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TDCPP mimics thyroid hormones associated with the activation of integrin $\alpha_v\beta_3$ and ERK1/2

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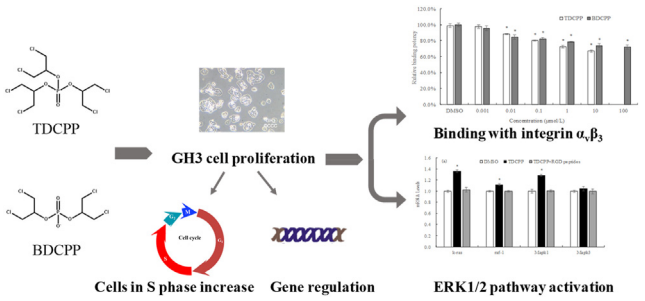
HIGHLIGHTS

- TDCPP and BDCPP induced GH3 cell proliferation.
- TDCPP and BDCPP showed strong binding with integrin $\alpha_v\beta_3$.
- TDCPP and BDCPP induced thyroid disruption that is associated with integrin $\alpha_v\beta_3$ and ERK1/2 activation.

GRAPHICAL ABSTRACT



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ABSTRACT

Tri(1,3-dichloropropyl) phosphate (TDCPP) potentially damages the thyroid system in humans and animals. However, knowledge of its toxic effects and underlying mechanisms is limited. The present study was conducted to determine the thyroid hormone-disrupting effects of TDCPP and its major metabolite, bis(1,3-dichloro-2-propyl) phosphate (BDCPP) in rat pituitary cell lines (GH3). TDCPP and BDCPP, that mimic the thyroid hormone (TH), promoted GH3 cell proliferation and modulated the progression of the cell cycle at 20 and 200 μmol/L, respectively. Similar to T3, TDCPP and BDCPP also significantly upregulated *c-fos* and downregulated *Tshβ* gene expression. Although the binding affinity of these chemicals for thyroid receptor β (TRβ) was not measured, significant competition between these chemicals to bind to the membrane thyroid hormone receptor (integrin $\alpha_v\beta_3$) was found, suggesting that TDCPP and BDCPP were strongly bound to integrin $\alpha_v\beta_3$. Results from a molecular docking analysis provided further evidence of strong binding affinities of TDCPP and BDCPP for integrin $\alpha_v\beta_3$, and the ligand binding site of Arg-Gly-Asp (RGD) was identified. Real-time PCR also supported the supposition that, after binding to integrin $\alpha_v\beta_3$, TDCPP and BDCPP may induce the activation of the extracellular signal-regulated protein kinase (ERK1/2) signal transduction pathway. Taken together, our data suggest that TDCPP and BDCPP have the ability to mimic THs and that the underlying mechanism might be associated with their interactions with integrin $\alpha_v\beta_3$ and the activation of the ERK1/2 pathway, providing new insight into the mechanism of TDCPP- and BDCPP-induced cytotoxicity.

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1. Introduction

Organophosphorus flame retardants (OPFRs) constitute a group of synthetic chemicals that are extensively used as flame retardants and added to many industrial products, such as plastics, furniture, paints, textiles, and electronics (Hou et al., 2016; USEPA, 2005). Since the worldwide bans and restrictions on the use of polybrominated diphenyl ethers (PBDEs) and typical brominated flame retardants (BFRs) were enforced, OPFRs have been increasingly used as alternatives to BFRs (Wang et al., 2018; Sühring et al., 2016). For example, tri(1,3-dichloropropyl) phosphate (TDCPP), a halogenated OPFR, has recently been the most frequently applied flame retardant in manufactured residential furniture (Betts, 2013; Hoffman et al., 2015). TDCPP is also typically used as a physical additive in polyurethane foam, which is the raw material for many daily products, and TDCPP can be released during use of these product through volatilization, abrasion and/or leaching (Hou et al., 2016). Thus, TDCPP is widely detected in various environmental matrices, such as surface and underground water (Wei et al., 2015), sediments, and indoor and outdoor air (Wang et al., 2018; van der Eede et al., 2012), with the concentrations ranging from several ng/L or ng/kg to mg/L or mg/kg. Moreover, humans are also exposed to TDCPP as indicated by frequent detection of their metabolites, such as bis (1,3-dichloro-2-propyl) phosphate (BDCPP) in urine, at levels has high as 541 μg/L (Hoffman et al., 2015). High exposure to TDCPP has been found in infants (Hoffman et al., 2015); therefore, it has become an emerging concern in environmental science fields (Wang et al., 2018).

Toxicological studies have shown that TDCPP might be a potential thyroid-disrupting chemical (TDC) (Xu et al., 2015; Godfrey et al., 2017). Xu et al. (2015) reported that TDCPP (100 μg/L) significantly reduced the concentrations of thyroid hormones (THs), including thyroxine (T4) and 3,5,3′-triiodothyronine (T3), in zebrafish. Wang et al. (2013) reported that TDCPP (300–600 μg/L) regulated the transcription of the genes involved in the hypothalamic–pituitary–thyroid (HPT) axis in zebrafish, causing changes to the THs, decreasing body weight and leading to malformations. Indeed, THs are involved in a large range of biological processes, and the disruption of THs might indicate ecologically relevant adverse outcomes. For instance, swim bladder development in fishes is controlled by THs, so the decline in THs induced by chemicals such as TDCPP may result in impaired swim bladder inflation (Godfrey et al., 2017; Hagenaars et al., 2014). In mammals, lungs comprise a similar system to that of the swim bladder under the control of THs, and thyroid disruption may affect the development of the lungs and/or contribute to some respiratory problems (Galambos et al., 2010).

The potential thyroid-disrupting mechanisms of TDCPP are not clear. Based on the results of molecular docking and in vitro assessments, Ren et al. (2016) demonstrated that TDCPP (50–100 μmol/L) could fit into the ligand-binding pocket of nuclear thyroid hormone receptor β (TRβ) and showed an agonistic potency towards TRβ in a luciferase reporter assay, such that thyroid disruption was partly attributed to the activation of TRβ.

The thyroid disruption by TDCPP might be also associated to membrane thyroid hormone receptor, such as integrin $\alpha_v\beta_3$. Integrin $\alpha_v\beta_3$ has been found as a typical membrane thyroid hormone receptor and mediated the nongenomic pathway of T4 that occurs outside the nucleus. The mechanism of nongenomic actions for T4 is well defined by initially binding to integrin $\alpha_v\beta_3$ and triggering intracellular signaling cascades involved in multiple developmental and physical events (Hammes and Davis, 2015; Bergh et al., 2005). Importantly, the nongenomic pathway may play not only an important role in TH action but also in the disruption by some TDCs. For example, the thyroid disruption by bisphenol A (BPA) might be partly attributed to integrin $\alpha_v\beta_3$ and its relative signal pathways, as observed in the African *Cercopithecus aethiops* monkey kidney cell line CV-1, which does not express a TR (Sheng et al., 2019); this work presented a good initial test useful for studying the nongenomic pathways of TDCs, although its use has rarely been reported.

The purpose of our study was to examine the thyroid disruption potential and mode of action (MoA) of TDCPP and its major metabolite, bis(1,3-dichloro-2-propyl) phosphate (BDCPP), considering the biotransformation of TDCPP. The T-screen, the cell cycle and apoptosis analysis were used to evaluate the thyroid disruption induced by TDCPP/BDCPP. The TRβ yeast assay was adopted to assess the interaction of the test chemicals with TRβ. Moreover, based on the hypothesis that thyroid disruptions caused by TDCPP/BDCPP could be associated with nongenomic pathway, the related gene expression, the interaction of the test chemicals with integrin $\alpha_v\beta_3$ and molecular docking simulation were conducted to identify the role of integrin $\alpha_v\beta_3$ and the downstream ERK1/2 signaling pathway in test chemicals-induced thyroid disruption.

2. Materials and methods

2.1. Chemicals

TDCPP (99%) and BDCPP (99%) were purchased from AccuStandard (CT, USA). T3 (95%), T4 (95%), dimethylsulfoxide (DMSO, 99.5%), and the RGD peptide (≥97%) were purchased from Sigma Chemical (MO, USA). PD98059 was purchased from Cell Signaling Technology (MO, USA). For all compounds, stock solutions were prepared in DMSO.

2.2. Cell culture

The rat pituitary tumor cell line (GH3) was obtained from the National Infrastructure of Cell Line Resource (Beijing, China). This cell line, the proliferation of which can be influenced by TDCs, is widely used to evaluate thyroid hormone disruption (Ghisari and Bonefeld-Jørgensen, 2005). The T-screen assay was developed based on this cell line and has been used for screening thyroid and anti-thyroid chemicals (Schriks et al., 2006).

The GH3 cells were cultured in F10 culture medium (Gibco, NY, USA) supplemented with 15% horse serum (HS, Gibco, New Zealand) and 2.5% fetal bovine serum (FBS, Gibco, Australia). The GH3 cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. T-screen assay

The T-screen assay was carried out to detect the thyroid-disrupting activity of the target chemicals by determining the level to which GH3 cells proliferated, as previously reported (Li et al., 2016). Briefly, GH3 cells were seeded into 96-well plates at a density of 2500 cells/well in 200 μL of serum-free F10 medium. After a 24-h incubation, the medium was replaced with test medium containing 0.5% target chemical and allowed to incubate for another 48 h (Ghisari and Bonefeld-Jørgensen, 2009). The tested concentrations of TDCPP/BDCPP ranged from 0.002 to 200 μmol/L. The viability of the GH3 cells was determined using an MTT cell proliferation detection kit (Nanjing Jiancheng Bio-Engineering Institute Co. Ltd., Nanjing, China) following the manufacturer's protocol. T3 (Supporting Information Fig. S2) and DMSO were used as the positive and negative control, respectively. The final concentration of DMSO in the samples used for biological assays did not exceed 0.5%.

2.4. Cytotoxicity test

A lactate dehydrogenase (LDH) cytotoxicity detection kit (Nanjing Jiancheng Bio-Engineering Institute Co. Ltd., Nanjing, China) was carried out following the manufacturer's protocol, and the LDH release was calculated as reported by Wen et al. to evaluate the cytotoxicity of GH3 cells induced by the test chemicals (Wen et al., 2015).

2.5. Cell cycle and apoptosis analysis

The effects of the test chemicals on the GH3 cell cycle and the extent of apoptosis were assessed with a NovoCyte flow cytometer (ACEA Biosciences Inc., CA, USA) as reported by Duan et al. (2012). The GH3 cells were seeded and exposed to the test chemicals as reported in Section 2.3. After exposure to the chemical, the GH3 cells were collected and treated following the protocol of the manufacturer of the cell cycle and apoptosis analysis kit (BD PharMingen, CA, USA). T3 (at a concentration of 20 nmol/L, Kong et al., 2018) and DMSO were used as the positive and negative control, respectively. The data were processed and analyzed using NovoExpress software (Version 1.2.4, ACEA Biosciences Inc., CA, USA). Experiments were repeated three times.

2.6. RT-PCR analysis

The GH3 cells were seeded and exposed to the test chemicals as reported in Section 2.3. Total RNA isolation, RNA quantification and cDNA synthesis were performed following previously described protocols (Kong et al., 2018). The cell proliferation-related *c-fos* gene, the TH secretion-related *Tshβ* gene and the ERK1/2-dependent *k-ras, Raf-1, Mapk1* and *Mapk3* gene were selected. The primers of the selected genes were designed with Primer 3 software (http://frodo.wi.mit.edu/) and synthesized by Sangon Biotech (Shanghai) Co. Ltd. (Shanghai, China, Supporting Information Table S1). T3 (at a concentration of 20 nmol/L, Kong et al., 2018) and DMSO were used as the positive and negative control, respectively (Supporting Information Fig. S5).

RT-PCR was performed on a Roche LightCycler® 480 instrument (Roche, CA, USA). The thermal cycler conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The Ct value for each targeted gene was normalized to that of β-actin based on the $2^{-\triangle\triangle Ct}$ method (Kong et al., 2018).

To identify the role of integrin $\alpha_v\beta_3$ in tested chemicals' effect on GH3 cells, the selected genes expression was examined after inhibiting the integrin $\alpha_v\beta_3$ by RGD peptides, which was a specific inhibitor of integrin $\alpha_v\beta_3$. In the inhibition assay, after co-exposure with RGD peptides (500 μmol/L), the GH3 cells were processed for further experiments (Davies et al., 2000; Liu et al., 2014; Skandalis et al., 2014).

2.7. TRβ yeast assay

The TRβ yeast assay was used to assess the interaction of the test chemicals with TRβ, as reported previously (Li et al., 2008). All the experiments were conducted in triplicate (or more) sets. Each assay group included the sample, the negative control (DMSO), and the positive control (T3, Supporting Information Fig. S6). The extent of the activity induced or inhibited by the test chemicals was calculated as previously shown by Li et al. (2008).

2.8. GH3 cell-based radioligand competitive binding assay

A GH3 cell-based radioligand competitive binding assay was used to assess the interaction of the test chemicals with integrin $\alpha_v\beta_3$. The $3PRGD_2$ kit was purchased from Medical Isotopes Research Center, Peking University. $Na^{99m}TcO_4$ was provided by the College of Nuclear Science and Technology, Beijing Normal University. The radiolabeling and synthesis of ^{99m}Tc-$3PRGD_2$ were performed as described previously (Ma et al., 2011). Details of the procedures used for determining the binding affinity of the tested chemicals for integrin $\alpha_v\beta_3$ are provided in the Supporting Information.

According to the report, T4 showed a stronger binding affinity than T3 for integrin $\alpha_v\beta_3$ (Bergh et al., 2005); therefore, T4 was selected as the positive control in the competitive binding assay (Supporting Information Fig. S7). The competition between TDCPP and BDCPP in binding to integrin $\alpha_v\beta_3$ was determined over ranges of concentrations from 0.001 to 10 μmol/L and 0.001–100 μmol/L, respectively. The results are expressed as relative binding strength as described in the Supporting Information to characterize the competition binding activity.

2.9. Molecular docking simulation

To understand the interactions between TDCPP/BDCPP and integrin $\alpha_v\beta_3$, a docking analysis was performed to simulate the potential binding mode of these chemicals with integrin $\alpha_v\beta_3$. The crystal structure of integrin $\alpha_v\beta_3$ was obtained from the Protein Data Bank (PDB, www.rcsb.org) as crystal 1L5G (3.2 Å), which shows the crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ in complex with an Arg-Gly-Asp (RGD) ligand (Kim et al., 2013). All small-molecule structures were built and the molecular docking studies were implemented with the Flexible Docking protocol (based on CHARMM, version 35b5) with Discovery Studio Visualizer 4.0 software (Accelrys Inc., CA, USA, Li et al., 2015). Amino acids within 4 Å in the RGD binding site were defined as flexible residues and the default parameters was used. The highest scored complex from the docking were considered for analysis, and the binding energy was calculated.

2.10. Statistical analysis

Only results at noncytotoxic concentrations were used for the statistical analyses. Significant differences between the control and exposure groups were evaluated using Dunnett's one-way analysis of variance (ANOVA). A p-value less than 0.05 was reported as significant. The statistical analysis was performed with SPSS software (Version 19.0, SPSS Inc., Chicago, IL, USA) on the mean values from at least three independent experiments. The results are reported as the mean ± standard deviation (SD).

3. Results

3.1. TDCPP/BDCPP induced thyroid disruption in GH3 cells

3.1.1. Cytotoxicity induced by TDCPP/BDCPP in GH3 cells

Cytotoxicity was not induced in the GH3 cells by TDCPP (0.002–20 μmol/L) or BDCPP (0.002–200 μmol/L, Supporting Information, Fig. S1). The data presented below refer only to the effects observed at concentrations that did not induce cytotoxicity.

3.1.2. TDCPP/BDCPP affected cell proliferation, cell cycle distribution and gene transcription of the GH3 cells

Changes in GH3 cell proliferation following exposure to TDCPP or BDCPP indicate that these chemicals might induce cell proliferation even at μmol/L exposure doses similar to that of T3 (Supporting Information, Fig. S2). As shown in Fig. 1, TDCPP (20 μmol/L) and BDCPP (200 μmol/L) increased GH3 cell proliferation by 1.22-fold and 1.16-fold ($p < 0.05$), respectively.

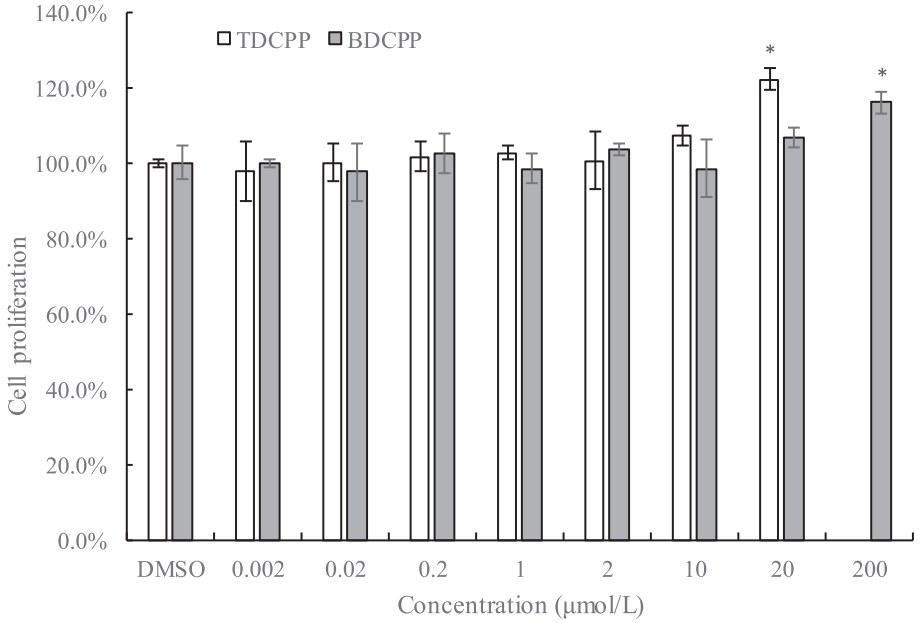

Fig. 1. TDCPP/BDCPP induced cell proliferation in the GH3 cells. * significantly different from the negative control ($p < 0.05$).

the results showed that TDCPP/BDCPP did not affect apoptosis (Supporting Information, Fig. S3). However, after a 48-h exposure, both TDCPP (20 μmol/L) and BDCPP (200 μmol/L) caused an increased in proportion of cells in the S phase compared to the number of S phase cells in the DMSO control ($p < 0.05$). The same trend was observed in cells exposed to T3 at a concentration of 20 nmol/L (Fig. 2).

The results showed that both of these chemicals upregulated the *c-fos* gene ($p < 0.05$, Fig. 3). Significant downregulation of the *Tshβ* genes was observed following exposure to TDCPP at a dose of 20 μmol/L or BDCPP at a dose of 200 μmol/L (Fig. 3), which are findings similar to those observed following T3 exposure.

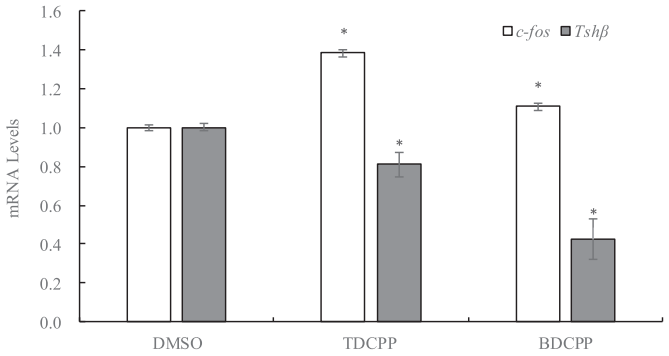

Fig. 3. Alterations in the gene expression of GH3 cells after exposure to TDCPP/BDCPP. * significantly different from the negative control ($p < 0.05$).

3.2. The mechanism of action of TDCPP/BDCPP-induced thyroid disruption in GH3 cells

3.2.1. The binding affinities of TDCPP/BDCPP for TRβ and integrin $\alpha_V\beta_3$

As shown in Fig. 4a, there was no significant difference between the values to indicate an induced activity of the tested chemicals or the negative control (DMSO, $p > 0.05$), suggesting that neither TDCPP nor BDCPP exhibited TRβ agonistic activities. After cells were incubated with TDCPP or BDCPP incubation and T3, inhibition

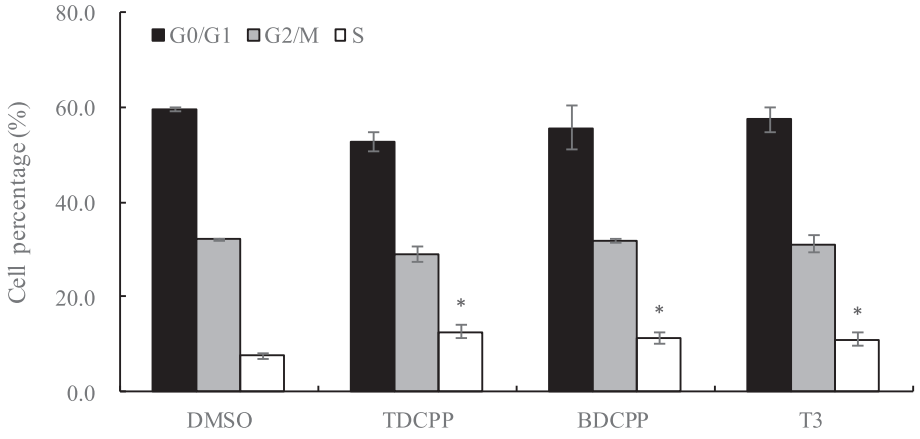

Fig. 2. Percentages of GH3 cells in each phase of the cell cycle after exposure to TDCPP/BDCPP. * significantly different from the negative control ($p < 0.05$).

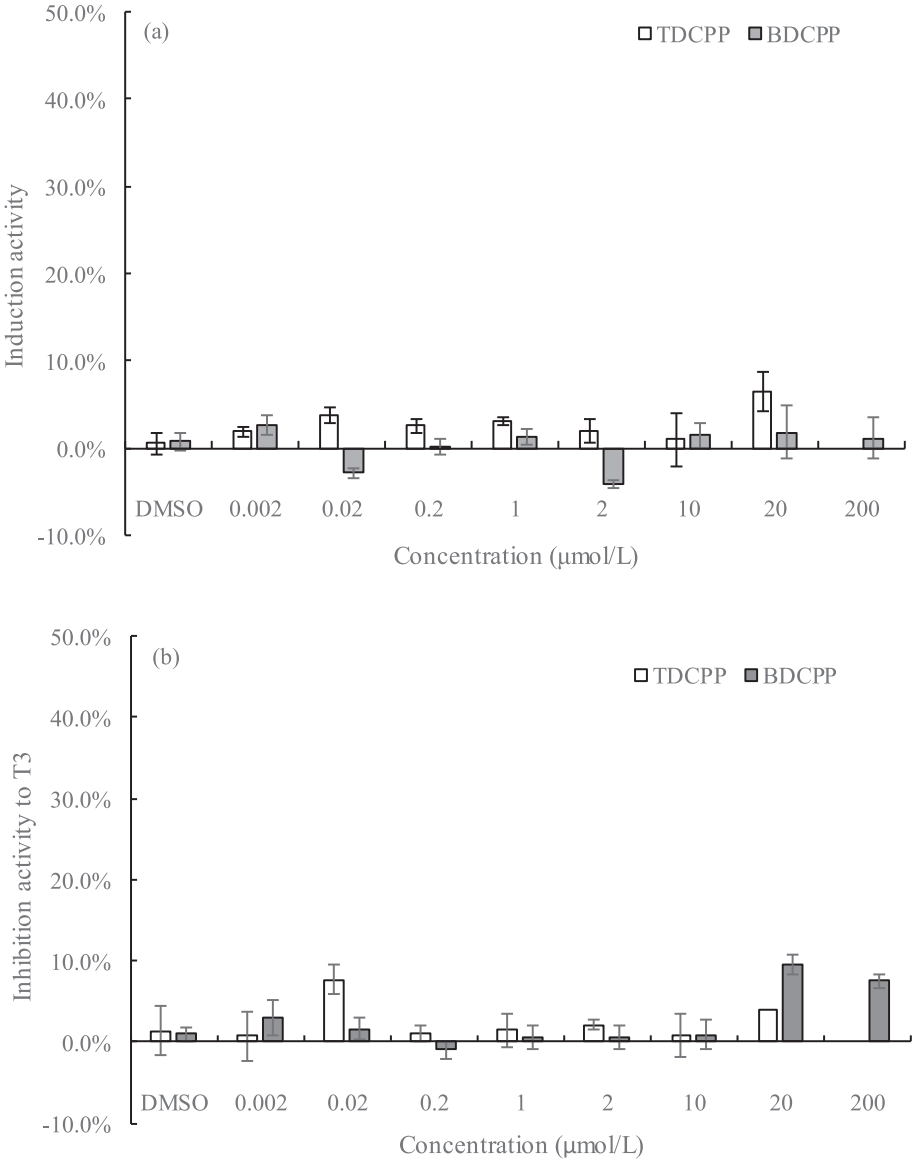

Fig. 4. The TRβ-disrupting activities of TDCPP/BDCPP, as determined by a recombinant TRβ yeast assay.

activity was determined to assess the competitive binding of these chemicals to TRβ in the presence of T3. The highest inhibition activity shown by TDCPP or BDCPP was lower than 10.0% (Fig. 4b), supporting the supposition that neither TDCPP nor BDCPP competitively bind to TRβ at the tested concentrations.

As shown in Fig. 5, similar competitive binding activities, but with different strengths, were observed for TDCPP and BDCPP. Significant inhibition of ^{99m}Tc-3PRGD$_2$ binding to GH3 cells was observed following exposure to TDCPP, even at doses as low as 0.01 μmol/L. With increased concentrations of TDCPP, more of the bound ^{99m}Tc-3PRGD$_2$ was displaced, and the radioactivity levels were reduced. For BDCPP, a slight but significant decrease in activity was observed at 0.01 μmol/L. All of these findings support the supposition that the both TDCPP and BDCPP have binding affinity for the membrane receptor integrin $\alpha_v\beta_3$.

Three typical compounds (standard ligands) with known binding affinity to integrin $\alpha_v\beta_3$ (T3, T4 and the RGD peptide) were selected and docked to integrin $\alpha_v\beta_3$ and the results were shown in Fig. S8, Fig. S9 and Fig. S10 in Supporting Information. Fig. S8 and Fig. S9 showed the docking views of T3, T4, RGD peptide, TDCPP and BDCPP with integrin $\alpha_v\beta_3$, respectively, as well as Fig. S10 showed the docking site of RGD peptide, TDCPP and BDCPP in integrin $\alpha_v\beta_3$, suggesting that these chemicals could dock to integrin $\alpha_v\beta_3$ at the binding site of RGD. Docking poses of three standard ligands (T3, T4 and the RGD peptide) showed residues of TYR A178 and ASN B215 participated in their hydrogen bond interactions with integrin $\alpha_v\beta_3$, as well as TYP A178 participated in a Pi interaction. Other residues e.g. ALA B218, TYR B122 etc. were also involved in the hydrogen bond interactions of three standard ligands with integrin $\alpha_v\beta_3$, which were similar to those reported by Kim et al. (2013) and listed in Table 1. TYR A178 also played an important role in the hydrogen bond interaction for both TDCPP and BDCPP, and TYR B166, ASP B179, ARG B216 and ALA A149 participated in the hydrogen bond interaction for BDCPP, suggesting that compared with TDCPP, there were more key amino acid residues at the binding site involved in the hydrogen bond interactions between BDCPP and integrin $\alpha_v\beta_3$. In the present study, the binding energy calculation showed that TDCPP showed a lower binding energy, of −55.68 kcal/mol, which was higher than that of the RGD peptide (−67.37 kcal/mol) and lower than that of T3 or T4 (−42.32 kcal/mol and −42.58 kcal/mol).

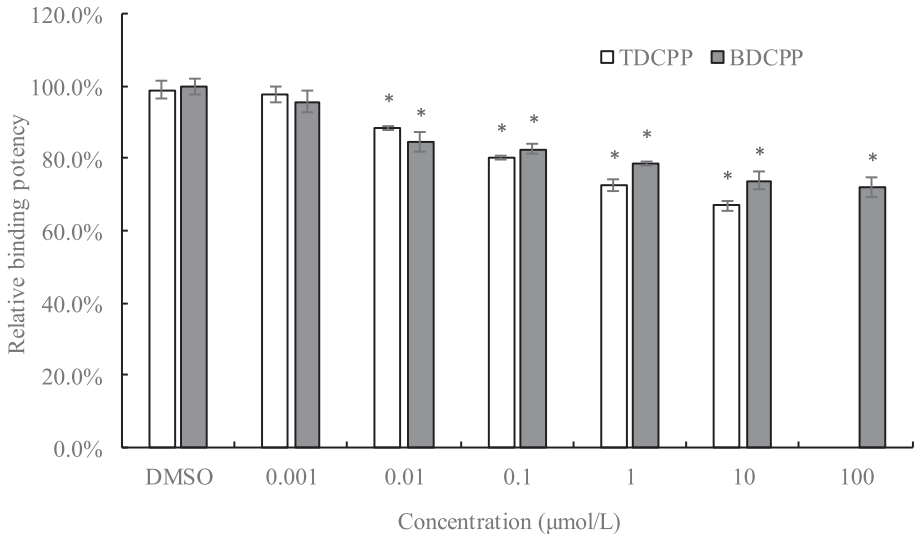

Fig. 5. The binding affinity of TDCPP/BDCPP for integrin $\alpha_v\beta_3$, as determined by the GH3 cell-based radioligand competitive binding assay.

Table 1
Structures and binding energies of the chemicals.

Chemicals	Formula	Binding energy (kcal/mol)	Hydrogen bond interaction	Pi interaction
T3	$C_{15}H_{12}I_3NO_4$	−42.32	TYR A178, ARG B214, ASN B215, and ASP A218	ARG B214, TYR A178
T4	$C_{15}H_{11}I_4NO_4$	−42.58	TYR A178, ASN B215, ASP A218, SER B123, TYR B122 and ALA B218	TYR A178
RGD peptide	$C_{12}H_{22}N_6O_6$	−67.37	TYR A178, ASN B215, ARG B214, ARG B216, ASP A217, GLU A121, and GLU A123	
TDCPP	$C_9H_{15}Cl_6O_4P$	−55.68	TYR A178	
BDCPP	$C_6H_{11}Cl_4O_4P$	−48.09	TYR A178, TYR B166, ASP B179, ARG B216, and ALA A149	

TYR: Tryptophan; ARG: Arginine; ASP: Aspartic acid; ALA: Alanine; SER: Serine; GLU: Glutamic acid; ASN: Asparagine.

BDCPP, the metabolite of TDCPP, had a higher binding energy (−48.09 kcal/mol) than its parent chemical.

3.2.2. TDCPP/BDCPP induced the thyroid disruption that is associated with activation of ERK1/2

The results showed no obvious change in the expression of *Mapk3* ($p > 0.05$). However, exposure to TDCPP (20 μmol/L) significantly upregulated *k-ras*, *Raf-1* and *Mapk1* by 1.4-, 1.1- and 1.3-fold, respectively (Fig. 6), compared to the control ($p < 0.05$), suggesting that ERK1/2 were activated. BDCPP (200 μmol/L) was also found to upregulate *k-ras* and *Raf-1* ($p < 0.05$).

An antagonist test conducted by treatment with an inhibitor elucidated the extent of TDCPP/BDCPP regulation on that ERK1/2 signaling pathways that are mediated by integrin $\alpha_v\beta_3$. After treatment with RGD peptide (integrin $\alpha_v\beta_3$ inhibitor), the overexpression of *k-ras*, *Raf-1* and *Mapk1* transcripts induced by TDCPP mostly disappeared (Fig. 6a), and the same trends were observed after was BDCPP was co-exposed with the RGD peptide (Fig. 6b).

4. Discussion

In this study, changes in the GH3 cell proliferation following the addition of TDCPP or its metabolite, BDCPP, showed that TDCPP and BDCPP induced effects similar to those of thyroid hormones. A similar result was reported by Ren et al. (2016), who found that TDCPP significantly enhanced GH3 cell proliferation, inducing a 1.2-fold cell proliferation increase at a concentration comparable to that of our study (50 μmol/L vs. the 20 μmol/L in this study). Our observations were also supported by a result obtained from zebrafish, in which TDCPP appeared to act in a manner similar to thyroid hormones in vivo (Godfrey et al., 2017).

In the present investigation, the cell cycle distribution and mRNA levels of the *c-fos* gene were affected after exposure to TDCPP or BDCPP, confirming the results from the cell proliferation assay. These chemicals promote the transition of GH3 cells from the G1 phase to the S phase, which may induce proliferation. Deng et al. (2017) reported that T3 can promote the proliferation of epicardial progenitor cells, and the potential underlying mechanism might be related to the accelerated progression of cells from the G1 phase to the S phase, supporting our results. Furthermore, the responses of the GH3 cells following exposure to TDCPP or BDCPP, including the upregulation of the *c-fos* gene, were similar to those induced by TH exposure. *c-fos*, a major component of activator protein 1 (AP-1), plays an important role in the regulation of cell proliferation (Brüsselbach et al., 1995). It has been reported that T4 increases the expression of *c-fos* in HeLa cells (Shih et al., 2004). Zhu et al. (2018) also demonstrated that phthalates increase the expression of *c-fos* and promote the proliferation of PC-3 and 22RV1 cells, suggesting that phthalates might induce cell proliferation through the activation of AP-1 in these cells. All of these results support the supposition that GH3 cell proliferation induced by TDCPP/BDCPP might be related to the modulated progression of the cell cycle through the G1/S transition and the upregulation of *c-fos*. This contention is also supported by Ronchetti et al. (2013), who suggested that modulated progression of the cell cycle by the simulation of cell cycle regulators is one cause of enhanced cell proliferation; another key component in the response to proliferative signals is the immediate-early gene *c-fos*, the expression of which is rapidly induced by mitogenic stimuli (Shaulian and Karin, 2002).

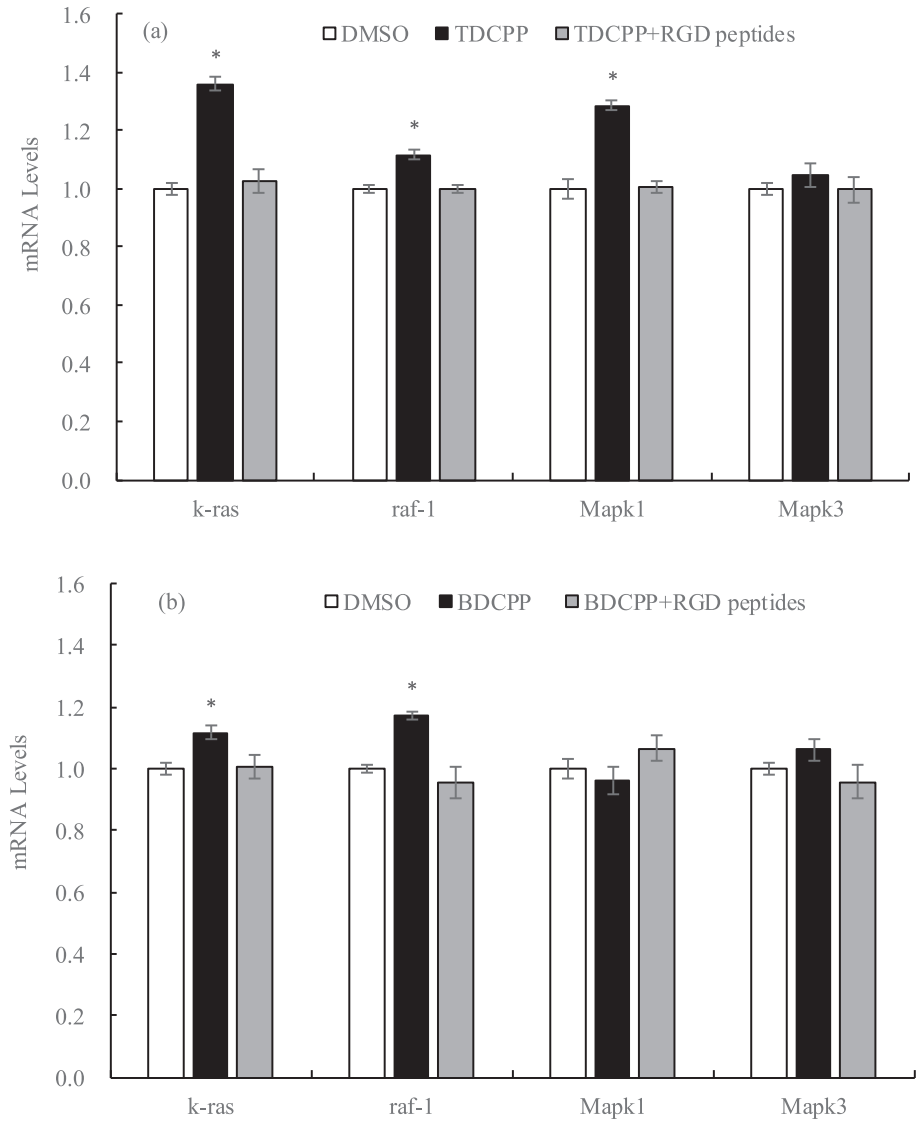

Fig. 6. Alterations of gene expression in the ERK1/2 pathway in the presence and absence of RGD peptides (integrin $\alpha_v\beta_3$ antagonist) and cotreatment with TDCPP or BDCPP in GH3 cells. * significantly different from the negative control ($p < 0.05$).

Thyroid-stimulating hormone (TSH), produced by the pituitary gland, is a primary hormone that regulates growth and metabolism by regulating the synthesis and secretion of THs (MacKenzie et al., 2009). Exposure to TDCPP/BDCPP leds to the significant down-regulation of the *Tshβ* gene in the GH3 cells, a finding that is consistent with the reports of previous studies (Lee et al., 2017). These observations in GH3 cells suggest that BPA and its analogs causes significant downregulation of the *Tshβ* gene in a manner similar to the effects of T3. The T3 downregulation of *Tshβ* gene expression might be attributed to a compensatory mechanism in response to increased TH levels. Based on these observations, TDCPP/BDCPP can regulate cell proliferation and gene expression in GH3 cells in a manner similar to that of T3, indicating that these chemicals could interact with the pituitary gland and could also lead to disruption of the synthesis of thyroid hormones. Compared to BDCPP, TDCPP displayed much stronger promotion of cell proliferation and regulation on the level of gene expression in GH3 cells, showing a more potency for thyroid disruption. Although there are dampening effects of TDCPP toxicity after metabolism (compared to BDCPP), it is urgent highly needs to carry out the relative toxicity mechanism research.

TRβ is the critical TR isoform for the T3-promoted proliferation of hepatocytes and pancreatic acinar cells (Kowalik et al., 2010; Brent, 2012). To study the mechanisms of TDCPP and BDCPP disruption, a recombinant TRβ yeast assay was used to observe the interaction of these chemicals with TRβ. The results suggested that TDCPP and BDCPP did not exhibit TRβ-antagonistic or TRβ-agonistic activity. These results are similar to those previously described by Kojima et al. (2013), who tested 11 OPFRs, including TDCPP, using the recombinant yeast assay and reported that none of the test compounds showed antagonistic or agonistic activity against TRα/β. However, Ren et al. (2016) reported the agonistic activity of TDCPP towards TRβ in concentrations greater than 25 μmol/L, as tested by a luciferase reporter gene assay. The observed differences in these results might be attributed to the different cell lines, expression plasmids adopted and the tested TDCPP concentrations.

The integrin $\alpha_v\beta_3$, a typical membrane receptor, has been recognized as a cell surface receptor for T4 and as having the initiation site for T4-induced activation of intracellular signal transduction (Davis et al., 2005, 2008). Given the important roles of

integrin $\alpha_v\beta_3$, previous mechanistic studies of TDCPP and BDCPP have focused on their actions mediated by TR pathways (Kojima et al., 2013; Ren et al., 2016), and studies of their effects mediated by integrin $\alpha_v\beta_3$ pathways are even more rare. The GH3 cell-based radioligand competitive binding assay was established based on the ^{99m}Tc-3PRGD$_2$ probe, with high affinity for integrin $\alpha_v\beta_3$, to measure the binding affinity of TDCPP and BDCPP for integrin $\alpha_v\beta_3$, showing the advantages of high sensitivity, stability as well as fast response. To the best of our knowledge, this is the first study to evaluate the binding strengths of TDCPP and BDCPP with integrin $\alpha_v\beta_3$ and to support the contention that these chemicals might interact with integrin $\alpha_v\beta_3$ even at a concentration as low as 0.01 μmol/L, providing a new direction for comprehensively understanding the mechanism of TDCPP- and BDCPP-induced thyroid disruption.

Molecular docking analysis was performed for TDCPP/BDCPP with integrin $\alpha_v\beta_3$, and it revealed a structural basis for the binding affinities of TDCPP/BDCPP for integrin $\alpha_v\beta_3$. Both TDCPP and BDCPP can be docked into the integrin $\alpha_v\beta_3$ binding site of RGD, in a manner similar to T4, suggesting that the TH-like activities induced by TDCPP/BDCPP towards integrin $\alpha_v\beta_3$ might be achieved by binding directly to the RGD binding site. Cody et al. (2007) revealed that the RGD binding site on integrin $\alpha_v\beta_3$ is utilized in the binding of THs, and they specified that the Arg side chain inserts into a narrow groove at the top of the propeller domain, the Asp protrudes in a cleft in the specific domain on integrin β_3, and the Gly residue lies at the interface between the α and β subunits (Xiong et al., 2002). As shown in Table 1, TDCPP had a lower binding energy than BDCPP, indicating that TDCPP exhibited the preferred mode of binding to the RGD recognition site on integrin $\alpha_v\beta_3$. This observation was in agreement with the results of our cell proliferation assay and gene transcription analysis, supporting the idea that the thyroid-disrupting effects of TDCPP dampen after metabolism. In the present study, all these results from experiments on the binding affinity analyses of TDCPP/BDCPP to TRβ or integrin $\alpha_v\beta_3$ and the molecular docking simulation indicated that the TDCPP- and BDCPP- induced thyroid-disrupting effects in GH3 cells might be linked with integrin $\alpha_v\beta_3$. To our knowledge, this is the first study that reports the effects of TDCPP/BDCPP involving integrin $\alpha_v\beta_3$.

TH binding to the membrane receptor integrin $\alpha_v\beta_3$ may lead to activation of the ERK1/2 signal transduction pathway (Davis, 2006). The results in this study described the activation of ERK1/2 after exposure to TDCPP/BDCPP and thus supported the idea that the activation might be a consequence of TDCPP/BDCPP exposure. The results that ERK1/2 activation was inhibited by the RGD peptide provide further evidence that integrin $\alpha_v\beta_3$ may take part in the ERK1/2 activation induced by TDCPP/BDCPP. The RGD peptide is an integrin $\alpha_v\beta_3$ antagonist that has no agonist function with integrin $\alpha_v\beta_3$ but instead blocks hormone binding at this site (Bergh et al., 2005). The addition of an integrin $\alpha_v\beta_3$ antagonist has been used to study the mechanism of action of THs via cell surface receptors. By adding RGD peptide, Davis (2006) demonstrated that TH binding at the membrane receptor integrin $\alpha_v\beta_3$ activates ERK1/2 and potentially promotes cell proliferation based on an inhibitor experiment, which is a finding consistent with our results. In fact, our previous work also indicated that the mechanisms underlying di-n-butylphthalate (DnBP) thyroid disruption might be related to integrin $\alpha_v\beta_3$ and ERK1/2 activation, and Sheng et al. (2019) also reported that integrin $\alpha_v\beta_3$ and its related signal pathways might participate in the thyroid-disrupting effects induced by BPA. It should be noted that only gene expression analyses were conducted in the Sheng et al. study, and the related protein expression will be analyzed in a future study. Taken together, the current and previous findings suggest that TDCPP and BDCPP have the ability to mimic THs, and this ability might be associated with their interaction with integrin $\alpha_v\beta_3$ and activation of the ERK1/2 pathway.

Actions of the nongenomically initiated thyroid hormone at integrin $\alpha_v\beta_3$ have been reported in recent years, and this new discovery is expected to prompt the exploration of the molecular mechanisms of environmental pollutants that disrupt the thyroid hormone system in humans and animals, although there have been few investigations or reports in this field to date. In fact, the T4 and T3 binding domains of integrin $\alpha_v\beta_3$ have been identified, and their function appears extensive, e.g., activating MAPK or ERK1/2, regulating the intracellular trafficking of TRα from the cytoplasm to the nucleus, controlling TRβ shuttling and promoting cell proliferation (Lin et al., 2009, 2011). Thus, studying the cell surface receptor integrin $\alpha_v\beta_3$ as the initiation site for TDCs might be an important research direction for studies of thyroid-disrupting mechanisms.

5. Conclusions

In the present study, the typical OPFR, TDCPP, and its metabolite BDCPP mimicked THs, inducing the proliferation of GH3 cells, changing the cell cycle distribution of these cells and regulating their gene expression. The potential mechanism of action of TDCPP/BDCPP involves binding to the membrane receptor integrin $\alpha_v\beta_3$ and activating the ERK1/2 pathway, providing new insight into the effects and underlying mechanism of TDCPP/BDCPP.

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.

CRediT authorship contribution statement

Jian Li: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - original draft. **Hedan Liu:** Data curation, Formal analysis, Methodology. **Na Li:** Data curation, Software, Writing - review & editing. **Jinsheng Wang:** Funding acquisition, Resources. **Liuting Song:** Investigation, Validation.

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Appendix A. Supplementary data

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