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DECHLORINATION AND DEMETHYLATION OF OCHRATOXIN A ENHANCE BLOCKING ACTIVITY OF PXR ACTIVATION, SUPPRESS PXR EXPRESSION AND REDUCE CYTOTOXICITY

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Abstract

The pregnane X receptor (PXR) has been established to induce chemoresistance and metabolic diseases. Ochratoxin A (OTA), a mycotoxin, decreases the expression of PXR protein in human primary hepatocytes. OTA is chlorinated and has a methylated lactone ring. Both structures are associated with OTA toxicity. The study was to test the hypothesis that structural modifications differentially impact PXR blocking activity over cytotoxicity. To test this hypothesis, OTA-M and OTA-Cl/M were synthesized. OTA-M lacked the methyl group of the lactone-ring, whereas OTA-Cl/M had neither the methyl group nor the chlorine atom. The blocking activity of PXR activation was determined in a stable cell line, harboring both PXR (coding sequence) and its luciferase element reporter. OTA-Cl/M showed the highest blocking activity, followed by OTA-M and OTA. OTA-Cl/M was 60 times as potent as the common PXR blocker ketoconazole based on calculated IC₅₀ values. OTA-Cl/M decreased by 90% the expression of PXR protein and was the least cytotoxic among the tested compounds. Molecular docking identified that OTA and its derivatives interacted with different sets of residues in PXR, providing a molecular basis for selectivity. Excessive activation of PXR has been implicated in chemoresistance and metabolic diseases. Downregulation of PXR protein expression likely delivers an effective mechanism against structurally diverse PXR agonists.

Keywords

Ochratoxin A; Mycotoxin; Ochratoxin A Derivatives; Pregnane X receptor; Chemoresistance; Metabolic diseases

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Conflict of interest

None

Declaration of interests

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.

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1. INTRODUCTION

All organisms including humans have developed defensive systems against chemical insults (Bolaji et al., 2019; Rekka et al., 2019; Shen et al., 2019; Waring, 2019). These systems consist of drug metabolizing enzymes and transporters. The expression of these genes is regulated, primarily at the level of transcription. The pregnane X receptor (PXR, NR1I2) is established as a master transcription factor that supports induced expression of these genes (Ihunnah et al., 2011; Pavék and Smutný, 2014; Chai et al., 2019). Structurally, PXR belongs to the nuclear hormone receptor superfamily (Zhang et al., 1999; Carnahan and Redinbo, 2005; Yoshinari, 2019). Like other nuclear receptors, PXR consists of a variable N-terminal domain, a highly conserved DNA-binding domain, a hinge region and a multifunctional C-terminal ligand-binding domain. The DNA-binding domain recognizes conical sequence AGG/TTCA (Carnahan and Redinbo, 2005; Yoshinari, 2019). The major portion of the ligand-binding domain is helical in structure, and the C-terminal helix (helix 12) is directly involved in switching from repressing to activating status of a target gene (Pavék, 2016; Fischer and Smieško, 2019). Binding to an agonist induces conformational changes of this helix, leading to a platform favoring association with coactivators, namely transactivation of target genes (Fischer and Smieško, 2019).

Activation of PXR has pharmacological, pathological and toxicological significance (Ihunnah et al., 2011; Chai et al., 2019; Yoshinari, 2019). Induction of drug-metabolizing enzymes and transporters via PXR determines the duration and intensity of drugs that are eliminated by these systems. For example, administration of the herbal antidepressant St John's wort, containing the potent PXR activator hyperforin, significantly increases the clearance of docetaxel (Goey et al., 2014). This chemotherapy drug is metabolized by cytochrome P450 3A4 (CYP3A4), a prototypic target of PXR (Maekawa et al., 2010). Furthermore, the expression of PXR is increased in a wide range of cancers (Miki et al., 2006; Chen et al., 2007; Hodnik et al., 2014). In addition to drug-drug interactions, excessive activation of PXR is an important factor contributing to metabolic diseases such as hepatic steatosis and diabetes (Ihunnah et al., 2011; Hakkola et al., 2016). PXR agonists significantly elicit postprandial hyperglycemia (Zhou et al., 2006; Hukkanen et al., 2014; Banerjee et al., 2015). In human hepatocytes, PXR agonists significantly induce lipid retention (Moya et al., 2010). Activation of PXR increases liver toxicity of acetaminophen (i.e., bioactivation), signifying the toxicological involvement of PXR in commonly used drugs (Cheng et al., 2009).

There is a need to develop PXR blockers to control metabolic diseases, to increase therapeutic efficacy, and in some cases to protect against bioactivation (Cheng et al., 2009; Li et al., 2013; Mani et al., 2013; Mooiman et al., 2014; Xu et al., 2014). Indeed, some PXR blockers have been described in the literature (Mani et al., 2013; Mooiman et al., 2014). These blockers are structurally diverse and usually have other pharmacological targets. For example, both metformin and ketoconazole are PXR antagonists (Mani et al., 2013; Mooiman et al., 2014). Metformin is a noncyclic compound and used to treat type II diabetes (Krausová et al., 2011). Ketoconazole, on the other hand, belongs to the class of imidazole heterocyclic compounds with potent antifungal activity (Lim et al., 2009).

Importantly, these compounds interact with their pharmacological targets at much lower concentrations than with PXR. As a result, their clinical use, as PXR blockers, has relatively limited efficacy. Indeed, clinical trials have demonstrated that therapeutic doses of ketoconazole cause little changes in PXR-mediated induction of CYP3A4 (Fuchs et al., 2013). Recently, Lin et al have reported that SPA70 selectively antagonizes PXR activation with a relatively high potency (Lin et al., 2017). This synthetic compound enhances the interaction between PXR and co-suppressor, pointing to the classic action of antagonists toward nuclear receptors.

Ochratoxin A (OTA) is a mycotoxin and present in a wide variety of food commodities including cereal, coffee and grape juice (Yeung et al., 2020). It has been reported that OTA, although there are exceptions (Zlender et al., 2009; Ayed-Boussema et al., 2012), downregulates a large number of drug-metabolizing enzymes and transporters (Stemmer et al., 2007; Vettorazzi et al., 2013; Marin-Kuan et al., 2006; Vettorazzi et al., 2019). In human primary hepatocytes, this mycotoxin has been shown to downregulate the expression of PXR protein (Doricakova and Vrzal, 2015). The downregulation is presumably achieved by inducing miR-148a, a microRNA that targets the 5'-untranslated region of human PXR (Doricakova and Vrzal, 2015). Toxicologically, OTA induces oxidative stress and causes DNA damage, primarily through its metabolites. Structurally, OTA contains an amide, has a lactone ring and is chlorinated (Fig 1). The amide bond undergoes hydrolysis and contributes to its rapidly metabolic elimination (Wu et al., 2011). The lactone ring is attached with a methyl moiety and also undergoes hydrolysis. However, the hydrolytic metabolite of the lactone is even more toxic than the parent compound, when injected into rats intravenously (Wu et al., 2011). The chlorine atom, on the other hand, is required for genotoxicity (Dai et al., 2003; Tozlovanu et al., 2006; Hadjeba-Medjdoub et al., 2012; Sharma et al., 2014; K szegi and Poór, 2016). Oxidative dechlorination of OTA leads to the formation of aryl radical, and glutathione is critical for neutralizing this free radical and yielding ochratoxin B with much lower toxicity (Fig.1).

The aim of the present study was to examine whether structural modifications selectively enhance PXR blocking activity and reduce cytotoxic potential. To test this possibility, two OTA derivatives were synthesized: OTA-M and OTA-Cl/M. OTA-M lacked the methyl group of the lactone-ring, whereas OTA-Cl/M had neither the methyl group nor the chlorine atom. To gain molecular insight into the blocking activity, a stably transfected cell line, harboring both PXR (coding sequence) and its luciferase element reporter, was used for the transactivation assay. Among OTA and its derivatives, OTA-Cl/M showed the highest blocking activity, at 60 times as potent as ketoconazole based on calculated IC₅₀ values. OTA-Cl/M decreased by 90% the expression of PXR protein, and was the least cytotoxic among OTA compounds. Molecular docking showed that OTA and its derivatives interacted with different sets of residues in PXR, and some of them also supported the interaction with SAP70 (Lin et al., 2017). The enhancement of PXR blocking activity and reduction of cytotoxicity, as shown by OTA-Cl/M, suggest that these two functional events can be structurally separated. Excessive activation of PXR has been implicated in chemoresistance and metabolic diseases (Ihunnah et al., 2011; Chai et al., 2019; Yoshinari, 2019). Downregulation of PXR protein expression likely delivers an effective mechanism against structurally diverse PXR agonists.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Ketoconazole, OTA, rifampicin (RIF) and anti-Flag antibody were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals had a purity of 98%. Dulbecco's modified eagle medium (DMEM) was from Life Technology (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone (GE Healthcare Bio-Sciences, Pittsburgh, PA). The luciferase kit was from Promega (Madison, WI). The goat anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce (Rockford, IL). Nitrocellulose membranes were from Bio-Rad (Hercules, CA). Unless otherwise specified, all other reagents were purchased from ThermoFisher Scientific (Fair Lawn, NJ).

2.2. Synthesis of OTA derivatives

Synthesis of OAT-Cl/M was completed with four major steps as outlined in Fig. 2A. Step 1: to a solution of lithium diisopropylamide (LDA, 2 eq) in anhydrous tetrahydrofuran (THF, 30 mL) under nitrogen cooled to -78°C was added dropwise a solution of dimethyl-2-hydroxyl-4-methylbenzene-1,3-dicarboxylate (2.0 g) in anhydrous THF (5 mL) at -78°C . After stirring for 20 min, paraformaldehyde (3 eq) was added dropwise at -78°C . The reaction was stirred at the same temperature for 30 min then warmed up to 0°C for 1 h. The reaction was quenched by adding acetic acid (1 mL), and then warmed to room temperature. The reaction was diluted with 2M HCl solution and extracted with ethyl acetate. The combined organic extracts were washed with water, brine, dried over Na_2SO_4 and concentrated. The residue was purified by silica chromatography to afford 468 mg (24%) of desired product 2. Step 2: Ester 2 (200 mg) obtained above was dissolved in MeOH (10 mL), to this solution was added LiOH (40 mg). The reaction was stirred at room temperature overnight to afford carboxylic acid 3 (100%), which was used without additional purification. Steps 3–4: Carboxylic acid 3 (100 mg) obtained above and L-phenylalanine tert-butyl ester (2 eq.) were dissolved in anhydrous N,N-dimethylformamide (3 mL), to this solution was added diethyl isopropyl ethyl amine (3 eq) followed by 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (2 eq) at 0°C . The reaction was stirred at room temperature for 2 h before quenched with water, and extracted with ethyl acetate. The combined organic extracts were washed with water, brine, dried over Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography to afford 120 mg of Boc-ester 4, which was dissolved in anhydrous CHCl_3 (2 mL). To this solution was added trifluoroacetic acid (1 mL). The reaction was stirred at room temperature for 2 h before ether was added to precipitate OTA-Cl/M (80 mg) as white solids.

Synthesis of OTA-M, as outlined in Fig. 2B, started with product 2 obtained above (Fig. 2A). Ester 2 (200 mg) was dissolved in anhydrous dichloromethane (10 mL). To this solution was added SOCl_2 (3 eq) at 0°C . The reaction was then warmed up to room temperature and stirred overnight. Upon completion, the reaction was concentrated under reduced pressure and the resulting residue was purified by silica gel chromatography to afford intermediate 5 (150 mg) as white solids. OAT-M was then prepared from 5, following similar procedure (steps 2–4 described in Fig. 2A). The chemical identities of the derivatives

were confirmed by mass- and NMR- (nuclear magnetic resonance) spectrometric analyses. Liquid chromatograph mass spectrometry (LCMS) was performed with HP 1110 Agilent LCMS using a quaternary G1311A pump coupled to a Micromass Platform LCZ detector. The gradient mobile phase consisted of 5 to 95% acetonitrile (v/v) over a period of time (10 min). ¹H NMR spectra were recorded on a Bruker UltraShield 400 MHz spectrometer. It should be noted that all chemicals used for the synthesis were purchased from Sigma-Aldrich unless otherwise specified. The purity was assessed to be at least 98% based on the analysis with high performance liquid chromatography with photodiode-array detection at different amount of synthesized compounds. The HPLC analysis was described previously (Tang et al. 2006).

2.3. Blocking of PXR activation

The activation of human PXR was performed in a stable line (hPXR-HRE) constitutively expressing human PXR and harboring a PXR element luciferase reporter (Song et al., 2004). This stable transfection was made in HEK293T cells (human embryonic kidney 293 cell line). This line was maintained in DMEM medium supplemented with 10% FBS and antibiotics (Zeocin, 300 µg/ml; blasticidin, 5 µg/ml). To determine the blocking activity of PXR activation, the hPXR-HRE cells were plated in 96-well plates at a confluence of 80%. After an overnight incubation, the cells were treated with RIF (10 µM) in the presence or absence of OTA or a derivative for 24 h in 10% delipided FBS without antibiotics. OTA or a derivative was tested at various concentrations (0–1 µM). These compounds were also tested for their effect on the basal activation (in the absence of RIF). As a control, ketoconazole (0–20 µM) was included for the basal and RIF-stimulated experiments. Cell lysates were then prepared and analyzed for luciferase activity. The signal was normalized with the amount of protein.

2.4. Western blotting and immunofluorescence staining

Cell lysates (5 µg) were resolved by 7.5% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) in a mini-gel apparatus and transferred electrophoretically to nitrocellulose membranes. After non-specific binding sites were blocked with 5% non-fat milk, the blots were incubated with the anti-Flag antibody. The primary antibody was localized with goat anti-rabbit IgG conjugated with horseradish peroxidase. Horseradish peroxidase activity was detected with a chemiluminescent kit (SuperSignal West Pico). The chemiluminescent signal was captured by Carestream 2200 PRO Imager (Shi et al., 2011). The immunofluorescence staining was performed as described previously (Marczak and Yan, 2017). hPXR-HRE cells were seeded in chamber slides at 40–80% confluence and cultured overnight. Media were replaced with fresh media containing OTA (1 µM), a derivative (1 µM), ketoconazole (10 µM) or DMSO. After an additional 24-h incubation, cells were washed with ice cold PBS and then fixed with 4% Paraformaldehyde for 10 min at pH 7.4. Cells were washed 3 times with PBS and permeabilization solution (0.1% Triton X-100) was added for 10 min. Chamber slides were incubated with 1% BSA (bovine serum albumin, 2 mg/mL) for 1 h to block nonspecific binding followed by incubation in the anti-FLAG monoclonal antibody (1:1000 dilution with PBS) for 30 min. The slides were washed five times with PBS. Subsequently, the slides were incubated with fluorescent isothiocyanate-conjugated anti-mouse IgG antibody for an

additional 30 min. The cell nuclei were counterstained with DAPI (5 µg/mL). Cells were then imaged using confocal microscope.

2.5. Cytotoxicity assay

hPXR-HRE cells were seeded into 96-well plates at a density of 5,000 cells/well. The lower seeding density was used to ascertain the potential of cell proliferation toxicity. After an overnight incubation, cells were treated with OTA, a derivative or ketoconazole. OTA or a derivative was tested at various concentrations (0–100 µM), whereas ketoconazole at 0–20 µM. After the cells were treated for 24 h, the medium was replaced with fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at a final concentration of 0.5 mg/ml. After 2-h incubation at 37°C, the medium was gently decanted, and dimethyl sulfoxide (100 µl/well) was added to dissolve formazan product. The optical density (OD) was determined at 570 nm.

2.6. Molecular docking

To gain molecular insight into the differential PXR blocking activities among OTA and its derivatives, we performed molecular modeling and docking studies, essentially as described previously (Xiao et al., 2013). The structure of PXR (5X0R) was retrieved from the Protein Data Bank (www.pdb.org). Subunit B and D as well as the inhibitor were removed by UCSF Chimera 1.11.2 (Pettersen et al., 2004). Autodock4.2 was used for docking simulation as described previously (Takagi et al., 2008; Xiao et al., 2013). Briefly, SPA70, generated by ChemDraw Professional 16.0, was used as a control to test if the Autodock4.2 system is comparable to the published molecular modeling results (Lin et al., 2017). After verification, docking program for OTA and its derivatives with PXR were performed, and structural confirmations were selected through Autodock4.2 according to their binding energies. Finally, by adopting Discovery Studio Client®, the saved structural confirmations were visualized, superimposed with PXR, and analyzed for residue interactions.

2.7. Other analyses

Protein concentrations were determined with BCA assay (Thermo Scientific Pierce Protein Biology) based on albumin standard. Data are presented as mean ± SD of at least four separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Statistical analyses were performed with SPSS-PASW Statistics 18. Significant differences were tested according to One-way ANOVA followed by a DUNCAN's test for comparison of means. In all cases, statistical significance was indicated by an asterisk or a line when *p* values were less than 0.05 or 0.01.

3. RESULTS

3.1. Synthesis of and blocking of PXR activation by OTA and its derivatives

Structurally, OTA has a chlorinated benzene attached with a methylated lactone ring (Fig 1). These two structures are associated with OTA toxicity (Tozlovanu et al., 2006; Hadjeba-Medjdoub et al., 2012; Sharma et al., 2014). To test whether structural modifications on these structures selectively enhance PXR blocking activity and reduce cytotoxic potential, we synthesized two OTA derivatives: OTA-M (demethylated) and OTA-Cl/M (dechlorinated

and demethylated). These derivatives were confirmed by mass- and NMR- (nuclear magnetic resonance) spectrometric analyses. Fig. 3 shows the results on OTA-Cl/M. This derivative had a retention time of 3:33 min (Fig. 3A). This compound had a m/z ratio of 355.03 $[M-H]^-$ (Fig. 3B), consistent with the predicted mass of OTA-Cl/M. The structure was confirmed by 1H NMR (Fig. 3C). Their effect on the functionality of PXR was tested in the hPXR-HRE cell line, stably transfected to express human PXR (Flag-tagged coding sequence only) and harbor a PXR element luciferase reporter (Song et al., 2004). This cell model allowed the blocking activity of PXR to be defined within the coding region, the PXR protein and its functionality. RIF, a prototypical activator of human PXR, was used as the stimulant (Cheng et al., 2009).

As shown in Fig. 4A, OTA at 0.1 μM slightly increased the reporter activity at both basal and stimulated conditions. The basal activity was increased by 17%, whereas the RIF stimulated activity increased by 10%. Increased concentrations of OTA to 0.5 and 1 μM blocked both basal and RIF-stimulated activity by 45 and 60%, respectively. In contrast, both derivatives OTA-M and OTA-Cl/M, at all concentrations used, significantly blocked the reporter activity (Figs. 4B and C) and the blocking was more profound than OTA. OTA-Cl/M at 0.1 μM blocked the basal and RIF-stimulated activity by 25 and 45%, respectively. In contrast, ketoconazole at 20 μM , a PXR antagonist (Lim et al., 2009), 200 times of the concentration of OTA-Cl/M, blocked the reporter activity by 44% only (Figs. 4C and 4D). Among the compounds tested, the IC_{50} values were calculated to be 0.39 μM for OTA-Cl/M, 0.55 μM for OTA-M, 0.78 μM for OTA and 22.38 μM for ketoconazole.

3.2. Suppressed expression of PXR protein by OTA and its derivatives

Both basal and RIF-stimulated activations were decreased, pointing to the suppression of PXR expression. To test this possibility, hPXR-HRE cells were treated with OTA or a derivative at 1 μM and the expression of PXR was determined by Western blotting. Likewise, ketoconazole (10 μM) was included as a control. As shown in Fig. 5A, OTA and its derivatives (1 μM) all suppressed the expression of PXR by 80 to 90%. Consistent with the blocking activity (Fig. 4), OTA-Cl/M caused the highest decrease of PXR expression. In contrast, ketoconazole at 10 times of the concentration of OTA and its derivatives, caused only a slight decrease (Fig. 5A). The decrease was confirmed by immunofluorescence staining (Fig. 5B). The expression of PXR in the stable cell line was driven by the CMV promoter, suggesting a negligible role of transcriptional regulation in the downregulated expression unless the CMV promoter was the target.

3.3. Cytotoxicity of OTA and its derivatives

Next, we tested whether OTA derivatives are similar as OTA in inducing cytotoxicity (general cellular toxicity). Cells were treated with OTA and its derivatives at various concentrations: 1, 10, 20, 50 and 100 μM and cytotoxicity was determined with MTT assay. These concentrations were much higher than those for the reporter assay. As shown in Fig. 6A, OTA and its derivatives at 1 μM , the highest concentration used for the reporter assay, caused no changes in cytotoxicity. At 10 μM , OTA-M but not OTA or OTA-Cl/M caused significant cytotoxicity. At 20 μM , significant cytotoxicity was detected with OTA-M and OTA but not OTA-Cl/M. In contrast, ketoconazole at 1 μM caused significant cytotoxicity

(Fig. 6B). Interestingly, ketoconazole at 1 and 10 μM caused similar cytotoxicity but increased concentration to 20 μM significantly increased cytotoxicity. The precise mechanism remains to be determined. It is likely that this antifungal agent uses more than one mechanisms that operate in a different concentration threshold. Phase contrast imaging of hPXR-HRE cells (HEK293T) showed profound morphological changes of cells treated with ketoconazole at 20 μM for 6 h. Cells were rounded and the nuclei were condensed, typical manifestations of apoptotic changes (Fig. 6C). Ketoconazole, although widely used in clinical setting, has unfavorable toxicological profiles as a PXR blocker (Venkatesh et al., 2011; Fuchs et al., 2013). In contrast, cells treated with OTA or its derivatives were morphologically normal (Fig. 6C) at the assay time-point.

3.4. Molecular docking

Both OTA-M and OTA-Cl/M, compared with OTA, were more potent in blocking PXR activation. In contrast, they exhibited differential potencies in cytotoxicity. OTA-M was more while OTA-Cl/M was less cytotoxic than the parent compound. To gain molecular insight, we performed molecular docking study. As shown in Fig. 7 and Table I, OTA and its derivatives made contacts with PXR through Van der Waals force, hydrogen bond, pi-sigma and alkyl/pi-alkyl interactions. OTA-M and OTA-Cl/M made contacts with the same amino acids: Leu 308, Val211, Met243, Pro227, Leu239, Glu321, Leu324, Leu240, Met323, His407 and Leu411. However, there was a notable difference. Specifically, OTA-Cl/M made the contact with His407 through Van der Waals force, whereas OTA-M made the contact through the alkyl interaction (Table I and Fig. 7). OTA, on the other hand, made contacts with 9 instead of 11 residues: Leu 308, Val211, Met243, Glu321, Leu324, Leu240, Met323, His407 and Leu319. With the exception of Leu319, all residues contacted by OTA were also contacted by OTA-Cl/M and OTA-M. The residues contacted by OTA-M and OTA-Cl/M but not by OTA were: Pro227, Leu239 and Leu411. The docking study also identified 9 residues that interacted with SPA70, a recently reported PXR blocker (Lin et al., 2017). Interestingly, 6 of them were identified to interact with OTA and its residues.

OTA made contacts with PXR, largely through the benzopyran structure. The interaction occurred at a leucine-rich region including Leu 308, Leu319, Leu324, Glu321 and Met323. The methyl group on the pyran ring interacted with all leucine residues in this region, whereas chlorine atom on the benzen ring interacted with two of them: Leu 308 and Leu324. In addition, Met323 made a contact with the chlorine atom as well. Other notable interactions were observed through Val211 and Leu240. The former interacted with hydroxyl moieties of the carboxylic acid or attached to the benzene ring. The later appeared to interact with the benzene ring of phenylalanine. The interactions with Val211 and Leu240 were also detected with OTA-M and OTA-Cl/M. On the other hand, there are noticeable differences between OTA and its derivatives, particularly related to the interactions with the benzopyran groups, the methyl moiety and the chlorine atom. For example, Glu321 interacted with the methyl moiety and the chlorine atom in OTA but with the lactone in the derivatives (Fig. 7). Even between OTA-M and OTA-Cl/M, some differences were observed. For example, OTA-M had the chlorine atom interact with Met323. In contrast, OTA-Cl/M had Met323 interact with the pyran ring.

4. DISCUSSION

Activation of PXR has been linked to chemoresistance, metabolic diseases and toxicological bioactivation (Ihunnah et al., 2011; Chai et al., 2019; Yoshinari, 2019). There is an increasing interest in developing blockers that target PXR activation. Given the fact that these clinical issues operate under different conditions, an ideal blocker would have high potency and selectively target PXR. In this study, we have shown that OTA and its derivatives efficaciously blocked PXR-directed reporter activation, and potent blocking activity was detected even at nanomolar concentrations with the derivatives. Importantly, the derivatives were shown to decrease the expression of the stably transfected PXR gene (i.e., coding sequence), and the decrease was correlated well with the blocking activity. Molecular docking identified that OTA and its derivatives interacted with different sets of residues in PXR or through different chemical bonds.

The high potency and efficacious suppression of PXR protein expression establish OTA derivatives as a unique class of PXR blockers. It has been reported that PXR activation is antagonized by therapeutic agents such as metformin (Mani et al., 2013), hormones or hormonal analogs such as diethylstilbestrol (Hodnik et al., 2015), health supplements such as sulforaphane (Zhou et al., 2007), and synthetic compounds such as SPA70 (Lin et al., 2017). These compounds have been shown or implicated to disrupt the interaction of PXR and steroid receptor coactivators, pointing to an involvement of classic mechanism of antagonism against nuclear receptors. On the other hand, these compounds, with an exception of SPA70, have a relatively low potency with an IC_{50} value ranging from micromolar to millimolar concentrations, diminishing their potential for clinical use. In this study, we have shown that OTA derivatives significantly blocked PXR activation with high potency (Fig. 4). OTA-Cl/M at 0.1 μ M, for example, blocked the basal and RIF-stimulated activation by 25 and 45%, respectively. In contrast, ketoconazole, a recognized PXR antagonist, required as much as 20 μ M to exert comparable blocking activity (Fig. 4)). SPA70, probably with the highest potency among reported PXR blockers, had an estimated IC_{50} of 0.5 μ M against RIF-stimulated activation (Lin et al., 2017).

Another major difference of OTA and its derivatives over the reported blockers is their inverse effect on the expression of PXR. The OTA compounds profoundly decreased the expression of PXR, whereas the opposite was true with some reported compounds, particularly related to SPA70 (Lin et al., 2017). For example, SPA70 increased the level of PXR protein by as much as two fold (Lin et al., 2017). In contrast, OTA-M and OTA-Cl/M decreased the expression of PXR by 80 and 90% (Fig. 5A), respectively. This decrease was detected when these derivatives were tested at a concentration of 1 μ M. This concentration caused no cytotoxicity (Fig. 6A). Interestingly, OTA, the parent compound, was less potent than OTA-M (demethylated derivative) in term of blocking PXR activation. However, OTA and OTA-M caused a comparable decrease in PXR expression. One explanation is that OTA but not OTA-M exerted agonist activity. Indeed, OTA at 0.1 μ M slightly increased the reporter activity at both basal and RIF-stimulated condition (Fig. 4A). Consistent with this observation, OTA has been shown to induce the mRNA levels of several PXR target genes (e.g., CYP3A4) in HK-2, a human proximal tubule cell line (Lee et al., 2018). However, no or only minimum induction of CYP3A4 mRNA, with an exception of two donors, was

detected in primary hepatocytes (Ayed-Boussema et al., 2012; Doricakova and Vrzal, 2015). Nevertheless, the expression of PXR protein in these primary hepatocytes was significantly decreased (Doricakova and Vrzal, 2015).

The precise mechanism(s) of the suppressed PXR expression by OTA and its derivatives remains to be determined. An early study demonstrated that OTA induced miR-148a, a microRNA that was shown to downregulate PXR expression. However, this microRNA targeted the 5'-UTR of PXR mRNA (Takagi et al., 2008). In addition, miR-148a seemed to be not correlated with CYP3A4 expression in in vivo studies (Wei et al., 2013). In this study, we have shown profound suppression of the PXR transgene (Fig. 5A). This transgene contained the coding sequence only and lacked the reported miR-148a target motif (Hodnik et al., 2015). In addition, the expression of the transgene was driven by the CMV but not the PXR native promoter, but yet profound downregulation of PXR protein expression was detected as seen in primary hepatocytes (Ayed-Boussema et al., 2012). On the other hand, actinomycin D, a transcription inhibitor, caused marginal changes in PXR mRNA expression, pointing to a minimal role of destabilizing PXR mRNA in the downregulation of PXR protein by OTA and its derivatives. Furthermore, the decreased expression of PXR protein in primary hepatocytes was not attenuated by MG132, a proteasome inhibitor (Doricakova and Vrzal, 2015). These results collectively suggest that OTA and its derivatives profoundly decrease the expression of PXR protein, most likely achieved through a non-proteasome mechanism with enhanced PXR protein degradation. Our molecular docking study identified that OTA and its derivatives made contacts with certain amino acids of PXR. Such complexes likely induced conformational changes favoring degradation. It should be emphasized that PXR signaling is regulated by cofactors and even in a genomic context-dependent manner (Song et al., 2004; Ihunnah et al., 2011; Mani et al., 2013). As a result, the level of PXR is essential but may not be sufficient to determine the expression of its target genes. OTA-Cl/M, among OTA and its derivatives, exhibited the most favorable property for blocking PXR activation over cytotoxicity. This derivative showed the largest margin of concentrations that blocked PXR activation over those that caused cytotoxicity (Figs. 4C, 5A and 6A). OTA-Cl/M at 1 μ M blocked PXR activation by as much as 90%. However, this derivative caused no cytotoxicity even at 20 μ M. In contrast, OTA-M and OTA were less potent in blocking PXR activation but more cytotoxic. As a matter of fact, OTA-M, the demethylated derivative, was the most toxic compound among these three OTA compounds (Fig. 6A). It remains to be determined on how the methyl group regulates the overall potential of OTA cytotoxicity. The methyl group is attached to the lactone ring and hydrolysis of the lactone represented toxicological activation. It is likely that the lactone-open metabolite with the methyl attachment is more reactive and delivers toxicity. On the other hand, the chlorine atom on the benzene is established to be critical for OTA toxicity, notably genotoxicity (Tozlovanu et al., 2006; Hadjeba-Medjdoub et al., 2012; Sharma et al., 2014). Elimination of this atom no longer caused DNA damage (Tozlovanu et al., 2006; Hadjeba-Medjdoub et al., 2012; Sharma et al., 2014). These findings suggest that the methyl group and the chlorine atom constitute a toxicological interplay. OTA-Cl/M is a dechlorination and demethylation derivative. Its large margin of efficacy over safety establishes that the toxicological potential and blocking activity toward PXR can be structurally separated.

In summary, our work points to several important conclusions. First, OTA and its derivatives potently downregulate the expression of PXR protein even at nanomolar concentrations, pointing to a broad application in blocking PXR activation. Second, the downregulation occurs with the transgene (coding sequence only). Molecular docking study identifies that OTA and its derivatives interact with certain residues in PXR. These findings suggest that the downregulation is mediated largely by facilitating protein degradation. Third, OTA-CI/M exhibits a large margin of efficacy-safety concentrations, suggesting that these two functional events can be structurally separated. Excessive activation of PXR has been linked to chemoresistance, metabolic diseases and toxicological bioactivation. There is an increasing interest in developing blockers that target PXR activation. This study has provided both experimental evidence and conceptual framework toward this objective.

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Abbreviation used:

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BSA	bovine serum albumin
CYP3A4	Cytochrome P450 3A4
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
LCMS	liquid chromatograph mass spectrometry
NMR	nuclear magnetic resonance
OTA	Ochratoxin A
OTA-M	demethylated OTA
OTA-CI/M	dechlorinated and demethylated OTA
PXR	pregnane X receptor
RIF	Rifampicin
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

5. REFERENCE

- Ayed-Boussema I, Pascussi JM, Zaied C, Maurel P, Bacha H, Hassen W (2012) Ochratoxin A induces Cyp3a4, 2b6, 3a5, 2c9, 1a1, and Cyp1a2 gene expression in primary cultured human hepatocytes: A possible activation of nuclear receptors. *Drug Chem Toxicol.* 35:71–80. [PubMed: 21834667]
- Banerjee M, Robbins D, Chen T (2015) Targeting xenobiotic receptors Pxr and Car in human diseases. *Drug Discov Today.* 20:618–28. [PubMed: 25463033]
- Bolaji OO, Adehin A, Adeagbo BA (2019) Pharmacogenomics in the Nigerian population: the past, the present and the future. *Pharmacogenomics.* 20:915–26. [PubMed: 31453771]

- Carnahan VE, Redinbo MR (2005) Structure and function of the human nuclear xenobiotic receptor PXR. *Curr Drug Metab.* 6:357–67. [PubMed: 16101574]
- Chai SC, Lin W, Li Y, Chen T (2019) Drug discovery technologies to identify and characterize modulators of the pregnane X receptor and the constitutive androstane receptor. *Drug Discov Today.* 24:906–15. [PubMed: 30731240]
- Chen Y, Tang Y, Wang MT, Zeng S, Nie D (2007) Human pregnane X receptor and resistance to chemotherapy in prostate cancer. *Cancer Res.* 67:10361–7. [PubMed: 17974979]
- Cheng J, Ma X, Krausz KW, Idle JR, Gonzalez FJ (2009) Rifampicin-activated human pregnane X receptor and CYP3A4 induction enhance acetaminophen-induced toxicity. *Drug Metab Dispos.* 37:1611–21. [PubMed: 19460945]
- Dai J, Wright MW, Manderville RA (2003) Ochratoxin A forms a carbon-bonded c8-deoxyguanosine nucleoside adduct: implications for c8 reactivity by a phenolic radical. *J Am Chem Soc.* 125:3716–7.
- Doricakova A, Vrzal R (2015) A food contaminant ochratoxin A suppresses pregnane X receptor (PXR)-mediated CYP3A4 induction in primary cultures of human hepatocytes. *Toxicology.* 337:72–8. [PubMed: 26341324]
- Fischer A, Smieško M (2019) Ligand pathways in nuclear receptors. *J Chem Inf Model.* 59:3100–9. [PubMed: 31246455]
- Fuchs I, Hafner-Blumenstiel V, Markert C, Burhenne J, Weiss J, Haefeli WE, Mikus G (2013) Effect of the CYP3A inhibitor ketoconazole on the PXR-mediated induction of CYP3A activity. *Eur J Clin Pharmacol.* 69:507–13 [PubMed: 22968811]
- Goey AK, Meijerman I, Rosing H, Marchetti S, Mergui-Roelvink M, Keessen M, Burgers JA, Beijnen JH, Schellens JH (2014) The effect of St John's Wort on the pharmacokinetics of docetaxel. *Clin Pharmacokinet.* 53:103–10. [PubMed: 24068654]
- Hadjeba-Medjdoub K, Tozlovanu M, Pfohl-Leschkowicz A, Frenette C, Paugh RJ, Manderville RA (2012) Structure-activity relationships imply different mechanisms of action for ochratoxin A-mediated cytotoxicity and genotoxicity. *Chem Res Toxicol.* 25:181–90. [PubMed: 22126095]
- Hakkola J, Rysä J, Hukkanen J (2016) Regulation of hepatic energy metabolism by the nuclear receptor PXR. *Biochim Biophys Acta.* 1859:1072–82. [PubMed: 27041449]
- Hodnik Ž, Peterlin Masic L, Tomasic T, Smodiš D, D'Amore C, Fiorucci S, Kikelj D (2014) Bazedoxifene-scaffold-based mimetics of solomonsterols A and B as novel pregnane X receptor antagonists. *J Med Chem.* 57:4819–33. [PubMed: 24828006]
- Hodnik Ž, Tomašič T, Smodiš D, D'Amore C, Mašič LP, Fiorucci S, Kikelj D (2015) Diethylstilbestrol-scaffold-based pregnane X receptor modulators. *Eur J Med Chem.* 103:551–62. [PubMed: 26408814]
- Hukkanen J, Hakkola J, Rysä J (2014) Pregnane X receptor (Pxr)--a contributor to the diabetes epidemic? *Drug Metabol Drug Interact.* 29:3–15. [PubMed: 24166671]
- Ihunnah CA, Jiang M, Xie W (2011) Nuclear receptor Pxr, transcriptional circuits and metabolic relevance. *Biochim Biophys Acta.* 1812:956–63. [PubMed: 21295138]
- K szegi T, Poór M (2016) Ochratoxin A: Molecular Interactions, Mechanisms of Toxicity and Prevention at the Molecular Level. *Toxins (Basel).* 8:111. [PubMed: 27092524]
- Krausova L, Stejskalova L, Wang H, Vrzal R, Dvorak Z, Mani S, Pavek P (2011) Metformin suppresses pregnane X receptor (Pxr)-regulated transactivation of Cyp3a4 gene. *Biochem Pharmacol.* 82:1771–80. [PubMed: 21920351]
- Lee HJ, Pyo MC, Shin HS, Ryu D, Lee KW (2018) Renal toxicity through AhR, PXR, and Nrf2 signaling pathway activation of ochratoxin A-induced oxidative stress in kidney cells. *Food Chem Toxicol.* 122:59–68. [PubMed: 30291945]
- Li H, Redinbo MR, Venkatesh M, Ekins S, Chaudhry A, Bloch N, Negassa A, Mukherjee P, Kalpana G, Mani S (2013) Novel yeast-based strategy unveils antagonist binding regions on the nuclear xenobiotic receptor Pxr. *J Biol Chem.* 288:13655–68. [PubMed: 23525103]
- Lim Y P, Kuo SC, Lai ML, Huang JD (2009) Inhibition of Cyp3a4 expression by ketoconazole is mediated by the disruption of pregnane X receptor, steroid receptor coactivator-1, and hepatocyte nuclear factor 4alpha interaction. *Pharmacogenet Genomics.* 19:11–24. [PubMed: 19077665]

- Lin W, Wang YM, Chai SC, Lv L, Zheng J, Wu J, Zhang Q, Wang YD, Griffin PR, Chen T (2017) SPA70 is a potent antagonist of human pregnane X receptor. *Nat Commun.* 8:741. [PubMed: 28963450]
- Maekawa K, Harakawa N, Yoshimura T, Kim SR, Fujimura Y, Aohara F, Sai K, Katori N, Tohkin M, Naito M., Hasegawa R, Okuda H, Sawada J, Niwa T, Saito Y (2010) Cyp3a4*16 and Cyp3a4*18 alleles found in east Asians exhibit differential catalytic activities for seven Cyp3a4 substrate drugs. *Drug Metab Dispos.* 38:2100–4. [PubMed: 20847137]
- Mani S, Dou W, Redinbo MR (2013) Pxr antagonists and implication in drug metabolism. *Drug Metab Rev.* 45:60–72. [PubMed: 23330542]
- Marczak MM, Yan B (2017) Circadian rhythmicity: A functional connection between differentiated embryo-chondrocyte-expressed gene 1 and small heterodimer partner. *Arch Biochem Biophys.* 631:11–18. [PubMed: 28797635]
- Marin-Kuan M, Nestler S, Verguet C, Bezençon C, Piguet D, Mansourian R, Holzwarth J, Grigorov M, Delatour T, Mantle P, Cavin C, Schilter B (2006) A toxicogenomics approach to identify new plausible epigenetic mechanisms of ochratoxin a carcinogenicity in rat. *Toxicol Sci.* 89: 120–34. [PubMed: 16251485]
- Miki Y, Suzuki T, Kitada K, Yabuki N, Shibuya R, Moriya T, Ishida T, Ohuchi N, Blumberg B, Sasano H (2006) Expression of the steroid and xenobiotic receptor and its possible target gene, organic anion transporting polypeptide-a, in human breast carcinoma. *Cancer Res.* 66:535–42. [PubMed: 16397270]
- Mooiman KD, Maas-Bakker RF, Moret EE, Beijnen JH, Schellens JH, Meijerman I (2014) Milk thistle's active components silybin and isosilybin: novel inhibitors of Pxr-mediated Cyp3a4 induction. *Drug Metab Dispos.* 41:1494–504.
- Moya M, Gomez-Lechon MJ, Castell JV, Jover R (2010) Enhanced steatosis by nuclear receptor ligands: A study in cultured human hepatocytes and hepatoma cells with a characterized nuclear receptor expression profile. *Chem Biol Interact.* 184:376–87. [PubMed: 20079722]
- Pavek P, Smutny T (2014) Nuclear receptors in regulation of biotransformation enzymes and drug transporters in the placental barrier. *Drug Metab Rev.* 46:19–32 [PubMed: 24020384]
- Pavek P (2016) Pregnane X receptor (PXR)-mediated gene repression and cross-talk of PXR with other nuclear receptors via coactivator interactions. *Front Pharmacol*7:456. [PubMed: 27932985]
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF chimera--a visualization system for exploratory research and analysis. *J Comput Chem.* 25:1605–12 [PubMed: 15264254]
- Rekka EA, Kourounakis PN, Pantelidou M (2019) Xenobiotic metabolising enzymes: Impact on pathologic conditions, drug interactions and drug design. *Curr Top Med Chem.* 19:276–91. [PubMed: 30706817]
- Sharma P, Manderville RA, Wetmore SD (2014) Structural and energetic characterization of the major DNA adduct formed from the food mutagen ochratoxin a in the nari hotspot sequence: Influence of adduct ionization on the conformational preferences and implications for the ner propensity. *Nucleic Acids Res.* 42:11831–45. [PubMed: 25217592]
- Shen Y, Shi Z, Yan B (2019) Carboxylesterases: Pharmacological inhibition, regulated expression and transcriptional involvement of nuclear receptors and other transcriptional factors. *Nucl Receptor Res.* 6:101435.
- Shi D, Yang D, Prinssen EP, Brian E. Davies BE, Yan B (2011) Surge in expression of carboxylesterase-1 during the post-natal stage enables a rapid gain of the capacity to activate the anti-influenza prodrug oseltamivir. *J Infect Dis.* 203:937–42. [PubMed: 21402544]
- Song X, Xie M, Zhang H, Li Y, Sachdeva K, Yan B (2004) The pregnane X receptor binds to response elements in a genomic context-dependent manner and its activator rifampicin selectively alters the bindings among target genes. *Drug Metab Dispos.* 32:35–42. [PubMed: 14709618]
- Stemmer K, Ellinger-Ziegelbauer H, Ahr HJ, Dietrich DR (2007) Carcinogen-specific gene expression profiles in short-term treated eker and wild-type rats indicative of pathways involved in renal tumorigenesis. *Cancer Res.* 67:4052–68. [PubMed: 17483316]

- Takagi S, Nakajima M, Mohri T, Yokoi T (2008) Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. *J Biol Chem.* 283:9674–80. [PubMed: 18268015]
- Tang M, Mukundan M, Yang J, Charpentier N, LeCluyse EL, Black C, Yang D, Shi D, Yan B (2006) Anti-platelet agents aspirin and clopidogrel are hydrolyzed by distinct carboxylesterases and clopidogrel is transesterified in the presence of ethyl alcohol. *J Pharmacol Exp Ther.* 319:1467–76. [PubMed: 16943252]
- Tozlovanu M, Faucet-Marquis V, Pfohl-Leszkowicz A, Manderville RA (2006) Genotoxicity of the Hydroquinone metabolite of ochratoxin A: structure-activity relationships for covalent DNA adduction. *Chem Res Toxicol.* 19:1241–7. [PubMed: 16978030]
- Venkatesh M, Wang H, Cayer J, Leroux M, Salvail D, Das B, Wrobel JE, Mani S (2011) In vivo and in vitro characterization of a first-in-class novel azole analog that targets pregnane X receptor activation. *Mol Pharmacol.* 80:124–35 [PubMed: 21464197]
- Vettorazzi A, van Delft J, López de Cerain A (2013) A review on ochratoxin a transcriptomic studies. *Food Chem Toxicol.* 59:766–83. [PubMed: 23747715]
- Vettorazzi A, Pastor L, Guruceaga E, López de Cerain A (2019) Sex-dependent gene expression after ochratoxin A insult in F344 rat kidney. *Food Chem Toxicol.* 123:337–48. [PubMed: 30449730]
- Waring RH (2020) Cytochrome P450: genotype to phenotype. *Xenobiotica.* 9–18. [PubMed: 31411087]
- Wei Z, Chen M, Zhang Y, Wang X, Jiang S, Wang Y, Wu X, Qin S, He L, Zhang L, Xing Q (2013) No correlation of hsa-miR-148a with expression of PXR or CYP3A4 in human livers from Chinese Han population. *PLoS One.* 8:e59141. [PubMed: 23527115]
- Wu Q, Dohnal V, Huang L, Ku a K, Wang X, Chen G, Yuan Z (2011) Metabolic pathways of ochratoxin A. *Curr Drug Metab.* 12:1–10. [PubMed: 21222585]
- Xiao D, Shi D, Yang D, Barthel B, Koch TH, Yan B (2013) Carboxylesterase-2 is a highly sensitive target of the antiobesity agent orlistat with profound clinical implications in the activation of anticancer prodrugs. *Biochem Pharmacol.* 85:439–47. [PubMed: 23228697]
- Xu F, Wang F, Yang T, Sheng Y, Zhong T, Chen Y (2014) Differential drug resistance acquisition to doxorubicin and paclitaxel in breast cancer cells. *Cancer Cell Int.* 14:538.
- Yeung AWK, Tzvetkov NT, Jó wik A, Horbanczuk OK, Polgar T, Pieczynska MD, Sampino S, Nicoletti F, Berindan-Neagoe I, Battino M, Atanasov AG (2020) Food toxicology: quantitative analysis of the research field literature. *Int J Food Sci Nutr.* 71:13–21. [PubMed: 31140340]
- Yoshinari K (2019) Role of nuclear receptors PXR and CAR in xenobiotic-induced hepatocyte proliferation and chemical carcinogenesis. *Biol Pharm Bull.* 42:1243–52. [PubMed: 31366862]
- Zhang H, LeCulyse E, Liu L, Hu M, Matoney L, Zhu W, Yan B (1999) Rat pregnane X receptor: molecular cloning, tissue distribution, and xenobiotic regulation. *Arch Biochem Biophys.* 368:14–22. [PubMed: 10415106]
- Zhou J, Zhai Y, Mu Y, Gong H, Uppal H, Toma D, Ren S, Evans RM, Xie W (2006) A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J Biol Chem.* 281:15013–20. [PubMed: 16556603]
- Zhou C, Poulton EJ, Grun F, Bammler TK, Blumberg B, Thummel KE, Eaton DL (2007) The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor. *Mol Pharmacol.* 71:220–9. [PubMed: 17028159]
- Zlender V, Breljak D, Ljubojevi M, Flajs D, Balen D, Brzica H, Domijan AM, Peraica M, Fuchs R, Anzai N, Saboli I (2009) Low doses of ochratoxin a upregulate the protein expression of organic anion transporters Oat1, Oat2, Oat3 and Oat5 in rat kidney cortex. *Toxicol Appl Pharmacol.* 239:284–96. [PubMed: 19538982]

Highlights

- The pregnane X receptor (PXR) is activated by structurally diverse PXR agonists
- Excessive activation of PXR is implicated in chemoresistance and metabolic diseases
- OTA-Cl/M was synthesized and found to block PXR functionality with low cytotoxicity
- The blocking activity is achieved by potent downregulation of PXR protein
- This novel mechanism points to a broad effect against diverse PXR agonists

Figure 1. Chemical structure of major metabolites of OTA

Oxidative dechlorination of OTA leads to the formation of aryl radical and formation of DNA adducts. However, the presence of glutathione (GSH) detoxifies it and forms OTB (ochratoxin B). Hydrolysis of the lactone leads to the formation of the open-lactone form of OTA (OP-OTA) with equal or more toxicity than OTA.

Figure 2. Synthesis of OTA derivatives

(A) Synthesis of OTA-CI/M and (B) Synthesis of OTA-M. These derivatives shared the intermediate of Compound 2 and the synthetic steps 2–4 (A). The major difference was chlorination of compound 2 for the synthesis of OTA-M. **Abbreviation** DCM, dichloromethane; DIPEA: diisopropylethylamine; DMF: N,N-dimethylformamide; HATU: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; LDA: lithium diisopropylamide; LiOH: lithium hydroxide; MeOH: methanol; SOCl₂: thionyl chloride; TFA: trifluoroacetic acid.

A

B

C

Figure 3. LCMS- and NMR-spectrometric analysis of OTA-CI/M

LCMS was performed with HP 1110 Agilent LCMS using a quaternary G1311A pump coupled to a Micromass Platform LCZ detector. OTA-CI/M (10 μ L at 1 mg/mL) was injected and separated on a Zorbax C18 column over a 10 min acetonitrile gradient (5–95%, v/v). (A) Representative chromatogram (UV absorption). (B) Mass-spectra in the negative ion mode. (C) ¹H NMR spectra were recorded on a Bruker UltraShield 400 MHz.

Figure 4. Blocking activity on PXR activation by OTA, OTA-CI/M, OTA-M and ketoconazole

The stably transfected cell line (hPXR-HRE) was plated in 96-well plates at a confluence of 80%. After an overnight incubation, the cells were treated with rifampicin (10 μM) in the presence or absence of OTA (A), OTA-M (B), OTA-CI/M (C) or ketoconazole (D). OTA and its derivatives were tested at 0–1 μM, whereas ketoconazole at 0–20 μM. The treated cells were cultured for 24 h in 10% delipided FBS without antibiotics. Cell lysates were then prepared and analyzed for luciferase activity. The signal was normalized with the amount of protein. Single and double asterisk symbols indicate statistical significance over vehicle control at $p < 0.05$ and $p < 0.01$, respectively.

A

B

Figure 5. Suppressed expression of PXR by OTA, OTA-CI/M, OTA-M and ketoconazole

(A) Western blotting hPXR-HRE cells were plated at a confluence of 80% and treated with OTA (1 μ M), OTA-CI/M (1 μ M), OTA-M (1 μ M), ketoconazole (10 μ M), or DMSO (0.1%) for 24 h. Cell lysates (5 μ g) were resolved by 7.5% SDS-PAGE in a mini-gel apparatus and transferred electrophoretically to nitrocellulose membranes. After non-specific binding sites were blocked with 5% non-fat milk, the blots were incubated with the anti-Flag antibody. The primary antibody was localized with goat anti-rabbit IgG conjugated with horseradish peroxidase. Horseradish peroxidase activity was detected with chemiluminescent. The

chemiluminescent signal was captured by Carestream 2200 PRO Imager. the relative intensities were quantified by the Carestream 2200 Analysis Software. (B) The immunofluorescence staining hPXR-HRE cells were seeded in chamber overnight and then treated with OTA (1 μ M), a derivative (1 μ M), ketoconazole (10 μ M) or DMSO. After an additional 24-h incubation, cells were washed and fixed with 4% Paraformaldehyde. Chamber slides were incubated with 1% BSA (2 mg/mL) for 1 h to block nonspecific binding followed by incubation in the anti-FLAG monoclonal antibody (1:1000 dilution with PBS) for 30 min. The slide was washed five times with PBS. Subsequently, the slide was incubated with fluorescent isothiocyanate-conjugated anti-mouse IgG antibody for an additional 30 min. The cell nuclei were counterstained with DAPI (5 μ g/mL). Cells were then imaged using confocal microscope. It should be noted that the software was switched to pseudoRED instead of typical pseudoBLUE for DAPI image to increase the contrast over the green. Arrows: nuclear localization. Bar: 20 μ m.

Figure 6. Cytotoxicity of OTA, its derivatives and ketoconazole

(A) Cytotoxicity of OTA and its derivatives hPXR-HRE cells were seeded into 96-well plates at a density of 5000 cells/well. After an overnight incubation, cells were treated with OTA or a derivative at various concentrations (0–100 μM). After the cells were treated for 24 h, the medium was replaced with fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at a final concentration of 0.5 mg/ml. After 2-h incubation at 37°C, the medium was gently decanted, and dimethyl sulfoxide (100 μl/well) was added to dissolve formazan product. The optical density (OD) was determined at 570 nm. Statistical

significance over vehicle is indicated by an asterisk symbol at $p < 0.05$. A line indicates statistical significance between two treatments at $p < 0.05$. (B) Cytotoxicity of ketoconazole Cells were seeded and treated with ketoconazole at 0–20 μM . Cell viability was determined as described above. Statistical significance over vehicle is indicated by a single ($p < 0.05$) or double ($p < 0.01$) asterisk symbol. (C) Morphological analysis phase contrast imaging of cells was taken after 6 h- treatment with DMSO (0.1%) or different chemicals (20 μM).

Figure 7. Molecular docking

The structure of PXR (5X0R) was retrieved from the Protein Data Bank (www.pdb.org). Autodock4.2 was used for docking simulation and structural confirmations were selected through Autodock4.2 according to their binding energies. The structural confirmations were visualized, superimposed with PXR, and analyzed for residue interactions by adopting Discovery Studio Client®.

Table I.

Amino acids interacting with OTA, derivatives and/or SPA70

Compound	211	212	227	239	240	243	299	307	308	319	321	323	324	407	411
OTA-CI/M	Val		Pro	Leu	Leu	Met			Leu		Glu	Met	Leu	His	Leu
OTA-M	Val		Pro	Leu	Leu	Met			Leu		Glu	Met	Leu	His	Leu
OTA	Val				Leu	Met			Leu	Leu	Glu	Met	Leu	His	
SPA70	Val	Ser	Pro				Trp	Cys	Leu		Glu	Met	Leu		

Note. The data are compiled from Fig. 7. Numbers represent the location of an amino acid. van der Waals interactions in light green, hydrogen bond in dark green, alkyl/Pi-alkyl in light purple, and Pi-Sigma in dark purple.