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New insights into the risk of phthalates: Inhibition of UDP-glucuronosyltransferases

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Abstract

Wide utilization of phthalates-containing products results in the significant exposure of humans to these compounds. Many adverse effects of phthalates have been documented in rodent models, but their effects in humans exposed to these chemicals remain unclear until more mechanistic studies on phthalate toxicities can be carried out. To provide new insights to predict the potential adverse effects of phthalates in humans, the recent study investigated the inhibition of representative phthalates di-n-octyl ortho-phthalate (DNOP) and diphenyl phthalate (DPhP) towards the important xenobiotic and endobiotic-metabolizing UDP-glucuronosyltransferases (UGTs). An *in vitro* UGTs incubation system was employed to study the inhibition of DNOP and DPhP towards

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Conflicts of interest

The authors have declared no conflict of interests.

UGT isoforms. DPhP and DNOP weakly inhibited the activities of UGT1A1, UGT1A7, and UGT1A8. 100 μM of DNOP inhibited the activities of UGT1A3, UGT1A9, and UGT2B7 by 41.8% ($p < 0.01$), 45.6% ($p < 0.01$), and 48.8% ($p < 0.01$), respectively. 100 μM of DPhP inhibited the activity of UGT1A3, UGT1A6, and UGT1A9 by 81.8 ($p < 0.001$), 49.1% ($p < 0.05$), and 76.4% ($p < 0.001$), respectively. *In silico* analysis was used to explain the stronger inhibition of DPhP than DNOP towards UGT1A3 activity. Kinetics studies were carried out to determine mechanism of inhibition of UGT1A3 by DPhP. Both Dixon and Lineweaver–Burk plots showed the competitive inhibition of DPhP towards UGT1A3. The inhibition kinetic parameter (K_i) was calculated to be 0.89 μM . Based on the $[I]/K_i$ standard ($[I]/K_i < 0.1$, low possibility; $1 > [I]/K_i > 0.1$, medium possibility; $[I]/K_i > 1$, high possibility), these studies predicted *in vivo* drug–drug interaction might occur when the plasma concentration of DPhP was above 0.089 μM . Taken together, this study reveals the potential for adverse effects of phthalates DNOP and DPhP as a result of UGT inhibition.

Graphical Abstract

Keywords

Phthalates; UDP-glucuronosyltransferases (UGTs); Di-n-octyl ortho-phthalate (DNOP); Diphenyl phthalate (DPhP)

1. Introduction

Phthalates, diesters of phthalic acids, have been widely used as plastisizers to increase the flexibility, pliability and elasticity of plastics (Wan et al., 2013). Phthalates have been also used in cosmetics, personal care products, food packaging and medical products (Wan et al., 2013). The huge production volume and wide application (approximately 6 million tons/year), have likely resulted in the presence of phthalates (Xie et al., 2007), air (Wang et al., 2012), soil (Kong et al., 2012), food (Fierens et al., 2013) and biosamples of human (e.g., blood, breast milk, urine, etc.) (Wan et al., 2013; Zimmermann et al., 2012; Lin et al., 2011). The questionable safety of phthalates, including estrogenic endocrinedisrupting activity (Chen et al., 2014), severely limits their use.

Phthalates are substrates of drug-metabolizing enzymes (DMEs), and thus can be eliminated through a series of phase I (e.g., hydrolysis, etc.) and phase II (e.g., glucuronidation, sulfation, etc.) metabolic reactions (Hauser and Calafat, 2005). Phthalates can also affect the

catalytic activities of drug-metabolizing enzymes (DMEs) (Aitio and Parkki, 1978). UDP-glucuronosyltransferases (UGTs), a superfamily of membrane proteins located into the endoplasmic reticulum (ER), are the important DMEs involved in the conjugation of a variety of drugs, herbal components, and pollutants with glucuronic acid (Rowland et al., 2013; Li et al., 2012). Additionally, UGTs can metabolize some endogenous substances. For example, UGT1A1 is the major enzyme involved in the metabolic elimination of bilirubin (Zheng et al., 2014). UGT1A3 can conjugate the bile acids (Erichsen et al., 2010). UGT2B17 is involved in the metabolism of testosterone (Zhu et al., 2015). Therefore, the inhibition of UGTs' activity might significantly disrupt the metabolism of xenobiotics and endogenous compounds.

A previous study revealed that the endocrine disrupting compound bisphenol A had inhibitory effects towards UGTs (Jiang et al., 2013). Therefore, a similar question was raised for phthalates, and thus the inhibition of UGT by phthalates was investigated in the current manuscript. Di-n-octyl ortho-phthalate (DNOP) and diphenyl phthalate (DPhP) were used as the representative phthalates. DNOP and DPhP and their metabolites can be detected in the plasma of humans exposed to these agents. However, since they share the similar structures, the inhibition of DNOP and DPhP towards UGTs could reflect the influence of both parent compound and their metabolites towards UGTs.

2. Materials and methods

2.1. Materials

4-Methylumbelliferone (4-MU), 4-methylumbelliferone- β -D-glucuronide (4-MUG), Tris-HCl, 7-hydroxycoumarin, and uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA) were purchased from Sigma-Aldrich (St Louis, MO). Recombinant human UGT isoforms (UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2B7) expressed in baculovirus-infected insect cells were obtained from BD Gentest Corp. (Woburn, MA, USA). All other reagents were of HPLC grade or of the highest grade commercially available.

2.2. Determination of phthalates' inhibition towards UGTs' isoforms

In vitro UGTs' activity determination was performed as previously described (Jiang et al., 2013; Fang et al., 2015). A 200 μ L incubation reaction mixture is consisted of recombinant human UGT isoforms, 5 mM of UDPGA, 5 mM of $MgCl_2$, 50 mM of Tris-HCl buffer (pH=7.4), and various concentrations of 4-MU as the substrate. Di-n-octyl ortho-phthalate (DNOP) and diphenyl phthalate (DPhP) were dissolved in the DMSO to make a stock solution of 20 mM, and various concentrations of working solutions were prepared through dilution with DMSO. The most optimal microsomal concentration and incubation time were first determined to generate a linear glucuronidation reaction. Before initiation of the reaction, 5 min pre-incubation was performed, and UDPGA was added to begin the metabolic reaction. The incubation temperature was 37 $^{\circ}C$, and an equal volume of ice-cold acetonitrile with 7-hydroxycoumarin (100 μ M) was employed to terminate the metabolic reaction. After the deproteinization at 20,000 $\times$ g for 10 min, 10 μ L of supernatant was used for high-performance liquid chromatography (HPLC) analysis. The HPLC system

(Shimadzu, Kyoto, Japan) contained a SCL-10A system controller, two LC-10AT pumps, a SIL-10A auto injector, a SPD-10AVP UV detector. Chromatographic separation was carried out using a C18 column (4.6 × 200 mm, 5 µm, Kromasil) at a flow rate of 1 mL/min and UV detector at 316 nm. The mobile phase consisted of acetonitrile (A) and H₂O containing 0.5% (v/v) formic acid (B). The following gradient condition was used: 0–15 min, 95–40% B; 15–20 min, 10% B; 20–30 min, 95% B. The calculation curve was generated by peak area ratio (4-MUG/internal standard) over the concentration range of 4-MUG 0.1–100 mM. The curve was linear over this concentration range, with an r^2 value > 0.99. The limits of detection and quantification were determined at signal-to-noise ratios of 3 and 10, respectively. The accuracy and precision for each concentration were more than 95%.

2.3. Molecular docking to explain the interaction between phthalates and UGT1A3

Till up to now, three dimensional structure of UGT1A3 remains unknown. Therefore, we constructed the homology model of UGT1A3 enzyme by homology modeling method. The amino acid sequence of UGT1A3 enzyme (ID:NP061966) was obtained from NCBI database. This target protein sequence was used for the homology modeling of the three dimensional structure of UGT1A3 enzyme. The templates for structural homology modeling included the crystal structures of oleandomycin glycosyltransferase (PDB code: 2iya), flavonoid 3-O glycosyltransferase (PDB code:2c1x), and hydroquinone glucosyltransferase (PDB code:2vce). MODELLER 9v14 program was used to predict the three dimensional structure of UGT1A3 enzyme according to the known crystal structures of homologous proteins. Alignment of target protein sequence with the three template proteins was used as the input for model script in MODELLER program, and twenty models were generated. These models were validated with the help of Modeller objective function and DOPE (Discrete Optimization Protein Energy) score build by MODELLER program. PROCHECK was used to check the qualities of predicted models.

To gain better insight for the interaction between di-nonyl ortho-phthalate (DNOP) and diphenyl phthalate (DPhP) with UGT1A3, docking studies were carried out. The autodock software along with autodock tool was utilized to generate the docking between flexible small molecule and rigid protein. DNOP and DPhP were docked into the cavity of UGT1A3 enzyme, respectively. The non-polar hydrogen atoms of UGT1A3 were merged, and subsequently the Kollman charges were added to the protein structure by AutoDock Tools. Gasteiger partial charges were assigned to the compounds. Then the defined grid box was set to cover the entire ligand-binding site. The value was set to 80 × 80 × 80 in X, Y and Z coordinate, and the grid point spacing for grid-box was 0.375 Å. Lamarckian Genetic Algorithm (LGA) was applied for protein-fixed ligand-flexible docking calculations. The LGA runs (ga_run parameter) were set to as standard 50 runs for each ligand. After the docking search completed, the best conformation with the lowest docked energy was analyzed for the interactions including binding energy, hydrogen bonds and hydrophobic contacts between inhibitors and enzyme.

2.4. Inhibition kinetic analysis

The inhibition kinetic type and parameters were determined for the inhibition of DPhP towards UGT1A3. Different concentrations of substrates and inhibitors were added in the

reaction mixture to determine the glucuronidation velocity of 4-MU. Dixon and Lineweaver–Burk plots were employed to evaluate the reversible inhibitory type, and second plot of slopes from Lineweaver–Burk plot over the concentrations of DPhP was utilized to calculate the K_i value.

2.5. In vitro-in vivo extrapolation (IVIVE)

In vitro-in vivo extrapolation (IV-IVE) was performed using the following equation

$$\text{AUC}_i/\text{AUC} = 1 + [\text{I}]_{\text{in vivo}}/K_i$$

The terms are defined as follows: AUC_i/AUC is the predicted ratio of *in vivo* exposure of xenobiotics or endogenous substances with or without the co-exposure of DPhP. $[\text{I}]_{\text{in vivo}}$ is the *in vivo* exposure concentration of DPhP, and the K_i value was *in vitro* inhibition kinetic parameters.

2.6. Data presentation and statistical analysis

Data were given as the mean value plus S.D., and statistical analysis was carried out using two-tailed one student t-test.

3. Results

3.1. Diphenyl phthalate (DPhP) exerts stronger inhibition than di-n-octyl ortho-phthalate (DNOP)

To investigate the inhibition of Diphenyl phthalate (DPhP) and Di-n-octyl ortho-phthalate (DNOP) towards UGT isoforms, 100 μM of compounds was used to initially screen the inhibitory potential. As shown in Fig. 1, DPhP and DNOP weakly inhibited the activity of UGT1A1, UGT1A7, and UGT1A8. 100 μM of DNOP inhibited the activity of UGT1A3, UGT1A9, and UGT2B7 by 41.8% ($p < 0.01$), 45.6% ($p < 0.01$), and 48.8% ($p < 0.01$), respectively. 100 μM of DPhP inhibited the activity of UGT1A3, UGT1A6, and UGT1A9 by 81.8% ($p < 0.001$), 49.1% ($p < 0.05$), and 76.4% ($p < 0.001$), respectively.

Given the important contribution of UGT1A3 towards the metabolism of endogenous substances, concentration-dependent inhibition behavior was determined, and the results (Fig. 2) showed that both DPhP and DNOP showed dose-dependent inhibition towards UGT1A3, and the inhibition capability of DPhP was significantly different from the inhibition of DNOP at 60 ($p < 0.05$), 80 ($p < 0.001$), and 100 μM ($p < 0.05$) of compounds.

3.2. Mechanism explanation of the stronger inhibition of DPhP than DNOP towards UGT1A3

DPhP and DNOP were docked into the binding pocket of UGT1A3 to explore the interaction between ligand and protein. The molecular docking method used for complex enzyme-inhibitor detected the following functional site residues in the target protein UGT1A3: Ser39, His40, Leu117, Met120, Arg174, Asn175, Gly309, Ser310, Val312, Gln358, His373, Gly375, Ser376, His377, Gly378, Glu381, Phe395, Gly396, Asp397, and Gln398. The residues in the binding site of UGT1A3 towards DNOP were given in Fig. 3. DNOP did not

form hydrogen bonds with UGT1A3. The binding site of DPhP to UGT1A3 is similar to that of complex UGT1A1-DNOP (Fig. 4). The binding site of UGT1A3 for binding with DPhP is composed of residues Gly38, Ser39, His40, Met116, Met120, Asn175, Gly309, Ser310, Val312, His373, Gly375, Ser376, His377, Gly378, and Gln398. The N atoms of residues Ser39 and Ser310 made hydrogen bonds with the DPhP. The hydrophobic interaction between these two compounds with UGT1A3 was also determined (Fig. 5). The residues in UGT1A3 involved in the hydrophobic interaction with DNOP were consisted of Ser39, His40, Arg174, Gly309, Gln358, His373, Gly375, His377, Gly378, Gly396, Leu117, Met120, Val312, and Phe395. The residues involved in the hydrophobic interaction with DPhP contained Ser39, His40, Gly309, Ser310, His373, Gly375, His377, Met116, Met120, and Val312.

The binding energy was also analyzed, and DNOP and DPhP reveals binding free energy of -6.94 and -7.82 , respectively, which was accordant with the experimental result $\text{DPhP} > \text{DNOP}$ in inhibitory capability.

3.3. Inhibition kinetics of DPhP towards UGT1A3

The inhibition kinetics was determined for the inhibition of UGT1A3 by DPhP due to its inhibition capability more than 80%. The intersection point was located in the second quadrant in Dixon plot (Fig. 6A), and the intersection point was located in the vertical axis in Lineweaver–Burk plot (Fig. 6B), indicating the competitive inhibition of DPhP towards UGT1A3. The fitting equation for the second plot was $y = 15.3x + 10$, and the inhibition kinetic parameter (K_i) was calculated to be $0.89 \mu\text{M}$.

4. Discussion

The regulation effect of phthalates on enzymes could mediate some of the adverse effects of these phthalates. Oral or intraperitoneal administration of phthalates results in decreased succinic dehydrogenase (SDH) activity (Lake et al., 1975). Phthalates were also reported to affect the metabolism of lipids through regulating peroxisome proliferator-activated receptor α (PPAR α)-mediated fatty acids metabolizing enzymes (Sarath Josh et al., 1982). The influence of phthalates on the drug-metabolizing enzymes (DMEs) was also reported. For example, di(2-ethylhexyl) phthalate (DEHP) produced a time-and route-dependent effect on the hepatic cytochrome P450 contents and activity of aminopyrine *N*-demethylase, aniline hydroxylase, alcohol dehydrogenase and high and low K_m aldehyde dehydrogenases when given orally or intraperitoneally.

Using DNOP and DPhP, the present study investigated the inhibition potential of phthalates towards an important phase II enzyme UGT using an *in vitro* incubation system. UGT1A3 was significantly inhibited by both phthalates, with DPhP exerting stronger inhibition than DNOP. *In silico* modeling revealed that substitution of octane by benzene significantly changed the inhibition potential towards UGT1A3; this was associated with a change of hydrogen bonding and hydrophobic interactions between the two compounds and the enzyme. DNOP didn't form hydrogen bonds with UGT1A3, while DPhP could form two hydrogen bonds with the residues Ser39 and Ser310 in UGT1A3. More hydrogen bonds formation of DPhP with UGT1A3 majorly might contribute to the stronger inhibition of

DPhP towards UGT1A3. UGT1A3 was an important drug-metabolizing enzyme catalyzing the glucuronidation of endogenous substances, such as estrogen and bile acids, and inhibition of UGT1A3 activity could disrupt the metabolic process of these important endogenous substances (Lankisch et al., 2008). Based on the $[I]/K_i$ standard ($[I]/K_i < 0.1$, low possibility; $1 > [I]/K_i > 0.1$, medium possibility; $[I]/K_i > 1$, high possibility), the *in vivo* drug–drug interaction might occur when the plasma concentration of DPhP rise above 0.089 μM . It should be noted that some other UGT isoforms were also inhibited by DNOP and DPhP can also be inhibited, such as the inhibition of DNOP towards UGT1A9 and UGT2B7, and DPhP towards UGT1A6 and UGT1A9. All these UGT isoforms play a key role in the metabolism of xenobiotics (e.g., propofol, zidovudine, etc.), therefore, the influence of DNOP and DPhP towards the metabolism of these compounds considered when these drugs are used therapeutically in patients also exposed to phthalates.

In conclusion, inhibition by phthalates DNOP and DPhP of UGTs was demonstrated. The extents of inhibition by DNOP and DPhP towards the activity of UGT1A3 could be explained with molecular docking model *in silico*. The inhibition kinetic type and parameters were determined for the inhibition of DPhP towards UGT1A3. Taken together, a new insight was given in the present study for the adverse effects of the phthalates DNOP and DPhP.

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HIGHLIGHTS

- Di-n-octyl ortho-phthalate (DNOP) and diphenyl phthalate (DPhP) exhibited strong inhibition towards UGT1A3.
- More hydrogen bonds contributed the stronger inhibition of DPhP towards UGT1A3 than DNOP.
- DPhP competitively inhibited UGT1A3 with the inhibition kinetic parameter (K_i) to be 0.65 μM .

Fig. 1. Initial screening of the inhibition of phthalates DNOP and DPhP towards the activity of UGT isoforms. 100 μ M of compounds was used, and two-tailed one student's t-test was used. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 2. Comparison of the inhibition capability of DNOP and DPhP towards UGT1A3 at multiple concentrations. Data were given as mean plus S.D. *, $p < 0.05$; ***, $p < 0.001$.

Fig. 3.
The binding site of DNOP to the active cavity of UGT1A3. The residues in the binding site were shown in stick.

Fig. 4.
The binding site of DPhP to active cavity of UGT1A3. The residues in the binding site were shown in stick. The hydrogen bonds formed between DPhP and UGT1A3 enzyme were shown in line.

Fig. 5.

The hydrophobic interaction between DNOP (A) and DPhP (B) with UGT1A3. The residues involved in the formation of hydrophobic interaction to ligands were shown.

Fig. 6. Determination of inhibition kinetics of DPhP towards UGT1A3-catalyzed glucuronidation of 4-MU. (A) Dixon plot to determine the inhibition of DPhP towards UGT1A3-catalyzed glucuronidation of 4-MU; (B) Lineweaver-Burk plot to determine the inhibition of DPhP towards UGT1A3-catalyzed glucuronidation of 4-MU; (C) Second plot to determine the inhibition of DPhP towards UGT1A3-catalyzed glucuronidation of 4-MU.