Mycotoxins were incubated for 0, 60, and 120 min with CYP1A2, 2C9, 2C19, 2D6, and 3A4 enzymes, and then the concentrations of ZEN or ZELs were determined. As the basis of comparison, CYPnull (produced using an empty vector under the same conditions as CypExpress CYP reagents) was also applied. CYP2C9, CYP2C19, and CYP2D6 did not affect the levels of ZEN and ZELs in the incubates. Both CYP1A2 and CYP3A4 caused time-dependent decrease in the concentrations of ZEN and β-ZEL (Fig. 3A and C). However, only CYP1A2 was able to reduce α-ZEL levels in the incubates (Fig. 3B).

3.3. Inhibition of OATPs by ZEN and ZELs

The inhibitory effects of ZEN, α-ZEL, and β-ZEL on the activity of multispecific OATPs were investigated in A431 cells engineered to overexpress one of the OATPs (1A2, 1B1, 1B3, or 2B1) and in their mock-transfected controls. Fluorescent dye uptake was examined using SR101 (in OATP1A2 assay) or pyranine (in OATP1B1/3 and OATP2B1 assays) test substrates validated earlier to identify OATP inhibitors (Patik et al., 2018; Bakos et al., 2020). ZEN, α-ZEL, and β-ZEL exerted inhibitory actions on each OATP tested (Fig. 4), their IC$_{50}$ values were typically in the 1–10 μM range (Table 2). ZEN (20 μM) caused 80–100% decrease in OATP-mediated dye uptake (Fig. 4), and it showed low micromolar (approximately 2 μM) IC$_{50}$ values for OATP1A2 and OATP2B1 (Table 2). α-ZEL (20 μM) almost completely abolished the transport activity of the OATPs examined, exerting its strongest inhibitory effects on OATP1A2 (IC$_{50}$ = 2.6 μM) and OATP2B1 (IC$_{50}$ = 3.0 μM). At 20 μM, β-ZEL caused 70% inhibition of OATP1B1 activity, while approximately 90% (OATP1A2 and OATP1B3) or even higher (OATP2B1) inhibitory effects were observed on the other OATPs tested (Fig. 4).

3.4. Effect of ZEN and ZELs on the viability of OATP-overexpressing vs. mock-transfected cells

By mediating the increased accumulation of certain toxic compounds, the activity of OATPs may result in elevated toxicity (Windt et al., 2019; Ungvári et al., 2023). To examine the potential involvement of OATPs in ZEN/ZEL-induced toxicity, the impacts of ZEN, α-ZEL, and β-ZEL on the viability of OATP-expressing and mock control cells were tested. Considering that higher levels of ZEN and ZELs can result in more dominant non-specific passive uptake, we applied 20 μM concentrations of the mycotoxins. Neither ZEN nor α-ZEL exhibited relevant cytotoxic effects on the mock control cells, while β-ZEL caused a moderate decrease in cell viability (Fig. 5). In A431 cells overexpressing OATPs, we did not observe statistically significant (p < 0.05) differences in cell viability compared to the mock controls.

4. Discussion

CYP enzymes and/or OATP transporters have major roles in the toxicokinetics of several xenobiotics, including certain mycotoxins like alternariol, aflatoxin B1, and ochratoxin A (Rushing and Selim, 2019; Wang et al., 2020; Fliszár-Nyúl et al., 2023). Nevertheless, the interactions of ZEN and ZELs with these proteins have been barely characterized. Therefore, using *in vitro* models, we examined the inhibitory effects of ZEN, α-ZEL, and β-ZEL on CYP enzymes and on OATP transporters, the CYP-mediated depletion of these mycotoxins, and the potential impacts of OATPs on the ZEN/ZEL-induced cytotoxicity.

ZEN showed slight or no inhibitory effects on CYP2C19 and CYP2D6, and it considerably decreased CYP1A2, CYP2C9, and CYP3A4 activity (Fig. 2 and Table 1). In an earlier *in vitro* study, ZEN did not inhibit CYP1A-catalyzed phenacetin deethylation, slightly inhibited CYP2D-catalyzed dextromethorphan demethylation, and strongly decreased CYP2C-catalyzed tolbutamide hydroxylation and CYP3A-catalyzed midazolam hydroxylation (Schelstraete et al., 2019). Nevertheless, our results (Fig. 3) and an earlier study (Pfeiffer et al., 2009) demonstrated the involvement of CYP1A2 in the metabolism of ZEN, supporting our observation that ZEN is both a substrate and an inhibitor of CYP1A2. Based on our current knowledge, no data are available regarding the inhibitory actions of α-ZEL and β-ZEL on CYP enzymes. ZELs induced considerably stronger impacts on CYP2C19 than ZEN (Fig. 2A); however, even ZELs proved to be only weak inhibitors of this enzyme. Similar to ZEN, ZELs showed strong inhibitory effects on CYP3A4 (IC$_{50}$ = 1–2 μM), while they were moderate inhibitors of CYP1A2 and CYP2C9
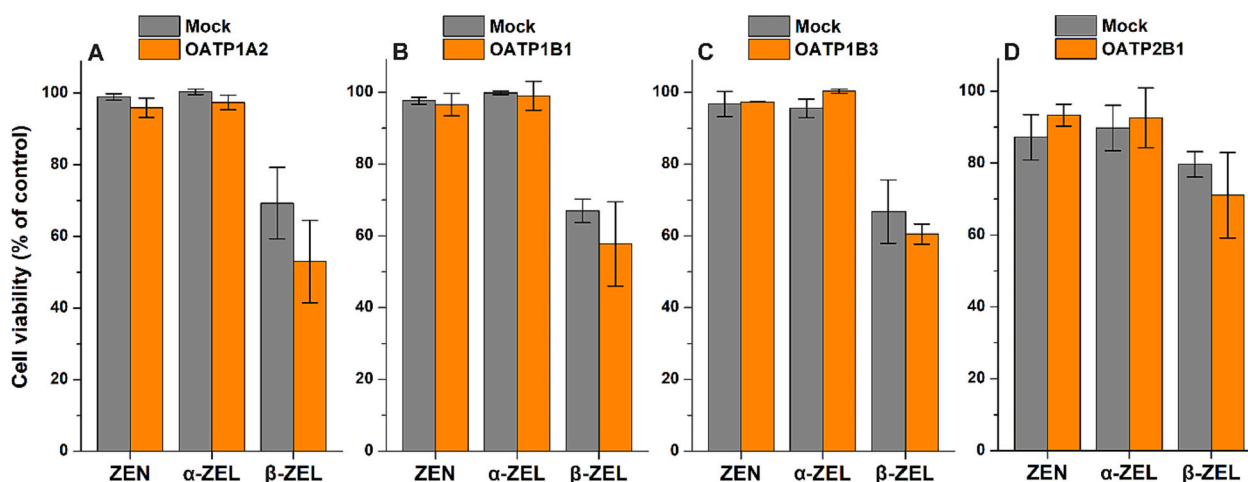

Fig. 5. Effects of ZEN and ZELs (each 20 μM) on the viability of OATP-overexpressing A431 cells *vs.* the mock controls. Cells transfected with OATP1A2 (**A**), OATP1B1 (**B**), OATP1B3 (**C**), or OATP2B1 (**D**), as well as mock-transfected controls, were plated in 96-well plates (5000 cells/well). After 24 h, the cells were treated with ZEN, α-ZEL, or β-ZEL (each 20 μM) in cell culture medium for 120 h. The influence of mycotoxins on cell viability (% of control ± SEM; n = 3) was assessed using a PrestoBlue-based cytotoxicity assay.

([Table 1](#)). Since the repeated *per os* treatment of pigs with ZEN (for two weeks, with doses equal to the maximal guidance levels) led to the increased C_{max} and AUC of midazolam (*per os*, single dose) (Schelstraete et al., 2019), it is reasonable to hypothesize that the high exposure to ZEN may affect the CYP3A4-mediated metabolism of certain drugs. In addition, the reduced metabolites of ZEN likely take part in the enzyme inhibition. On the other hand, previous studies demonstrated that ZEN is a CYP inducer (Ayed-Boussema et al., 2011; Duca et al., 2012; European Food Safety Authority (EFSA), 2017), which can cause a counter-regulation during the chronic exposure to the mycotoxin. Therefore, further *in vivo* studies are required for the proper evaluation of ZEN/ZEL-CYP3A4 interactions.

Based on the mycotoxin depletion experiments, CYP1A2 takes part in the biotransformation of ZEN, α-ZEL, and β-ZEL ([Fig. 3](#)). Furthermore, the concentrations of ZEN and β-ZEL were also decreased during their incubation with CYP3A4, while α-ZEL levels were not affected by the latter CYP enzyme. A previous report examined the CYP-catalyzed hydroxylation of ZEN and α-ZEL in human microsomes, where significant involvement of CYP1A2 and the lower importance of CYP3A4 have been described (Pfeiffer et al., 2009). In another study with human microsomes, CYP2C8 and CYP3A4/5 supported the formation of hydroxylated ZEN metabolites, while these derivatives were not detected after the incubation with CYP1A2 (Bravin et al., 2009). Some of these results are controversial; however, considering the data available, it is reasonable to hypothesize that CYP1A2 and/or CYP3A4 are the most important CYP enzymes in the oxidative biotransformation of ZEN, α-ZEL, and β-ZEL. Due to the CYP-catalyzed formation of catechol then quinone metabolites, these reactions may have high importance in the genotoxic impacts of ZEN (Fleck et al., 2012).

As it has been reported, ZEN and α-ZEL can inhibit organic anion and cation uptake mediated by human organic anion transporters (OAT1–4) and organic cation transporters (OCT1–2), respectively (Tachampa et al., 2008). Furthermore, the direct transport of ZEN, α-ZEL, and β-ZEL by ABCC1, ABCC2, and ABCC3 efflux transporters has also been described (Videmann et al., 2009). In another study, ZEN was identified as a substrate/inhibitor of the ABCG2 transporter (Xiao et al., 2015). The multispecific OATPs (1A2, 1B1, 1B3, and 2B1) are crucial determinants of pharmacokinetics by regulating the ADME of their drug substrates (Kovacsics et al., 2017) and have been identified as victims of drug-drug and food-drug interactions (Kalliokoski and Niemi, 2009; Kaci et al., 2023). Our study demonstrated for the first time that ZEN, α-ZEL, and β-ZEL can decrease the transport activity of OATP1A2, 1B1/3, and 2B1 ([Fig. 4](#)). Furthermore, we found that ZEN and ZELs can be considered as relatively strong inhibitors of OATP1A2 and OATP2B1 ($IC_{50} = 1.5–3.2$ μM). Based on their IC_{50} values ([Table 1](#)), ZEN and α-ZEL display comparable inhibitory activity towards OATP1A2 and OATP2B1, while β-ZEL emerges as the most effective on OATP1A2 transport ($IC_{50} = 1.5$ μM). Furthermore, ZEN and ZELs were moderate inhibitors of OATP1B1 and OATP1B3 ([Table 1](#)). In humans, the total plasma concentrations of ZEN, ZELs, and their glucuronide conjugates are from the nanomolar to the low micromolar range (Pillay et al., 2002). Since glucuronic acid conjugates are the dominant forms in the circulation (European Food Safety Authority (EFSA), 2017), it is unlikely that ZEN or ZELs can significantly affect the OATP-mediated tissue uptake of drugs. Nevertheless, the high exposure to ZEN may result in considerably higher levels of the mycotoxin in the intestinal tract compared to the circulation, leading to its potential inhibitory effect on OATP2B1-mediated drug absorption. OATPs did not modify the cytotoxic impacts of ZEN and ZELs (each 20 μM) in A431 cells ([Fig. 5](#)). Nevertheless, this observation does not prove the absence of OATP-mediated uptake of these mycotoxins. Therefore, additional experiments are required to further characterize the potential direct uptake of ZEN and ZELs by OATPs.

5. Conclusions

In summary, the interactions of ZEN, α-ZEL, and β-ZEL with CYP (1A2, 2C9, 2C19, 2D6, and 3A4) enzymes and OATPs (1A2, 1B1, 1B3, and 2B1) were examined employing *in vitro* models. ZEN and ZELs showed relatively strong inhibitory effects of CYP3A4, they were moderate inhibitors of CYP1A2 and CYP2C9, and they caused weak or negligible impacts on CYP2C19 and CYP2D6. Based on enzyme depletion experiments, ZEN and ZELs are inhibitors but not substrates of CYP2C9. Furthermore, CYP1A2 and CYP3A4 take part in the metabolism of ZEN and β-ZEL, while only CYP1A2 is involved in the biotransformation of α-ZEL. ZEN and ZELs are relatively strong inhibitors of OATP1A2 and OATP2B1, and they produced moderate inhibitory effects on OATP1B1 and OATP1B3. Nevertheless, the viability of mock and OATP-overexpressing A431 cells did not show statistically significant differences after the treatment with 20 μM concentrations of ZEN or ZELs. The above-listed results promote the better understanding of the toxicokinetics and the potential pharmacokinetic interactions of ZEN and ZELs.

CRediT authorship contribution statement

Hana Kaci: Writing – original draft, Investigation, Formal analysis. **Ágnes Dombi:** Investigation, Formal analysis. **Patrik Gömbös:** Investigation, Formal analysis. **András Szabó:** Methodology, Investigation, Funding acquisition. **Éva Bakos:** Investigation, Formal analysis. **Csilla Özvegy-Laczka:** Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Conceptualization. **Miklós Poór:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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