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Dichlorodiphenyltrichloroethane metabolites inhibit DNMT1 activity which confers methylation-specific modulation of the sex determination pathway☆

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ABSTRACT

Dichlorodiphenyltrichloroethane (DDT) poses a significant health risk to humans which is associated with genomic DNA hypomethylation. However, the mechanism and biological consequences remain poorly understood. *In vitro* assays confirmed that the DDT metabolites 2,2-bis(p-chlorophenyl)-acetic acid (DDA) and 1-chloro-2,2-bis-(p-chlorophenyl)ethylene (DDMU), but not other DDT metabolites, significantly inhibited DNA methyltransferase 1 (DNMT1) activity, leading to genomic hypomethylation in cell culture assays. DNMT1 as a target for DNA hypomethylation induced by DDT metabolites was also confirmed using cell cultures in which DNMT1 was silenced or highly expressed. DDA and DDMU can modify methylation markers in the promoter regions of sexual development-related genes, and change the expression of *Sox9* and *Oct4* in embryonic stem cells. Molecular docking indicated that DDA and DDMU bound to DNMT1 with high binding affinity. Molecular dynamic simulation revealed that DDA and DDMU acted as allosteric modulators that reshaped the conformation of the catalytic domain of DNMT1. These findings provide a new insight into DDT-induced abnormalities in sexual development and demonstrate that selective binding to DNMT1 by DDA and DDMU can interfere with human DNMT1 activity and regulate the expression of the *Sox9* and *Oct4* genes.

1. Introduction

Dichlorodiphenyltrichloroethane (DDT) is an efficient organochlorine pesticide that was widely used in agriculture in the 1950s–1980s (Eskenazi et al., 2018). Although it has been listed in the Stockholm Convention because of environmental concerns, it is still used in developing countries, such as India and South Africa, to control insects and infectious diseases (Huang et al., 2013). Current annual global usage of DDT for vector control is about 3000–3500 tonnes (Henk et al., 2017). DDT is persistent in different environmental matrices because it is fat-soluble and difficult to degrade (Aichner et al., 2013; Huang et al., 2013). Human exposure to DDT can be via contaminated food, as well as water and dust (Yu et al., 2012). Children can also be exposed to maternal DDT *in utero* and through breastfeeding (Gascon et al., 2015; Zhou et al., 2012). Our previous work showed that the median concentration of DDT and its metabolites (DDTs) in cord sera approached 389 ng/g (lipid weight) and that food consumption was the main route of exposure to DDT (Wang et al., 2013; Yu et al., 2012; Zhang et al., 2018). Thus, the persistent high burden of DDT in serum should not be neglected in human health.

A large body of evidence indicates that DDT can be a potential health hazard, and it has been associated with reproductive failure and developmental retardation, tumorigenesis, and neurodegeneration (Harada et al., 2016; He et al., 2015; IARC, 1991). DDT can undergo transformation into a serial of metabolites, such as dichlorodiphenyldichloroethane (DDD), dichlorodiphenyl-

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dichloroethylene (DDE), 1-chloro-2,2-bis-(p-chlorophenyl) ethylene (DDMU) and 2,2-bis(p-chlorophenyl)-acetic acid (DDA), hereafter referred to as DDTs (Yu et al., 2011). *In vitro* studies have shown that DDTs can bind to nuclear receptors and exhibit estrogen-like and anti-androgenic effects (Tohyama et al., 2015; Zhang et al., 2016). They can also bind to human follitropin receptors and activate the cyclic AMP pathway (Munier et al., 2016). In recent years, there has been growing evidence that DDTs exert toxic effects by perturbing epigenetic modification, especially genomic DNA methylation status. Population studies have shown that genomic methylation levels in leukocyte DNA are significantly associated with serum burdens of p,p'-DDD, o,p'-DDT, and p,p'-DDT (Itoh et al., 2014; Rusiecki et al., 2008). Moreover, DDT represses the transcriptional activity of DNA methyltransferase (DNMT) 3A in the hypothalamus and contributes to DNA hypomethylation in young male rats (Shutoh et al., 2009). DDT can also give rise to low-density cytosine-phospho-guanine (CpG) features in regions of DNA methylation even after multiple generations (Skinner et al., 2018).

DNA methylation, which occurs mainly at the CpG site, plays an important role in regulating gene transcription (Horvath et al., 2018). Abnormal modification of DNA methylation has been found extensively in cancer and other human diseases, even in the initial stages of occurrence (Poirier et al., 2015; Qazi et al., 2017). Consequently, the association between DNA methylation and occurrence of disease has attracted attention and it has become a hot topic in etiological research (Klutstein et al., 2016). DNA methylation homeostasis is regulated by two clusters of enzymes. One cluster is the DNMTs, which catalyze the addition of methyl groups to genomic DNA, and include DNMT1, DNMT3A and DNMT3B. The other cluster is the DNA dioxygenases, which act as erasers of DNA modification by methylation. The ten-eleven translocations (TETs) family of DNA dioxygenases contains TET1, TET2 and TET3 (Koch et al., 2018). Previous studies have shown that chemical pollutants and some small designer molecules can influence the activities of DNMTs and TETs and modify epigenetic status (Hu et al., 2014; Xu et al., 2014). Chua et al. (2019) found that 1-(1,1-biphenyl-3-yl)-4-amino-5-chloropyrimidin-2(1H)-one was an allosteric inhibitor of TETs. Phenolic hormones found in the environment, such as bisphenol A and nonylphenol, can also affect DNMT activity (Xu et al., 2014). DDTs show structural homology with some of the allosteric modulators of DNMTs. This suggests that DNMTs could be potential targets for DDT-induced abnormality of DNA methylation.

Ligand binding triggers specific conformational changes in the activity domains of DNMTs, and thus disturbs the dynamics of DNA modification by methylation. In the laboratory, approaches such as surface plasmon resonance and crystal structure characterization can provide information about binding between ligand and proteins, but such methods are time consuming, laborious, and inefficient (Aristotelous et al., 2015; Moraleja et al., 2015). Virtual screening methods can be an alternative for investigating toxic mechanisms (Hevener et al., 2018; Zhang et al., 2016). Among virtual screening tools, toxicophore moieties that provide qualitative information about structural features of chemicals with specific toxic properties are a promising method for predicting the potential risk of environmental pollutants (Singh et al., 2016). Quintessential toxicophore features usually include hydrogen bond acceptors or donors, and hydrophobic and aromatic groups. Based on these chemical features, some active pollutants retrieved by toxicophore screening are similar to predicted potential targets (Singh et al., 2016). For example, Kortagere et al. (2008) found that chlorine had the highest affinity for leucine, followed by bromine for arginine and fluorine for glycine. However, identification of the toxicophore moiety for persistent organic pollutants to specific target is still unusual. We thus considered it desirable to map the toxicophores of DDTs that target DNA-methylation-associated elements.

In the present study, *in vitro* and *in silico* approaches were adopted to reveal the mechanism and biological consequences of abnormal DNA methylation induced by DDTs. Firstly, the interactions between DDTs and DNMT1 were identified and confirmed by *in vitro* enzymatic activity assay and in cell cultures. Secondly, the potential effects of exposure to DDTs on the modulation of DNA methylation and the expression patterns of sexual determination genes were also investigated in a human cell culture. Finally, the mechanisms by which DDMU and DDA bind to DNMT1 were explored by molecular docking and molecular dynamic simulation.

2. Materials and methods

2.1. Chemicals and reagents

DDT and its metabolites o,p′-DDT, p,p′-DDT, o,p′-DDE, p,p′-DDE, o,p'-DDA, and p,p'-DDA were purchased from AccuStandard (Billerica, MA, USA). Other metabolites o,p′-DDMU and p,p′-DDMU were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The oligonucleotide poly(dI-dC)•poly(dI-dC) was purchased from Shenggong Biotechnology (Shanghai, China). Calf intestine alkaline phosphatase and recombinant human DNMT1 were purchased from New England Biolabs (Beverly, MA, USA). The total protein quantification kit, nuclease P1 (from *Penicillium citrinum*), snake venom phosphodiesterase I, dimethyl sulfoxide (DMSO) and decitabine were obtained from Sigma (St. Louis, MO, USA). The EpiQuik™ nuclear extraction kit I and DNA methyltransferase activity/inhibition assay kit were obtained from EpiGentek (Brooklyn, NY, USA).

2.2. In vitro DNMT1 activity assay

Inhibition of DNMT1 activity by DDTs was determined as described previously (Hu et al., 2014; Iglesias et al., 2016). The assay for DNMT1 activity was carried out in duplicate in a total volume of 10 μL reaction solution for 30 min at 37 °C. The reaction solution contained 1 μg poly(dI-dC)•poly(dI-dC) DNA and 2.5 U recombinant DNMT1 mixed in reaction buffer (50 mM Tris-HCl, 80 μM S-adenosyl-L-methionine, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, pH = 7.8). The assays were carried out in 96-well plates with compounds being screened at doses of 8, 40, 200, 1000, 5000, and 25,000 nM. Methyltransferase reactions were quenched by addition of cold isopropanol (−78 °C). The tubes were centrifuged at 13,400×*g* at 4 °C and the precipitates washed twice with cold ethanol. The modified DNA was dried naturally at room temperature. Negative controls with no DNA substrate were performed for all experiments.

The ratio of 5-mdC to dC in modified DNA samples was used as an indicator of the degree of contamination of DNMT1 activity. Quantification of 5-mdC and dC in DNA samples was carried out using liquid chromatography coupled with tandem mass spectrometry (Hu et al., 2014).

2.3. Cell culture

The human hepatic carcinoma cell (HepG2), non-small cell lung cancer (A549) cell, and embryonic stem (BG01V) cell lines were purchased from the Shanghai Cell Resource Center, Chinese Academy of Sciences. HepG2 cells were maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum in a humidified incubator in 5% CO_2 at

37 °C. A549 cells were cultured in F−12K supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) GlutaMAX™ in a humidified incubator in 5% CO_2 at 37 °C. Embryonic stem (BG01V) cells were cultured in a mixture of DMEM and Ham's F-12 medium supplemented with fetal bovine serum (5%, v/v). Antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) were added to protect against bacterial contamination.

2.4. siRNA transfection

A sequence-specific siRNA duplex against DNMT1 (si-DNMT1) and a negative control (si-NC) were synthesized by the Guangzhou Ribobio Corporation (Guangzhou, China). Oligonucleotides for si-DNMT1 and the negative control were dissolved in sterile water and then diluted in Opti-MEM medium (Gibco, Grand Island, NY, USA). siRNA was transfected into HepG2 and A549 cells (final concentration 50 pM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Cells transfected with siRNA duplexes were cultured in six-well plates for 48 h and DNMT1 silencing was validated by quantitative RT-PCR. The si-DNMT1 and si-NC sequences are given in Supplementary Information.

2.5. Plasmid construction and transfection

The transcript of the DNMT1 gene was amplified using cDNA as a template. The PCR product was inserted into the pEX-3 expression vector to construct the plasmid pEX-3-DNMT1 and insertion was validated by sequencing. pEX-3-DNMT1 (50 ng) or empty vector was transfected into HepG2 and A549 cells using Lipofectamine 2000 in accordance with the manufacturer's protocol. Expression of plasmids in HepG2 and A549 cells was monitored using quantitative real time-PCR.

2.6. DDT exposure, DNA methylation and measurement of DNMT activity

DDTs were dissolved in DMSO to the required concentrations. Experiments were performed when the cells had grown to approximately 80% confluence. The normal cells and DNMT1 knock-down and over-expressed HepG2 and A549 cells were treated with various concentrations (8, 40, 200, 1000, 5000, and 25,000 nM) of decitabine, o,p'-DDT, p,p'-DDT, o,p'-DDE, p,p'-DDE, o,p'-DDMU, p,p'-DDMU, o,p'-DDA, and p,p'-DDA in DMSO (0.1% DMSO, v/v).

After 48 h of treatment, the cells were collected, and genomic DNA was extracted from cell samples using a genomic DNA kit (Tiangen Biotech, China). DNA concentration and quality were measured using a NanoDrop 2000 (Thermo Fisher, Grand Island, NY, USA). Enzymatic hydrolysis of DNA and liquid chromatography coupled with triple quadrupole mass spectrometry detection were performed as described previously (Hu et al., 2014). The experimental procedures of global DNA methylation determination are listed in Supplementary Information. Nuclear proteins were isolated and then quantified from HepG2 and A549 cells exposed to DDTs or the untreated controls, and then 15 μg of nuclear protein was used to measure total DNMT activity using a DNMT1 activity assay kit according to the manufacturers' instruction (EpiGentek, Brooklyn, NY).

2.7. DNA methylation of promoters of Sox9 and Oct4

Genomic DNA was subjected to bisulfite conversion according to the manufacturer's instructions (EpiGentek, Brooklyn, NY, USA). Approximately 100 ng of bisulfite-treated DNA template was used for PCR amplification. The PCR amplicons were purified by gel electrophoresis, collected, and digested for methylated cytosine quantification (Hu et al., 2014). The experimental procedures and primers used for methylation-specific PCR are listed in Supplementary Information.

2.8. Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and digested with DNase I to eliminate genomic DNA contamination. Total RNA was reverse transcribed to cDNA. SYBR Premix Ex Taq™ (Takara, Dalian, China) was used for PCR amplification, and real-time fluorescence detection was carried out on a 7500 Real-Time PCR machine (Applied Biosystems, Foster City, CA. USA). Quantification of expression of target genes was based on the $2^{-\Delta\Delta}$Ct method (Applied Biosystems). To detect the expression of DNMT1 and *Sox9*, we used primers specific for DNMT1, *Sox9*, *Oct4*, and, as a reference standard, *β-actin* (Supplementary Information).

2.9. Molecular docking

A virtual screening strategy was used for discovery of the potential target for DDT-induced DNA abnormal methylation. Molecular docking studies were performed to investigate binding between DDTs and DNMT1. The crystal structure of DNMT1 (PDB ID: 4WXX) was extracted from the RCSB Protein Data Bank (http://www.rcsb.org/). The Autodock 4.2 program was adopted to carry out the molecular docking; docking was centered on the catalytic domain with a genetic algorithm with a 60 × 60 × 60 Å grid box to detect possible binding pockets. Detailed descriptions of homology modeling and model evaluation procedures are provided in Supplementary Information.

2.10. Molecular dynamic (MD) simulation

MD simulations were performed using GROMACS 5.1.5. The best orientations of ligand-receptor complexes derived from molecular docking were used for simulation. Simulation of binding of DDA and DDMU with human DNMT1 was performed. A CHARMM27 all-atom force field was used to develop a topology file for DNMT1. An explicit solvent-simple point charge model (SPC216 water molecules) was used for DNMT1 receptor solvation in a water molecular box. The boundary of the water molecular box and DNMT1 protein was set to a length of 1 nm. The total charges of the two complexes were neutralized by adding Na^+ and Cl^- to achieve a final salt concentration of 0.1 M. The next stage followed 5.0×10^4 steps of the steepest-descent method to make the whole system energetically minimized. The simulation was performed at a constant temperature of 300 K and pressure of one atm for 100 ps according to the V-rescale method and Parrinello-Rahman method respectively (Martonák et al., 2003). When the heated system was equilibrated at 300 K, the next step was to release the ligand restraints. The system was run continually with 5.0×10^6 steps for 10 ns for data collection.

2.11. Statistical analysis

SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used to analyze data and data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was chosen for assessment of the difference in the data between the control and DDTs-exposed groups. In the present study, $p < 0.05$ was defined as indicating statistical significance.

3. Results and discussion

3.1. DNMT1 activity inhibition assays

To investigate further the effects of the four DDT metabolites on DNMT1 enzymatic activity, they were assessed by reaction with DNMT1 coupled with mass spectrometry. Decitabine was used as a positive control. With decitabine at 40 nM, DNMT1 activity was decreased to 74% compared with the negative control group. o,p'-DDA, p,p'-DDA, o,p'-DDMU and p,p'-DDMU inhibited human DNMT1-mediated DNA methylation in a dose-dependent manner (Fig. 1). For example, the DNMT1 activity decreased from 85% with 40 nM o,p'-DDA to 79% with 1 μM o,p'-DDA. In comparison, o,p'-DDMU showed a more potent inhibition of DNMT1-mediated DNA methylation. A dose of 200 nM o,p'-DDA decreased the DNMT1 activity to 82%, while o,p'-DDMU inhibited activity to 76% at the same concentration (Fig. 1A and B). Compared with other DDT metabolites, o,p′-DDE and p,p′-DDE only slightly decreased DNMT1-mediated methylation, and 200 nM p,p'-DDE only decreased DNMT1 activity to 93% ($p > 0.05$). Although genomic DNA hypomethylation induced by DDT has been reported in epidemiological studies (Consales et al., 2016; Itoh et al., 2014; Rusiecki et al., 2008), o,p′-DDT and p,p′-DDT did not show significant inhibition of DNMT1 activity ($p > 0.05$) (Fig. S1). Moreover, results from a cell-based DNMT activity assay showed that DDTs in cells could also inhibit DNMT activity (Fig. 1C), and the data were consistent with those from the *in vitro* DNMT1 inhibition assay. These lines of evidence suggested that DDA and DDMU negatively modulated DNMT1 activity.

The inhibition of DNMT1 activity by DDT metabolites was also in agreement with binding affinity values derived from docking models. All these results demonstrated that DDA and DDMU markedly decrease the DNA methylation activity of human DNMT1.

3.2. DDTs trigger genomic DNA hypomethylation

Encouraged by the *in vitro* results, we also evaluated the effects of DDTs and metabolites on the genomic DNA methylation levels in HepG2 and A549 cells. The decline in genomic DNA methylation in HepG2 cells exposed to DDTs is shown in Fig. 2. It decreased from 5.45% ± 0.70%–2.00% ± 0.19% under 2.5 μM decitabine exposure, with decitabine being the positive control. Exposure to o,p'-DDT, p,p'-DDT, o,p'-DDA, p,p'-DDA, o,p'-DDMU, and p,p'-DDMU also altered genomic DNA methylation levels in HepG2 cells (Fig. 2 and S2). When cells were exposed to 2.5 μM o,p′-DDT, p,p′-DDT, o,p′-DDA, p,p′-DDA, o,p′-DDMU, or p,p′-DDMU, genomic DNA methylation was reduced to 77.8%–82.4% compared with the control group ($p < 0.05$). In contrast, the genomic DNA methylation levels of HepG2 cells in o,p′-DDE and p,p′-DDE exposure groups were in the range 5.25%–5.40%, and there were no obvious changes in genomic DNA methylation levels ($p > 0.05$). Similar results for global DNA hypomethylation were found in A549 cells (Fig. S2). The decrease in DNA methylation induced by DDT could therefore have been caused by DDT metabolism, while DDA and DDMU were the most potent inhibitors.

To explore the role of DNMT1 in genomic DNA hypomethylation induced by DDTs, we compared the cell culture data with data from the *in vitro* DNMT1 activity inhibition assay. The potential DNA hypomethylation in cell culture was consistent with that in the DNMT1 activity inhibition assay, except in the case of the parent compound DDT. The decrease in DNA methylation in cultured cells exposed to DDT could have been due to DDT biotransformation (IARC, 1991). mRNA silencing and overexpression tools were used to confirm the above findings. The expression of DNMT1 in HepG2

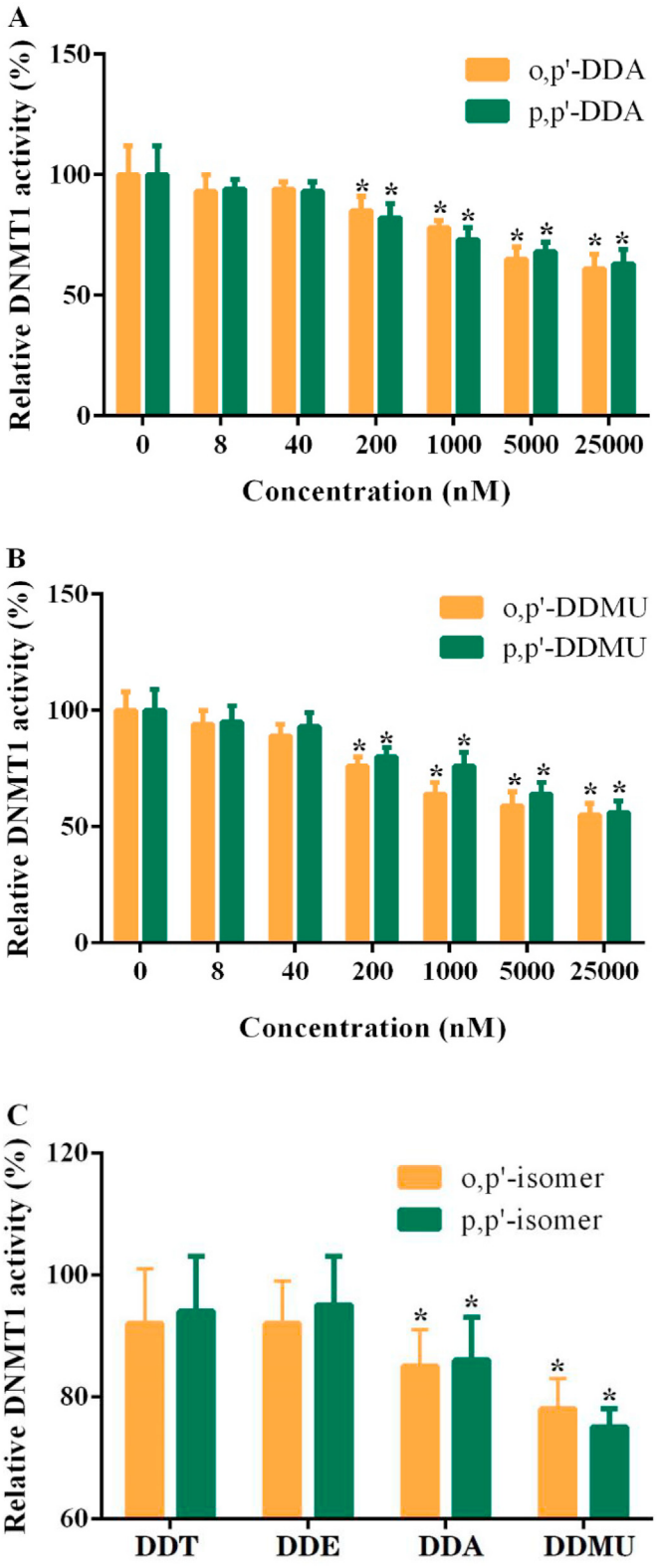

Fig. 1. Effects of DDTs on the activity of DNMT1 in cells and in an *in vitro* assay. (A) and (B) inhibition of DNMT1 activity induced by DDTs in an *in vitro* DNMT1 assay, (C) inhibition of DNMT activity caused by DDTs in a HepG2 cell based assay. The activity of the blank control group was set at 100%, and the data are expressed as mean ± SD, n = 5. * Significant difference with $p < 0.01$.

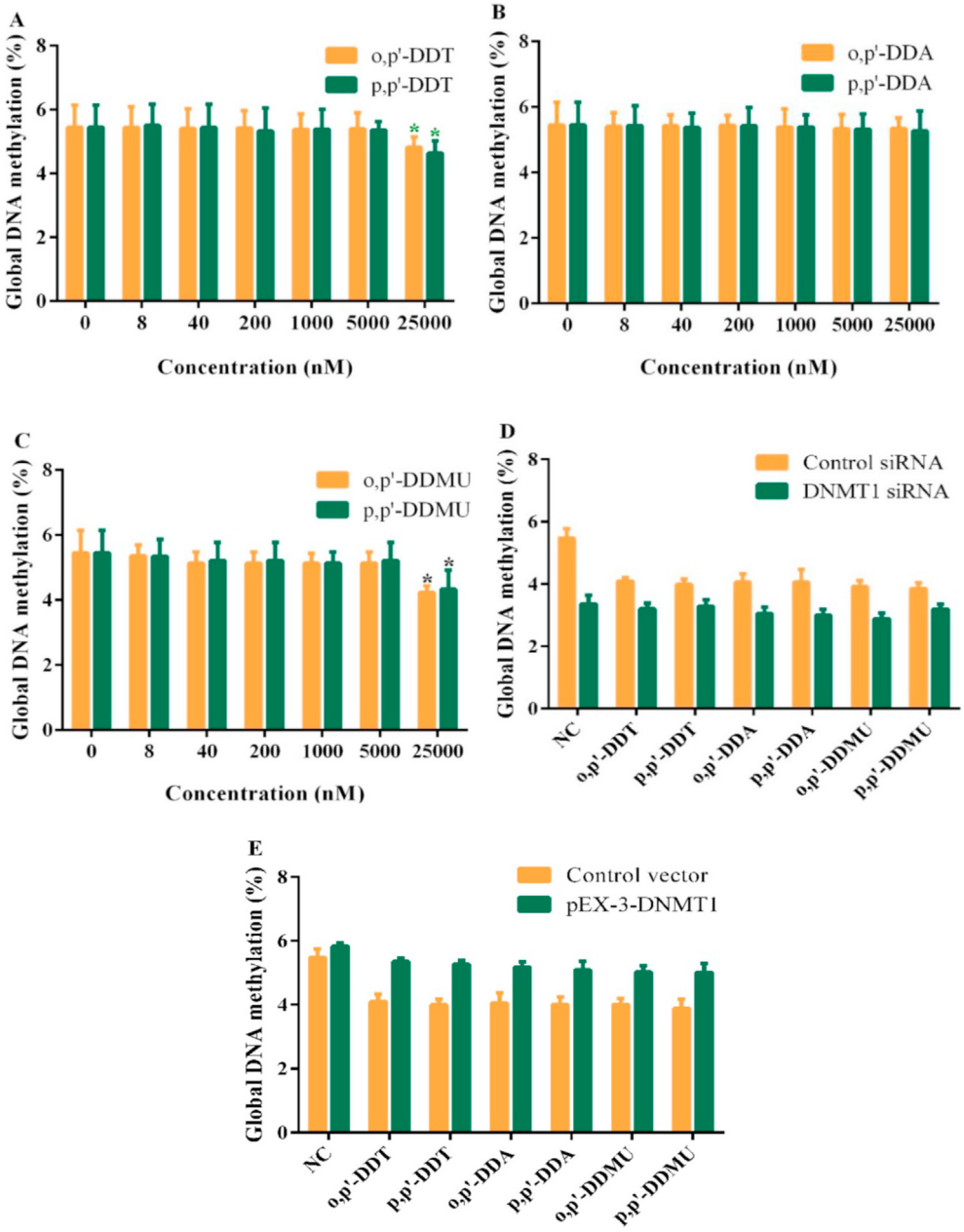

Fig. 2. Effects of DDTs on the level of global DNA methylation in HepG2 cells. The experimental results were expressed as the percentage global DNA methylation level of the human embryonic stem cells in the exposed groups compared to that of the blank control group. (A), (B) and (C) Effects of, respectively, DDT, DDA and DDMU on DNA hypomethylation in HepG2 cells. (D) Effects of silencing *DNMT1* in HepG2 cells on DDTs-induced DNA hypomethylation. (E) Effects of DNA methylation induced by DDTs in HepG2 cells with DNMT1 overexpression. The value for the blank control group was set at 100%, and the data are expressed as mean ± SD, n = 5. * Significant difference with $p < 0.01$.

and A549 cells was knocked down and upregulated using transfected siRNA and the pEX-3-DNMT1 plasmid respectively. Transfection with si-DNMT1 led to an approximately 67%–75% decrease in DNMT1 gene expression compared with that of controls, while transfection of pEX-3-DNMT1 caused approximately fourfold upregulation of DNMT1 gene expression (Fig. S3). DNA methylation levels in DNMT1 knock-down cells were only 61.4% and 68.3% compared with those in normal HepG2 and A549 cells respectively. Exposure to DDTs could further decrease DNA methylation, but the changes were not obvious (Fig. 2E). In contrast, a high level of expression of DNMT1 in cells reversed the decrease in genomic DNA methylation induced by exposure to DDTs (Fig. 2E). For example, o,p'-DDMU (2.5 μM) exposure decreased genomic DNA methylation to 77% compared with the control group, but only to 85.3% in HepG2 cells with increased expression of DNMT1. Thus, we believe that DNMT1 plays a critical role in DNA hypomethylation induced by DDTs.

DNA methylation, which is the most widely studied epigenetic event, changes chromatin structure and DNA stability, and inhibits the transcription and regulation of related genes, potentially leading to diseases such as cancer (Koch et al., 2018). DDT-induced DNA hypomethylation has been observed in previous studies (Itoh et al., 2014; Rusiecki et al., 2008; Shutoh et al., 2009; Skinner et al., 2013). However, the exact mechanism is poorly understood. Our data showed that DDT-induced genomic DNA hypomethylation can be attributed to DDTs, including DDA and DDMU, and not to DDT itself.

The data showed that o,p'-DDA, p,p'-DDA, o,p'-DDMU, and p,p'-DDMU exert significant inhibitory effects on the activity of DNMT1. However, o,p'-DDE and p,p'-DDE have no inhibitory effect on DNMT1 activity and cannot change genomic DNA methylation values. Previous research has shown that p,p'-DDE has no effect on the catalytic activity of DNMT1 and DNMT3A (which carries out *de novo* DNA methylation) in Atlantic salmon (Olsvik et al., 2018), and this is consistent with our results. The present study confirmed that DDA and DDMU caused a significant decrease in genomic DNA methylation in HepG2 and A549 cells, and the decrease in DNA methylation could be attributed to inhibition of DNMT1 activity by DDT metabolites.

3.3. Methylation specific expression of Sox9 and Oct4 genes

The methylation status of the promoters of specific genes can be important features in the regulation of gene expression, so the DNA methylation patterns and transcriptional activities of the sexual determination genes *Sox9* and *Oct4* in response to DDA and DDMU exposure were also explored. The results showed that DDA and DDMU could lead to hypermethylation of *Sox9*, while they caused hypomethylation of *Oct4* (Fig. 3 and S4). A dose response relationship was observed between DDA and DDMU exposure and hypermethylation of the *Sox9* gene promoter. The methylation level of this promoter was increased to 135% at 40 nM o,p'-DDMU compared with that in the control group, and it reached up to 182% at 5000 nM o,p'-DDMU (Fig. 3A). On the other hand, exposure to DDA and DDMU caused significant hypomethylation in the promoter region of the *Oct4* gene (Fig. 3B). The expression levels of the *Sox9* and *Oct4* genes were also determined. The levels of *Sox9* transcription decreased with an increase in DDA and DDMU concentrations (Fig. 3 and S5). For example, the levels of *Sox9* expression were decreased to 45% compared with the control group when exposed to 5000 nM of o,p'-DDMU (Fig. 3C). Moreover, significant induction (1.3−3.9 times) of *Oct4* expression was observed in embryonic stem cells in response to DDA and DDMU exposure.

Sox9 and *Oct4*, which are important during development, exhibit different expression patterns both spatially and temporally (Pamnani et al., 2016). *Sox9* expression is completely restricted to Sertoli cells and is important for the development of the male gonads. *Oct4* is fully expressed at the pre-implantation stage and then expression is strictly restricted to the blastocyst and the implantation process (Pan et al., 2002). In the present study, the hypermethylation in the promoter region of *Sox9* gene were observed, which could block the access of transcription factors to the DNA double strand, thus reducing the transcriptional activity of specific gene and leading to gene silencing. Abnormal DNA methylation patterns in the promoter regions of *Sox9* and *Oct4* were found in embryonic stem cells exposed to DDA or DDMU. DNA methylation is a mechanism that is crucial in controlling sexual determination via precise regulation of gene expression. Previous studies have shown that there is no DNA methylation in the *Sox9* promoter at the fetal stage of testicular development, whereas there is extensive hypermethylation in the *Oct4* promoter (Pamnani et al., 2016). This study also found that the level of *Sox9* expression were decreased to almost half the normal level. *Sox9* protein can activate the gene encoding the testis protein on the Y chromosome (the *TSPY* gene), and thus regulate the development of the testis in males. Clinical research found that low levels of *Sox9* expression were frequently present in patients with testicular dysgenesis syndrome, bisexual symptoms and gonadoblastoma (Buell-Gutbrod et al., 2011; Katoh-Fukui et al., 2015). Two-to four-fold increases in expression of the *Oct4* gene were also observed after DDA or DDMU exposure. This high level of *Oct4* expression may maintain the pluripotency of embryonic stem cells and interrupt the process of sexual differentiation (Pan et al., 2002). These findings indicate that DDA and

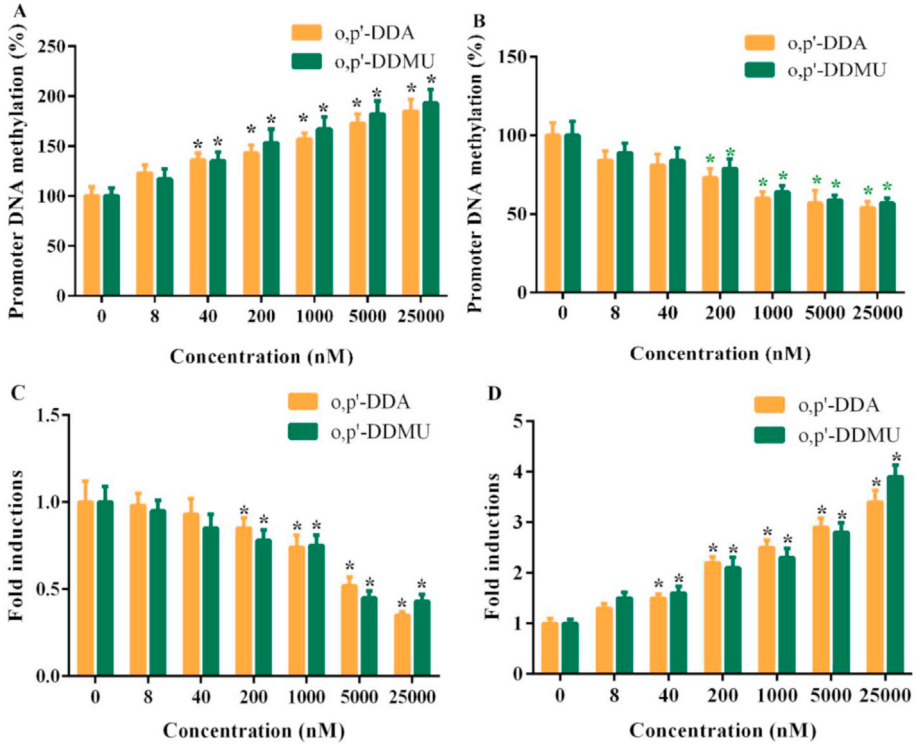

Fig. 3. Effects of DDTs on the level of promoter DNA methylation and expression of *Sox9* and *Oct4* genes in embryonic stem cells. The experimental results are expressed as the relative values for the embryonic stem cells in the exposed groups compared to that of the blank control group. (A) DNA methylation of *Sox9* promoter; (B) DNA methylation of *Oct4* promoter; (C) relative expression of *Sox9* and (D) relative expression of *Oct4*. The data are expressed as mean ± SD, n = 5. * Significant difference with $p < 0.01$.

DDMU can change DNA methylation patterns and disrupt the sexual determination pathway via abnormal expression of the *Sox9* and *Oct4* genes.

3.4. Molecular docking and molecular dynamics studies

An inverse docking strategy was used to screen candidate receptors of DDTs against human DNA methylation homeostasis**;** the 3D structures of DNMT1 was collected from currently available data in the Protein Data Bank or optimized by homology modeling (Fig. S6). Defining the 6.0 Å space around each ligand on the surface of the protein, a ready-to-dock protein pocket matrix was constructed for the DDTs in each of these pockets (Fig. 4). Decitabine-DNMT1 complex was used as a reference ligand-receptor complex for screening as it is a previously-characterized receptor of DDTs (Yoo et al., 2012) whose 3D structure has been resolved. DDA-DNMT1 and DDMU-DNMT1 complexes were ranked as potential candidates and placed immediately behind the decitabine-DNMT1 complex (Fig. 4). Moreover, hydrogen bonds between the carboxyl group of o,p'-DDA and the Asn1229 (aspartic acid) residue of DNMT1 were formed with a 2.96 Å hydrogen bond distance (Fig. 4). In contrast, we did not find any other strong interactions or hydrogen bonds in docked models of DDT and DDE (Figs. S7 and S8). These results suggested that DDA and DDMU bind strongly at the pocket sites of DNMT1.

The binding energy (ΔG) values for DDTs with DNMT1 ranged from −5.0 to −7.6 kcal/mol (Table S1). ΔG values for o,p′-DDA, p,p′-DDA, o,p′-DDMU, and p,p′-DDMU were only slightly lower than those of decitabine, suggesting that these metabolites bind strongly to DNMT1. According to the Autodock software, a ligand might have a higher binding affinity for the receptor if its ΔG value is lower (Trott et al., 2010). Higher ΔG values (from −5.0 to −5.9 kcal/mol) for DDT and DDE compared with DNMT1 indicated that they could not enter the catalytic domain of DNMT1. DDA and DDMU docked deeply into the catalytic pocket of DNMT1 and this was stabilized by a combination of polar and non-polar interactions (Fig. 4). The hydrophobicity of DDA and DDMU played a crucial role in DNMT1 binding, as the amino acid residues Asn1229, Gly874, Glu819, and Gly798 located in the inner surface of the ligand-docked domain of human DNMT1 formed **a** hydrophilic cavity (Table S2). DDA and DDMU both showed electron donor and acceptor interaction between their electron-deficient side chains in the C-bridge and Asn1229 of the DNMT1-ligand binding domain. Of these amino acids, Asn1229 is a key residue in the catalytic domain of DNMT1, suggesting that DDA and DDMU may interrupt the catalytic activity of DNMT1 (Yoo et al., 2012). Taken together, the bioinformatics screen showed that DDA-DNMT1 and DDMU-DNMT1 binding complexes likely act as triggers of DDTs-induced abnormal DNA methylation.

To explore the mechanisms of DDTs binding to DNMTs, the molecular dynamics of the ligand-DNMT1 complex were simulated. The root-mean-square deviation (RMSD) plot of each docking complex was compared with the initial structure (Fig. 5A and S9). A jump in RMSD in both DDA and DDMU was shown within 0.5 ns, which indicated structural relaxation around the initial docking complexes. The patterns of the RMSD plots of the two docking complexes differed in the initial 2.5 ns; hence, the binding of DDA and DDMU was affected by the stability of the complexes. Up to 8 ns, the two ligand-DNMT1 complexes had the most similar pattern of RMSD, suggesting a similar mode of stability for the two docking complexes. In addition, a higher RMSD for the o,p'-DDMU docked complex than for the o,p'-DDA docked complex was observed, which indicated that o,p'-DDMU triggers the maximum possible DNMT1 ligand-binding conformation (Table S3).

The fluctuation of each residue in each ligand-DNMT1 complex was then assessed by root-mean-square fluctuation (RMSF). o,p'-DDA and o,p'-DDMU exhibited high fluctuations in residues 900−1000 with an RMSF $\leq$0.1 nm for the ligand-docked complex, which may have been due to interaction between functional groups (Fig. 5B and S10). High fluctuation values show greater flexibility, while low fluctuation values show limited motion during MD simulation (Ul Haq et al., 2017). It is generally believed that the amino acid residues near the DDTs are more flexible than those located on the junction. We therefore believe that DDA and DDMU show high stability when combined with DNMT1, and the fluctuations of residues at active cavities are the most marked.

To investigate conformational changes in overall structure and binding domain for each docking complex, snapshots of 0, 5 and 10 ns simulation were visualized and analyzed. The o,p'-DDA-DNMT1 and o,p'-DDMU-DNMT1 snapshots from the MD trajectories were collected to explore structural changes in the catalytic domain of DNMT1 (Figs. 4C and 5D). The snapshots did not show any obvious changes in DNMT1 secondary structure (Fig. S11). The four residues in the C-terminal catalytic domain of DNMT1, P1224, S1226, E1266, and R1312, constitute the core catalytic domain for transfer of the methyl group from s-adenosylhomocysteine to cytosine base (Du et al., 2016). From the snapshots, it was clear that the relative position of the four residues was dramatically changed (Fig. S11). For the o,p'-DDA-DNMT1 complex, the distance of the functional groups between C1226 and R1312 began at 10.8 Å and then reduced to 7.8 Å at 5 ns and 8.1 Å at 10 ns. Moreover, the distance of the functional groups between P1224 and E1266 began at 6.4 Å and then reduced to 4.1 Å at 5 ns and then 3.8 Å at 10 ns for the o,p'-DDMU-DNMT1 complex. These snapshots indicated that DDA and DDMU induced conformational changes in the 3D structure of the methyltransferase domain and acted as allosteric modulators for human DNMT1 structure. Moreover, theses changes in structural conformation probably reduce the catalytic activity of DNMT1.

3.5. Toxicological implications of DDT-induced DNA hypomethylation and reproductive toxicity

DNMT1 is a key modifier of epigenetic regulation. Several studies on epigenetic regulation of DNMT1 by endogenous hormones have indicated the crucial biological role of crosstalk between endogenous hormones and epigenetic regulation. Among the compounds that have the potential to disturb dynamic epigenetic regulation, artificial hormones such as bisphenol A and phthalates are the most common (Hu et al., 2019). Estrogen is regarded as an ancient form of hormone and orthologs of estrogen have been found in low-level life forms (Thornton et al., 2003). It has been speculated that estrogenic effects are a consequence of abnormal DNA methylation caused by human hormones (Rusiecki et al., 2008; Zhang et al., 2011). It has been shown that DDT significantly decreases levels of DNMT1, DNMT3A and DNMT3B RNA in male Wistar rats, indicating that transcriptional down-regulation is associated with DNA hypomethylation (Shutoh et al., 2009). Our results now provide strong evidence that alterations in DNMT1 activity play an important role in DDT-induced abnormal DNA methylation. DDA and DDMU readily bind to the catalytic domain of DNMT1 and induce deformation of the chromatin modifier DNMT1. DDA-DNMT1 and DDMU-DNMT1 interactions are the initial events in genomic DNA hypomethylation induced by DDTs.

DDTs are frequently detected in human blood and placenta, even though their usage has been prohibited for more than four decades. The concentrations of total DDTs in the Chinese population are still high and they are also elevated in some other areas, such as Eastern Europe, Central America and other parts of Eastern

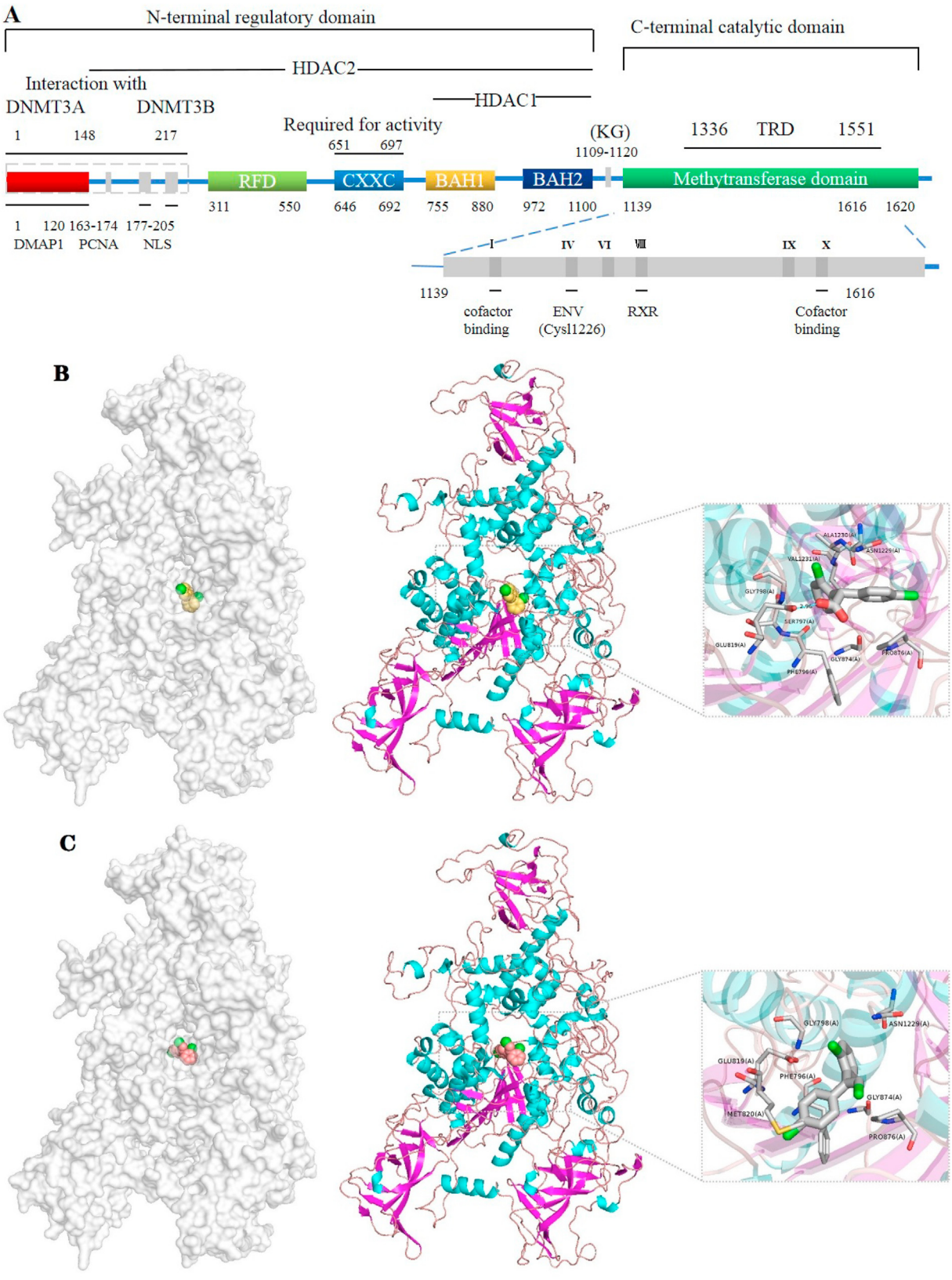

Fig. 4. Molecular docking between o,p'-DDA and o,p'-DDMU and DNMT1. (A) Color-coded domain architecture and sequence numbering of human DNMT1; (B) and (C) molecular docking results for o,p'-DDA and o,p'-DDMU with human DNMT1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

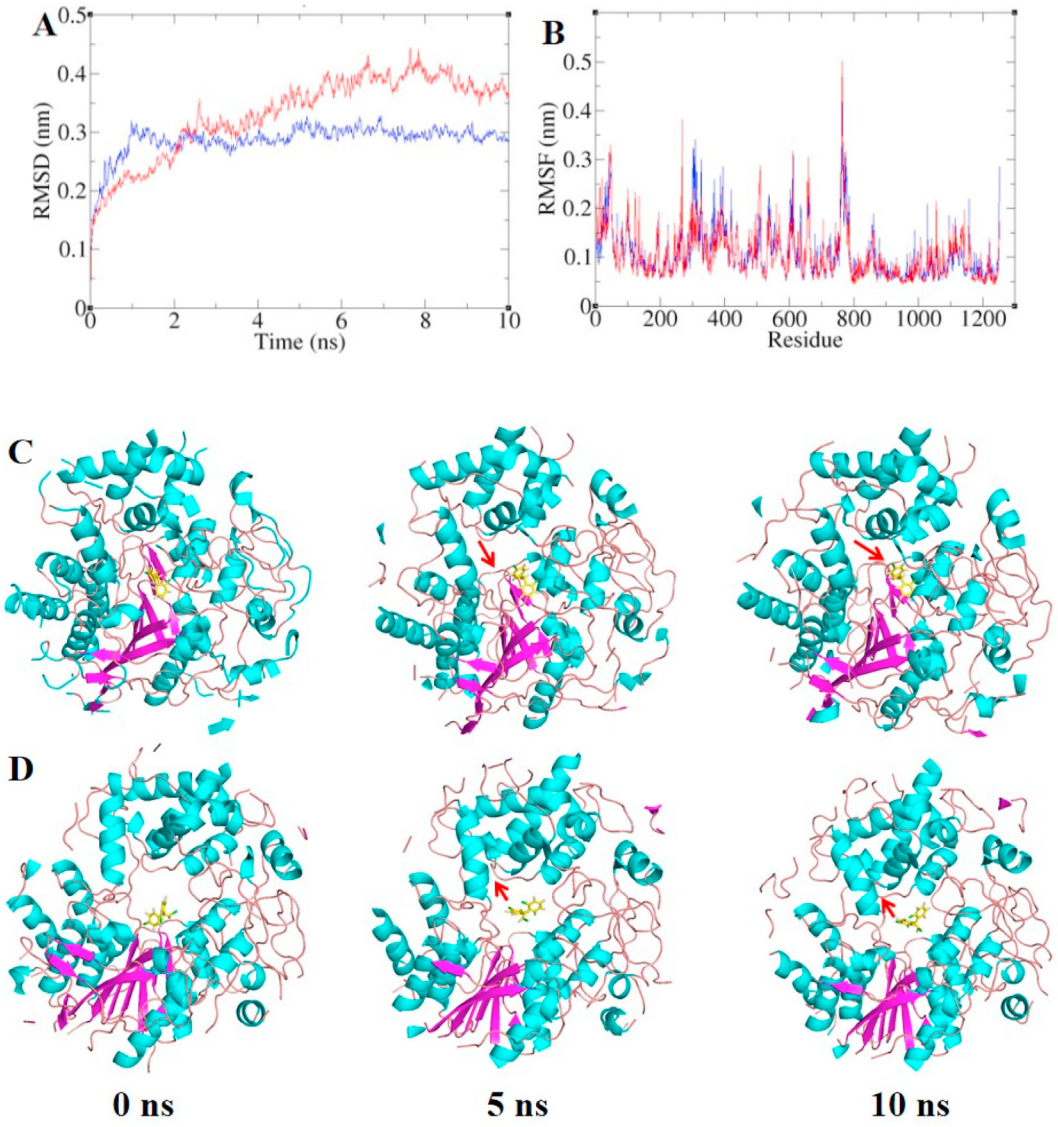

Fig. 5. MD stimulation results of docking o,p'-DDA and o,p'-DDMU with DNMT1. (A) Backbone root-mean-square deviation plot of docked states for o,p'-DDA and o,p'-DDMU with human DNMT1; (B) root-mean-square fluctuation plot of docked states for o,p'-DDA and o,p'-DDMU with human DNMT1; (C) and (D) Conformational changes of active domain in DNMT1 associated with o,p'-DDA and o,p'-DDMU respectively. Observed conformational change in binding pattern of DNMT1 with the apo form of o,p'-DDA and o,p'-DDMU over the course of 100-ns simulations. Time 0 represents the start of the production phase while 100 ns represents the end.

Asia (Koureas et al., 2019). For example, a study reported large concentrations of ∑DDTs in umbilical cord sera in China, with median and 95th percentile concentrations of 1.38 and 5.77 μg/g lipid weight respectively (Xu et al., 2017). On the basis of our study, DDA and DDMU can disrupt DNMT1 activity even at nanomolar levels. Although our data showed that DDE did not significantly inhibit DNMT1 activity, DDE contributes most to the level of ∑DDTs in the environment and in human tissue. DDE can be further biotransformed into other metabolites including DDA and DDMU. The ∑DDTs load in serum should therefore be considered when assessing the risk of DDT-induced abnormality of DNA methylation. Moreover, according to the toxicophore features that we developed, attention should be given to several other chemical pollutants that may impose health risks by affecting the activity of DNMT1, such as low-brominated diphenyl ethers and metabolites of polycyclic aromatic hydrocarbons. If genomic DNA methylation decreases by 0.1%, meaning that millions of methylated cytosine bases are lost from the human genome, it can dramatically change the frequency of gene transcription frequency, as was shown for *Sox9* and *Oct4* in our study. Although there are obvious limitations to the parallel, it is noteworthy that some of the DDT concentrations reported in serum are equal to or higher than the lowest concentrations that we found to be effective in interrupting DNA methyltransferase activity.

4. Conclusion

This study demonstrated, for the first time, that DDA and DDMU are human DNMT1 agonists that are the most potent DNMT1 inhibitors among DDT and its metabolites. DDA and DDMU have agonistic effects on human DNMT1. DDA and DDMU may be able to disrupt DNMT1-mediated DNA methylation at low concentrations. Molecular docking simulation showed that DDA and DDMU could fit into the DNMT1 ligand-binding pocket and induce conformational changes in the catalytic domain. Our study suggests that disruption of DNMT1 and hence the methylation patterns of sexual

determination genes may be one of the mechanisms by which DDTs induce adverse reproductive outcomes.

Credit author statement

Junjie Hu: performed exposure experiments and collected samples, wrote the paper together. Yan Yang. performed exposure experiments and collected samples. Xiaomei Lv: conducted the biochemical and molecular experiments. Zhilang Lao: conducted the in silico study. Lili Yu: performed the statistical analysis. wrote the paper together.

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.

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Appendix A. Supplementary data

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