



# Cisplatin increases carboxylesterases through increasing PXR mediated by the decrease of DEC1

Minqin Xu, Lihua Zhang, Lan Lin, Zhiyi Qiang, Wei Liu, Jian Yang<sup>✉</sup>

Department of Pharmacology, Nanjing Medical University, Nanjing, Jiangsu 211166, China.

## Abstract

*cis*-Diamminedichloroplatinum (CDDP) is widely used for the treatment of various solid cancers. Here we reported that CDDP increased the expression and enzymatic activities of carboxylesterase 1 (CES1) and carboxylesterase 2 (CES2), along with the upregulation of pregnane X receptor (PXR) and the downregulation of differentiated embryonic chondrocyte-expressed gene 1 (DEC1) in human hepatoma cells, primary mouse hepatocytes, mouse liver and intestine. The overexpression or knockdown of PXR alone upregulated or downregulated the CES1 and CES2 expression, respectively. The increases in CES1 and CES2 expression levels induced by CDDP abolished or enhanced by PXR knockdown or overexpression, implying that CDDP induces carboxylesterases through the activation of PXR. Likewise, the overexpression or knockdown of DEC1 alone significantly decreased or increased PXR and its targets. Moreover, the increases of PXR and its targets induced by CDDP were abolished or alleviated by the overexpression or knockdown of DEC1. The overexpression or knockdown of DEC1 affected the response of PXR to CDDP, but not vice versa, suggesting that CDDP increases carboxylesterases by upregulating PXR mediated by the decrease of DEC1. In addition, CDDP did not increase *DEC1* mRNA degradation but suppressed DEC1 promoter reporter activity, indicating that it suppresses DEC1 transcriptionally. The combined use of CDDP and irinotecan had a synergistic effect on two cell lines, especially when CDDP was used first.

**Keywords:** *cis*-diamminedichloroplatinum, pregnane X receptor, differentiated embryonic chondrocyte-expressed gene 1, carboxylesterase 1, carboxylesterase 2, irinotecan

## Introduction

Cisplatin or *cis*-diamminedichloroplatinum (CDDP) is one of the most effective and pioneering metal-based chemotherapeutic drugs<sup>[1]</sup>. It is widely used for the treatment of various solid cancers, such as cancers of head and neck, bladder, lung, cervical, colon and others<sup>[1–3]</sup>. Besides its strong antitumor activity and long-lasting efficacy, CDDP has some side effects,

such as drug resistance and serious adverse reactions including nausea, vomiting, nephrotoxicity, hepatotoxicity, ototoxicity, and neurotoxicity<sup>[4–6]</sup>. The CDDP-induced toxic effects mentioned above are correlated with cytochrome P450 (P450 or CYP), such as CYP2E1 and CYP3A4/5 that produce reactive oxygen species<sup>[7]</sup>. In addition, CDDP can increase a systemic clearance, resulting in a reduced efficacy and even drug resistance due to the induction of its drug

<sup>✉</sup>Corresponding author: Jian Yang, Department of Pharmacology, Nanjing Medical University, Nanjing, Jiangsu 211166, China. Tel: +86-25-86869408, E-mail: [jianyang@njmu.edu.cn](mailto:jianyang@njmu.edu.cn).

Received: 05 March 2023; Revised: 17 May 2023; Accepted: 23 May 2023; Published online: 15 November 2023

CLC number: R96, Document code: A

The authors reported no conflict of interests.

This is an open access article under the Creative Commons Attribution (CC BY 4.0) license, which permits others to distribute, remix, adapt and build upon this work, for commercial use, provided the original work is properly cited.

metabolizing enzymes (DMEs), such as CYP450<sup>[8]</sup>. Therefore, the efficacy and toxicity of CDDP are closely related to DMEs.

The liver and intestine are among the primary sources of DMEs and play a determinant role in drug metabolism<sup>[9]</sup>. The transactivation by two major xenobiotic nuclear receptors, the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), is mainly responsible for the increased expression of these genes<sup>[10]</sup>. Carboxylesterases constitute a group of enzymes that catalyze the hydrolysis of drugs containing functional groups, such as carboxylic acid ester, amide, and thioester<sup>[10-11]</sup>. The liver strongly expresses two major carboxylesterases, including human carboxylesterase 1 (CES1) and human carboxylesterase 2 (CES2), whereas the gastrointestinal tract mainly expresses CES2<sup>[10,12]</sup>. In addition to the difference in tissue distribution, the two enzymes hydrolyze distinct drugs. For instance, CES1 rapidly hydrolyzes clopidogrel, an antithrombotic agent<sup>[12]</sup>; while CES2 rapidly hydrolyzes irinotecan (CPT11), an antitumor drug<sup>[12]</sup>. CDDP can increase CYP3A4 and p-gp through activating PXR<sup>[13-14]</sup>. However, the effect of CDDP on other drug-metabolizing enzymes, such as carboxylesterases, remains uncertain.

Human differentiated embryonic chondrocyte-expressed gene 1 (DEC1), also named mouse stimulated with retinoic acid 13 (STRA13), and rat split and hairy-related protein (SHARP), is one of the basic helix-loop-helix transcriptional factors. It is correlated with cell differentiation, proliferation, biological rhythm and homeostasis of metabolism<sup>[15-16]</sup>. DEC1 can be combined with retinoid X receptor alpha (RXR $\alpha$ ), which impedes the formation of heterodimer of RXR and other nuclear receptors, such as PXR that leads to the repression of PXR function<sup>[17]</sup>. In addition, DEC1 represses CYP3A4 expression directly through binding to proximal promoter of CYP3A4<sup>[18]</sup>.

In the present study, we found that CDDP increased CES1 and CES2 expressions accompanied by increasing PXR but decreasing DEC1 expression. It was hypothesized that CDDP increased CES1 and CES2 expressions and enzymatic activities through increasing PXR expression mediated by the decrease of DEC1 expression *in vitro* and *in vivo*. According to the fact that hydrolysis of CPT11 is required for its cytotoxicity, which is activated by hydrolyzed by CES2<sup>[12]</sup>, it was assumed that the concurrent use of CDDP and CPT11 would enhance the activation of CPT11, thus making a synergistic effect. The outcome could contribute to guiding clinical rational combination of CDDP with CPT11 for the patients

who suffered solid cancers, and is worth further studying and clinical trials.

## Materials and methods

### Reagents and materials

CDDP (Cat. #PHR1624), p-nitrophenylacetic acid (PNPA; Cat. #N20204-100G), clopidogrel hydrogen sulfate (Cat. #Y0001333), irinotecan hydrochloride (Cat. #I1406), rifampicin (RIF; Cat. #557303), and phenobarbital (Cat. #PHR8843) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM; Cat. #12430054) and TRIzol Reagent (Cat. #15596018CN) were from Invitrogen (Carlsbad, CA, USA). Trypase was from NCM Biotech (Cat. #C100C1, Suzhou, China). Monloney murine leukemia (MLV) reverse transcriptase (Cat. #M3681), RNase inhibitor (Cat. #N2111) and Luciferase Assay Reagent (Cat. #E1910) were purchased from Promega (Madison, WI, USA). Fetal bovine serum (FBS) was from Hyclone Laboratories (Cat. #SH30084.03, Logan, Utah, USA). The anti-CES1 was from Abcam (Cat. #ab45957, Cambridge, UK). The anti-CES2, anti-mPXR, and anti-CYP3A4 were kindly provided by Dr. Bingfang Yan (University of Rhode Island, Kingston, RI, USA). PXR shRNA and packaged lentiviral vectors containing *DEC1* shRNA (three viral strains) or lentiviral vectors (as control, shRNA) were from Genechem (Shanghai, China). The reporter constructs (pGL3-DEC1-1.3kb-Luc, pGL3-DEC1-1.1kb-Luc), DEC1 expressed construct and PXR expressed construct were kindly provided by Dr. Bingfang Yan. Restore PLUS Western Blot Stripping Buffer was from Thermo Fisher Scientific (Waltham, MA, USA). Antibody against DEC1 was from Santa Cruz (Cat. #sc-101023, Santa Cruz, CA, USA), antibody against  $\beta$ -actin was from Bioworld (Cat. #BS6007M, St. Louis Park, USA), and antibodies against CYP3A4 and PXR were from Abcam (Cat. #ab3572 and #ab118366, respectively). Goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase was from Proteintech Group (Cat. #SA00001-1, Chicago, Illinois, USA), and BCA protein assay kit were from Pierce Chemical (Cat. #23227, Pierce, Rockford, IL, USA). ECL Western blotting detection system was from Vazyme Biotech Co., Ltd. (Cat. #E412-02, Nanjing, Jiangsu, China). GenJet DNA Vitro Transfection Reagent (Ver. II) was from SignaGen Laboratories (Cat. #SL100488, Gaithersburg, MD, USA). All other chemicals were obtained from Sigma-Aldrich.

### Cell culture and treatment

Hepatoma (HepG2) and colon adenocarcinoma

(SW480) cells were from American Type Culture Collection (Manassas, VA, USA). Cells were seeded at the density of  $5 \times 10^6$  cells/well (6-well plates for protein level),  $5 \times 10^5$  cells/well (12-well plates for mRNA level), or 5 000 cells/well (96-well plates for methylthiazolyldiphenyl-tetrazolium bromide [MTT] assay) in DMEM with 5% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin in a humidified environment with 5% CO<sub>2</sub> at 37 °C overnight. The cells were treated with CDDP (0, 1.25, 2.5, 5, and 10 μmol/L) for 24 h, and the treated cells were cultured in a 1% serum-reduced medium. RIF (10 μmol/L) was treated as a positive control.

### Primary mouse hepatocytes culture

Primary mouse hepatocytes were isolated from male ICR mice at the age of four weeks (obtained from the experimental animal center of Nanjing medical University, Nanjing, China), referred to the two-step perfusion method as was described previously with some modification<sup>[19]</sup>. The isolated primary mouse hepatocytes were plated at the density of  $4 \times 10^6$  cells/well into collagen coated 6-well plates and were maintained at 37 °C, in a humidified atmosphere of 5% CO<sub>2</sub> for 4 h to allow attachment. Primary mouse hepatocytes were then washed by PBS and supplied with fetal bovine serum-free medium. After being continually cultured for 2 days with a change of fresh medium, primary mouse hepatocytes were treated with CDDP (0, 1.25, 2.5, 5, and 10 μmol/L) for another 24 h. PB (1 mmol/L) was treated as a positive control.

### Animals and drug treatment

Thirty male ICR mice (4-week-old, 20–25 g weight, either sex) were obtained from Jiangsu Province's Medical Experimental Animal Center (Nanjing, Jiangsu, China). Mice were kept under environmentally controlled conditions (ambient temperature, 22 °C; humidity, 40%) in a 12-h light/dark cycle with food and water *ad libitum*. After being domesticated for one week, mice were divided into three groups (control, CDDP-2.5, and CDDP-5, with 10 mice in each group). Mice in the CDDP-2.5 and CDDP-5 groups were injected intraperitoneally with CDDP 2.5 or 5 mg/(kg·day) for 3 days, respectively, and mice in the control group were injected intraperitoneally with the same volume of normal saline. The CDDP dosage in mice was converted from human dosage using body surface area normalization<sup>[20]</sup> and referred to the previous studies<sup>[21]</sup>. By the time of 24 h after the last injection for sacrifice, mice were intraperitoneally injected with ketamine (1 mL/kg at 100 mg/mL). When mice were

completely anesthetized (approximately 5 min), surgery was performed to expose the livers. The liver was perfused with PBS through the portal vein to remove blood. The upper intestine of mice was isolated and washed with cold PBS. The perfused liver and washed intestine were then divided into two parts, with one part being immediately used for preparing total RNA and the other one frozen at –80 °C for preparing S9 fraction.

*Dec1*<sup>+/-</sup> C57BL/6 mice (RBRC04841) were obtained from the BRC (RIKEN BioResource Center, Japan). Heterozygous adult male mice (*Dec1*<sup>+/-</sup>) were crossed with adult female mice (*Dec1*<sup>+/-</sup>) to generate homozygous mice (*Dec1*<sup>+/+</sup> and *Dec1*<sup>-/-</sup>)<sup>[22]</sup>. Double checks (after birth and before the experiment) were applied to make sure the correct mouse genotype. The mouse genotype identification was presented in **Supplementary Fig. 1** (available online). Totally, 24 mice of two types (*Dec1*<sup>+/+</sup>, *Dec1*<sup>-/-</sup>, *n* = 12 in each group, regardless of sex) were used. The total RNA and S9 fraction of the liver and intestine were prepared as described below.

The use of animals was approved by the Institutional Animal Care and Use Committee (IACUC, approval No. IACUC-2202043) of Nanjing Medical University. The procedures followed the guidelines for the care and use of animals established by the IACUC. Every effort was made to minimize animal suffering and to reduce the number of animals used for experiments.

### Preparation of S9 fractions

The frozen livers were thawed in homogenization buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L KCl and 2 mmol/L EDTA), and then homogenized with a six-pass Teflon mortar and pestle driven by a Wharton stirrer. Homogenates were centrifuged at 10 000 *g* at 4 °C for 20 min. After that, S9 fraction of the liver (supernatant) was assayed for the hydrolysis of PNPA and for Western blotting.

### Quantitative reverse transcription-PCR (qRT-PCR) assay

Total RNA was extracted by using TRIzol and checked by 1.5% agarose gel electrophoresis for quality control. The first-strand cDNA was synthesized by using the total RNA (1 μg) at 25 °C for 10 min, 42 °C for 50 min, and 70 °C for 10 min by using oligo (dT15) and M-MLV reverse transcriptase. qRT-PCR was performed by using FastStar Universal SYBR Green Master with the 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The primers were shown in **Table 1**. All data were normalized to the *GAPDH* or *ACTB*.

**Table 1 The primers for human genes and mouse genes**

Genes	Forward (5'→3')	Reverse (5'→3')
<b>Human</b>		
<i>CES1</i>	ACCCCTGAGGTTTACTCCACC	TGCACATAGGAGGGTACGAGG
<i>CES2</i>	CATGGCTTCCTGTATGATGGT	CTCCAAAGTGGGCGATATTCTG
<i>PXR</i>	CGAGCTCCGCAGCATCA	TGTATGTCCTGGATGCGCA
<i>DEC1</i>	GGCGGGGAATAAAACGGAGCGA	CCTCACGGGCACAAGTCTGGAA
<i>CYP3A4</i>	TCAATAACAGTCTTCCATTCTCAT	CTTCGAGGCGACTTCTTTCA
<i>GAPDH</i>	GTATGTCGTGGAGTCTACTGGTGTG	GGTGCAGGATGCATTGCTGACATTC
<b>Mouse</b>		
<i>Ces1d</i>	GGCATCAACAAGCAAGAGTTTGGC	CTTTTGGTGAGGTGATCTGTCCC
<i>Ces1e</i>	TTCAAGGATGTCAGACCACC	AACACATTTTTTTTGATACAGGGTA
<i>Pxr</i>	GATGGAGGTCTTCAAATCTGCC	GGCCCTTCTGAAAAACCCCT
<i>Stral3</i>	ACGGAGACCTGTCAGGGATG	GGCAGTTTGTAAGTTTCCTTGC
<i>Cyp3a11</i>	CTTTCCTTCACCCTGCATTCC	CTTTCCTTCACCCTGCATTCC
<i>Actb</i>	TAAAGACCTCTATGCCAACACAGT	CACGATGGAGGGGCCGACTCATC

### Enzyme activity assay

The total hydrolytic activity was spectrophotometrically determined with standard substrate PNPA. The treated cell suspension was sonicated by a sonifier (Nanjing, China), and the cell debris was precipitated by centrifugation at 12 000 *g* at 4 °C for 15 min. The supernatants or S9 fractions of mouse liver were assayed for hydrolytic activity toward PNPA as described previously<sup>[9]</sup>. A sample cuvette (1 mL) contained 10 µg of cell lysates or S9 fractions from the liver diluted in 100 mmol/L potassium phosphate buffer (pH 7.4), and substrate (1 mmol/L) at room temperature. Reactions were initiated by adding PNPA (10 µL of 100 mmol/L stock in acetonitrile), and the hydrolytic rate was recorded from an increase in absorbance at 400 nm. The extinction coefficient (E400) was determined as 13 mmol/(L·cm). Several controls were conducted including incubation with no protein.

### Cell viability and morphology

Cell viability was detected by the MTT assay. HepG2 cells were seeded into 96-well plates at the density of 5 000 cells per well overnight. Cells were treated with CDDP (0, 6.25, 12.5, 25, 50, and 100 µmol/L) for 24 h. The same volume of PBS was as the control. Then, 20 µL of 5 mg/mL MTT was added to the cells, and the cells were incubated at 37 °C for another 4 h. The culture medium was discarded, and 0.1 mL DMSO was used to dissolve the precipitate. The absorbance at 570 nm [*D*(570 nm)] was measured using an Automated Microplated Reader ELx800

(BioTek, Winooski, VT, USA).

After treatment with or without CDDP (5 µmol/L) for 12 h, the cells were washed twice with DMEM and treated with oseltamivir (100 µmol/L), clopidogrel (100 µmol/L), or CPT11 (40 µmol/L), respectively, for another 24 h. The morphological changes were observed and images were taken under an inverted light microscope (Nikon, Japan, at 22 °C). Subsequently, the cells were subjected to the MTT assay.

For the synergistic effect assay, HepG2 or SW480 cells were seeded into 96-well plates at a density of 5 000 cells per well overnight. The cells were treated with CPT11 (0, 5, 10, 20, and 80 µmol/L) alone or together with CDDP (5 µmol/L) for 24 h; or the cells were treated with CDDP (5 µmol/L) for 2 h first, and then treated with CPT11 (0, 5, 10, 20, and 80 µmol/L) (including CDDP) for another 22 h. After that cell viability was determined by using MTT, IC<sub>50</sub> of CPT11 was calculated. For overexpression or knockdown experiments, transfected cells (vector, sh-*DEC1*, OE-*DEC1*), (vector, si-*PXR*, OE-*PXR*), or (vector, OE-*DEC1* + si-*PXR*, sh-*DEC1* + OE-*PXR*) were seeded into 96-well plates at a density of 5 000 cells per well overnight. The cells were initially treated with CDDP (5 µmol/L) for 2 h, followed by treatment with CPT11 (0, 5, 10, 20, and 80 µmol/L) (including CDDP) for another 22 h. After that cell viability was determined by using MTT, and the IC<sub>50</sub> of CPT11 was calculated.

### Modulation of PXR or DEC1 expression by RNA interference and overexpression

For the RNA interference experiment, HepG2 or



SW480 cells were plated in 6-well plates overnight at the density of  $6 \times 10^5$  cells/well in DMEM supplemented with 5% FBS. Transfection was conducted with GenJet (Ver II). With the transfection mixtures containing 1  $\mu$ g of si-*PXR* construct or an equal amount of corresponding vector, the transfected cells were maintained for 48 h. Alternatively, the cells were lentivirally transduced with the most effective virus strains (LV-sh*DEC1* or LV-Con). After 12 h of infection with LV-sh*DEC1* or LV-Con (multiplicity of infection of 20), the purified *DEC1* knockdown cells were obtained by continued screening with medium containing puromycin. The transfected or infected cells were treated with CDDP (5  $\mu$ mol/L) or DMSO (0.1%, v/v) for 24 h. Subsequently, the cells were harvested, and the protein was extracted. The expressions of CES1, CES2, CYP3A4, and PXR was determined by Western blotting analysis.

For overexpression, HepG2 cells were transfected with 1  $\mu$ g of *PXR* (or *DEC1*) construct or an equal amount of corresponding vector. After 24 h of incubation, the transfected cells were treated with the same treatment as mentioned above. Western blotting analysis was used to determine the expressions of CES1, CES2, CYP3A4, and PXR. The cell lysates (60  $\mu$ g for knockdown, 10  $\mu$ g for overexpression) were detected for the efficiency of knockdown and overexpression of PXR (or *DEC1*) by Western blotting analysis.

### Luciferase assay

*DEC1* promoter reporters (1.3 kb and 1.1 kb) were donated by Dr. Yan from the University of Rhode Island. HepG2 cells were plated in 48-well plates in DMEM with 10% FBS at the density of  $1 \times 10^5$  cells per well. The transfection was conducted by GenJet DNA Vitro Transfection Reagent (Ver. II). The transfection mixtures contained 50 ng of a reporter plasmid (pGL3-*DEC1*-1.3-Luc or pGL3-*DEC1*-1.1-Luc) along with 5 ng of pRL-TK. After incubation at 37 °C for 12 h, the transfected cells were treated with either CDDP (5  $\mu$ mol/L) or the same volume of PBS for another 24 h. Cells were washed twice with PBS and then lysed by passive lysis buffer (1 $\times$ ; Promega). The collected cells were performed to freeze/thaw two cycles. The reporter enzyme activities were determined with the dual-luciferase reporter assay system. This system contained two substrates, the firefly luminescence and Renilla luminescence, which were used to determine the activities of two luciferases sequentially. The firefly luciferase activity, reflecting the reporter activity, was measured by mixing an aliquot of 10  $\mu$ L lysates with Luciferase Assay Reagent II (Promega). After that, the firefly luminescence was quenched, and the Renilla luminescence was activated simultaneously by

injecting Stop & Glo reagent (Promega) to the sample tubes. The firefly luminescence signal intensity was normalized based on the intensity of Renilla luminescence signal as normalized luciferase activity. The ratio of CDDP and PBS treatment normalized luciferase activity representing relative luciferase activity.

### Western blotting analysis

Cell (HepG2 or primary mouse hepatocytes) lysates (80, 40, 20, or 10  $\mu$ g) or S9 fractions of mouse liver or intestine (30  $\mu$ g) were resolved by 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride membrane pretreated with methanol. After non-specific binding sites were blocked with 5% non-fat milk, the blots were incubated with an antibody against CES1 (1 : 2 000), CES2 (1 : 2 000), CYP3A4 (1 : 2 000), CYP3A11 (1 : 2 000), *DEC1* (1 : 2 000), human PXR (1 : 1 000), mouse PXR (1 : 1 000), or  $\beta$ -actin (1 : 5 000). The primary antibodies were subsequently localized with goat anti-rabbit IgG conjugated with horseradish peroxidase. Horseradish peroxidase activity was detected with a chemiluminescent kit (Pierce). The protein bands were visualized with an enhanced chemiluminescence detection system. To eliminate the systematic errors between the sample adding and the different gels, the target proteins and the internal reference protein ran out on the same membrane. Therefore, membranes were horizontally stripped or cut and then reprobbed. For example, samples were loaded on the same gel with a marker for detecting CYP3A4, CES1, and CES2. Then, they were electrophoretically transferred to a PVDF membrane. The membrane was horizontally cut along with 50 kDa into two parts. One membrane (> 50 kDa) was detected for CYP3A4, and then it was washed three times with TBS and stripped with Restor PLUS Western Blot Stripping Buffer for 15 min at 37 °C with gentle shaking to remove the blot. After being washed three times with TBS, the membrane (> 50 kDa) was detected for CES1 and CES2 successively with Western blotting. The other membrane (< 50 kDa) was detected for  $\beta$ -actin. Protein levels were quantified by density analysis using Image Analysis software (NIH), and expressed as interest protein/ $\beta$ -actin. Protein concentrations were determined with the BCA protein assay based on albumin standard.

### Statistical analysis

Data were represented as means  $\pm$  standard deviation. Statistical analysis for multiple comparisons was performed using SPSS software (SPSS, version 22, Chicago, IL, USA). The significance was determined by one-way or two-way analysis of

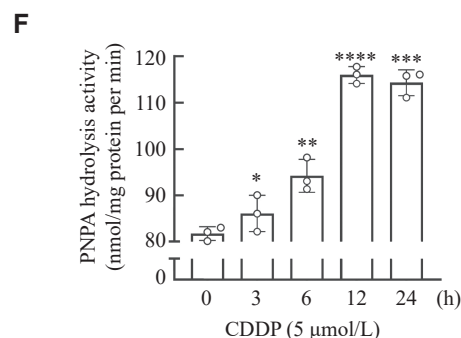
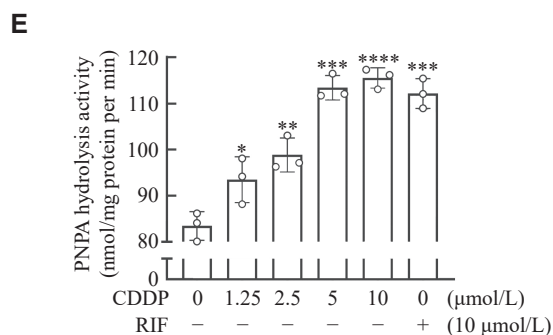
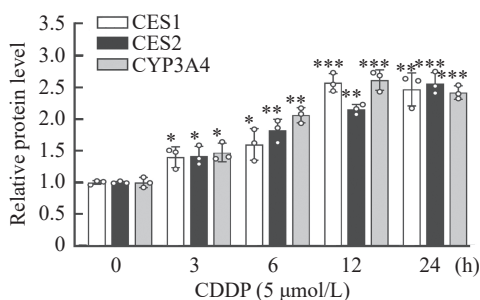
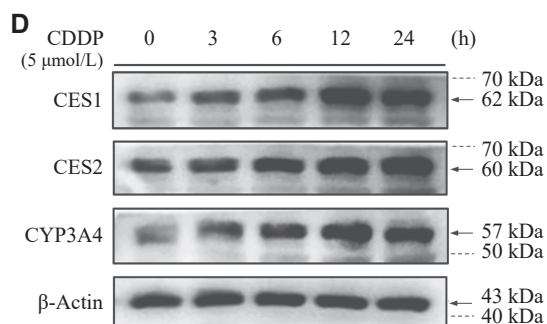
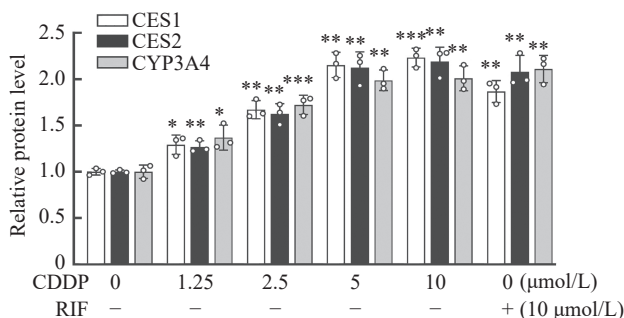
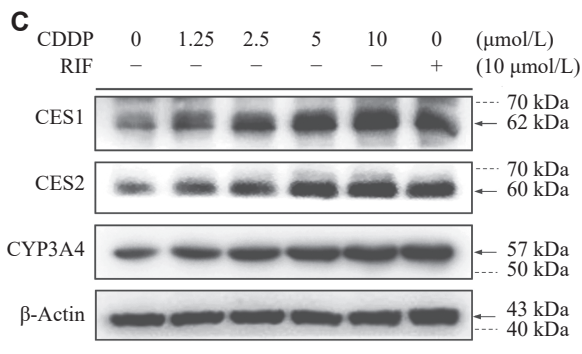
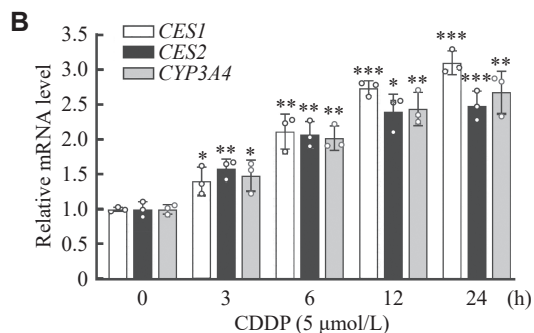
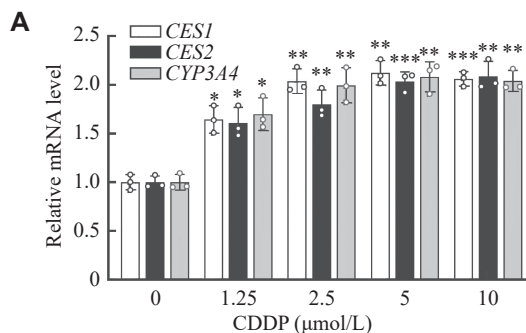
variance, followed by Tukey's post hoc test, and the paired comparisons were analyzed by Student's *t*-test. The differences were considered statistically significant when  $P < 0.05$ .

**Results**

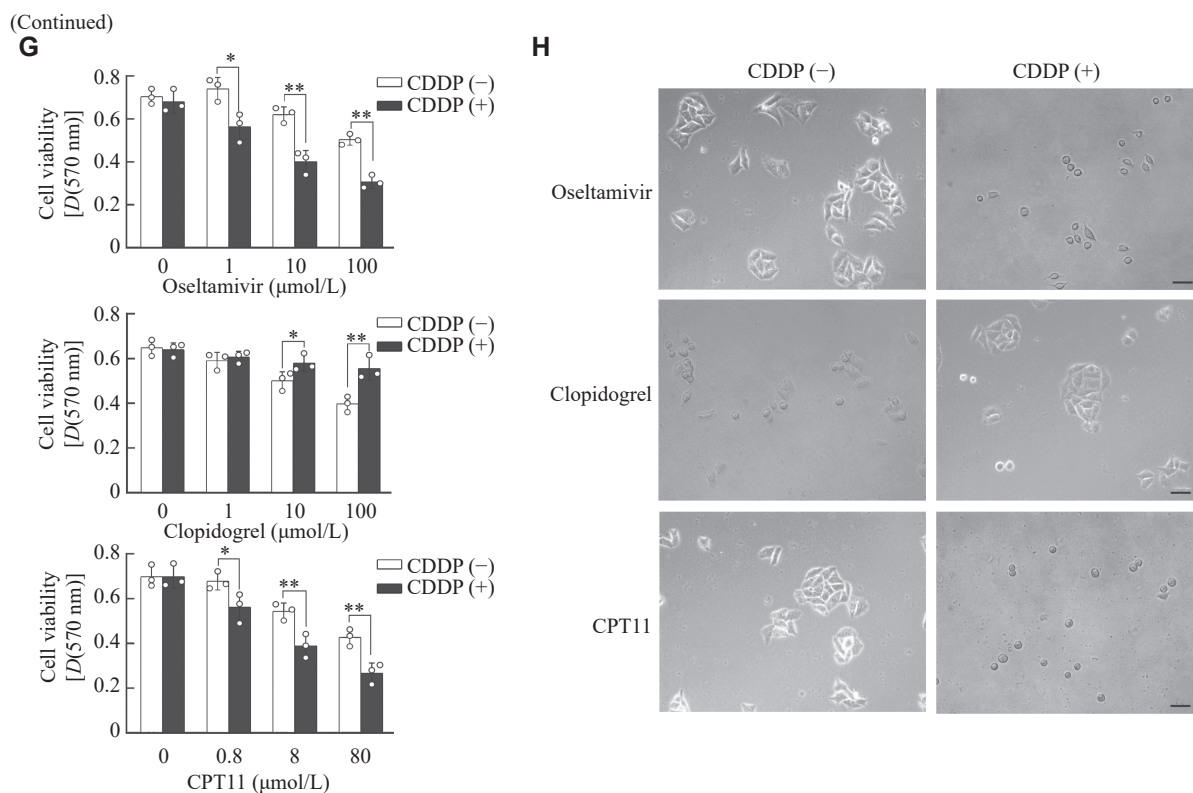
**CDDP induced the expression and activities of CES1 and CES2 in HepG2 cells**

First, we tested whether CDDP (6.25–100  $\mu\text{mol/L}$ )

affected the cell viability and determined the dose range of CDDP. As shown in **Supplementary Fig. 2A** (available online), when the dosage of CDDP exceeded 25  $\mu\text{mol/L}$ , the cell viability dropped below 40% in HepG2 Cells. Therefore, the dose range of CDDP was set between 1.25  $\mu\text{mol/L}$  and 10  $\mu\text{mol/L}$ . HepG2 cells were treated with CDDP (1.25, 2.5, 5, and 10  $\mu\text{mol/L}$ ), or the same volume of PBS for 24 h or just one dose of CDDP (5  $\mu\text{mol/L}$ , non-cytotoxic, **Supplementary Fig. 2B**, available online) for



(Continued)



**Fig. 1** Induction of CES1 and CES2 expression as well as their activities in HepG2 cells. A–F: HepG2 cells were treated with various concentrations (0, 1.25, 2.5, 5, and 10  $\mu\text{mol/L}$ ) of CDDP or RIF (10  $\mu\text{mol/L}$ , as a positive control) for 24 h or with the same concentration (5  $\mu\text{mol/L}$ ) of CDDP for 0, 3, 6, 12, and 24 h. mRNA and protein levels of CES1, CES2, and CYP3A4 were detected by qRT-PCR (A and B) and Western blotting (C and D), respectively. *Gapdh* was used as a reference gene for qRT-PCR and  $\beta$ -actin was used as a loading control for Western blotting. The overall hydrolysis activities of cell lysates were determined with standard substrate PNPA (E and F). G: HepG2 cells were pretreated with or without CDDP (5  $\mu\text{mol/L}$ ) for 12 h and then treated with oseltamivir, clopidogrel, or CPT11 for another 24 h. Cell viability was determined by MTT assay. H: Morphological analysis of the HepG2 cells treated with oseltamivir (100  $\mu\text{mol/L}$ ), clopidogrel (100  $\mu\text{mol/L}$ ), or CPT11 (80  $\mu\text{mol/L}$ ) for 48 h with or without CDDP (5  $\mu\text{mol/L}$ ) pretreatment. Scale bar: 50  $\mu\text{m}$ . The data are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). The significance was determined by one-way analysis of variance, followed by Tukey's post hoc test and the paired comparisons were analyzed by Student's *t*-test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$  vs. the control group (0  $\mu\text{mol/L}$  or 0 h) or comparisons shown in the figure. Abbreviations: CDDP, *cis*-diamminedichloroplatinum; RIF, rifampicin; CES1, carboxylesterase 1; CES2, carboxylesterase 2; CYP3A4, cytochrome P450 3A4; PNPA, p-nitrophenylacetic acid; CPT11, irinotecan.

corresponding times (0, 3, 6, 12, and 24 h). As shown in **Fig. 1**, the treatment with CDDP consistently increased both mRNA and protein levels of CES1, CES2, and CYP3A4 in a dose- and time-dependent manner (**Fig. 1A–1D**). RIF also increased protein expressions of CYP3A4, CES1, and CES2 (**Fig. 1C**). Here, CYP3A4 acted as a positive control induced by CDDP<sup>[17]</sup>. Consistent with the increases in both mRNA and protein levels of CES1 and CES2, the overall hydrolysis of PNPA significantly increased both in a dose- and time-dependent manner (**Fig. 1E and 1F**). It was noticed that the most significant induction of CDDP was at 5  $\mu\text{mol/L}$  (**Fig 1A and 1C**), whereas some cytotoxicity was detected in the cells treated with 12.5  $\mu\text{mol/L}$  of CDDP, based on the MTT assay (**Supplementary Fig. 2A**) and microscopic observation. Therefore, 5  $\mu\text{mol/L}$  of CDDP (non-cytotoxic, **Supplementary Fig. 2B**) was chosen to investigate the mechanism in the following experiments.

Because PNPA was not a specific substrate for carboxylesterase isozymes, we subsequently investigated toxicological consequences of the increase of CES1 and CES2 induced by CDDP. According to previous studies, CES1 and CES2 differed markedly in the hydrolysis of oseltamivir (antiviral drug), clopidogrel (antiplatelet drug), and CPT11 (anticancer agent)<sup>[12,23]</sup>. CES1 rapidly hydrolyzes oseltamivir and clopidogrel, whereas CES2 mainly hydrolyzes CPT11. Importantly, the hydrolysis of oseltamivir and clopidogrel is represented a higher or a lower toxicity than their parent drugs, respectively; while the hydrolysis of CPT11 is represented a higher toxicity than its parent drug. HepG2 cells were first treated with CDDP (5  $\mu\text{mol/L}$ ) for 12 h, washed twice with DMEM, and then treated with oseltamivir, clopidogrel or CPT11. After incubation for another 24 h, cell viability was measured by the MTT assay, and morphologic changes were detected under a microscope before the

MTT assay. The cells pretreated with CDDP alone showed no difference in cell viability, compared with those non-pretreated with CDDP (**Fig. 1G**). However, cells pretreated with CDDP followed with oseltamivir (1, 10, and 100  $\mu\text{mol/L}$ ), clopidogrel (1, 10, and 100  $\mu\text{mol/L}$ ) or CPT11 (0.8, 8, and 80  $\mu\text{mol/L}$ ) presented statistically significant cell viability changes at the corresponding concentration, compared with those non-pretreated with CDDP (**Fig. 1G**). For example, the cells pretreated with CDDP exhibited a significant decrease in cell viability when exposed to oseltamivir at corresponding concentration, compared with those without CDDP (**Fig. 1G**, top). Oppositely, the cells pretreated with CDDP exhibited a significant increase in cell viability when exposed to clopidogrel at 10  $\mu\text{mol/L}$ , compared with that non-exposed to CDDP (**Fig. 1G**, middle), implying that CDDP induced CES1 activity. In addition, the cells pretreated with CDDP exhibited a significant decrease in cell viability when exposed to CPT11, compared with that in non-pretreated cells (**Fig. 1G**, bottom), suggesting that CDDP increased CES2 activity. The changes in cell morphology were consistent with those in cell viability. As shown in **Fig. 1H**, under a bright field, cells pretreated with CDDP were spread, and projects were well extended, while the cells without CDDP pretreatment were round, isolated, and aggregated when exposed to 100  $\mu\text{mol/L}$  oseltamivir for 48 h (**Fig. 1H**, top). Conversely, cells pretreated with CDDP and exposed to 100  $\mu\text{mol/L}$  clopidogrel for 48 h showed opposite results (**Fig. 1H**, middle). Likewise, when exposed to 80  $\mu\text{mol/L}$  CPT11 for 48 h, cells without CDDP pretreatment were morphologically normal, whereas cells pretreated with CDDP were isolated, round and shrank (**Fig. 1H**, bottom). These data indicated that CDDP induced the CES1 and CES2 expression as well as their activities in HepG2 cells.

#### **Inverse regulation of PXR and DEC1 expression by CDDP in HepG2 cells**

PXR is involved in the transcriptional regulation of CYP3A4 and CESs<sup>[24]</sup>, and DEC1 represses PXR by bounding to RXR $\alpha$ <sup>[17]</sup>. To explore the roles of PXR and DEC1 in the increase of CES1, CES2, and CYP3A4 expressions induced by CDDP, we examined the expressions of PXR and DEC1 in HepG2 cells treated with CDDP. As shown in **Fig. 2**, CDDP stimulated PXR but inhibited DEC1 expression at both mRNA and protein levels in a dose- and time-dependent manner (**Fig. 2A–2D**), suggesting that CDDP increased CES1, CES2, and CYP3A4 expressions along with increasing PXR but decreasing DEC1 expression.

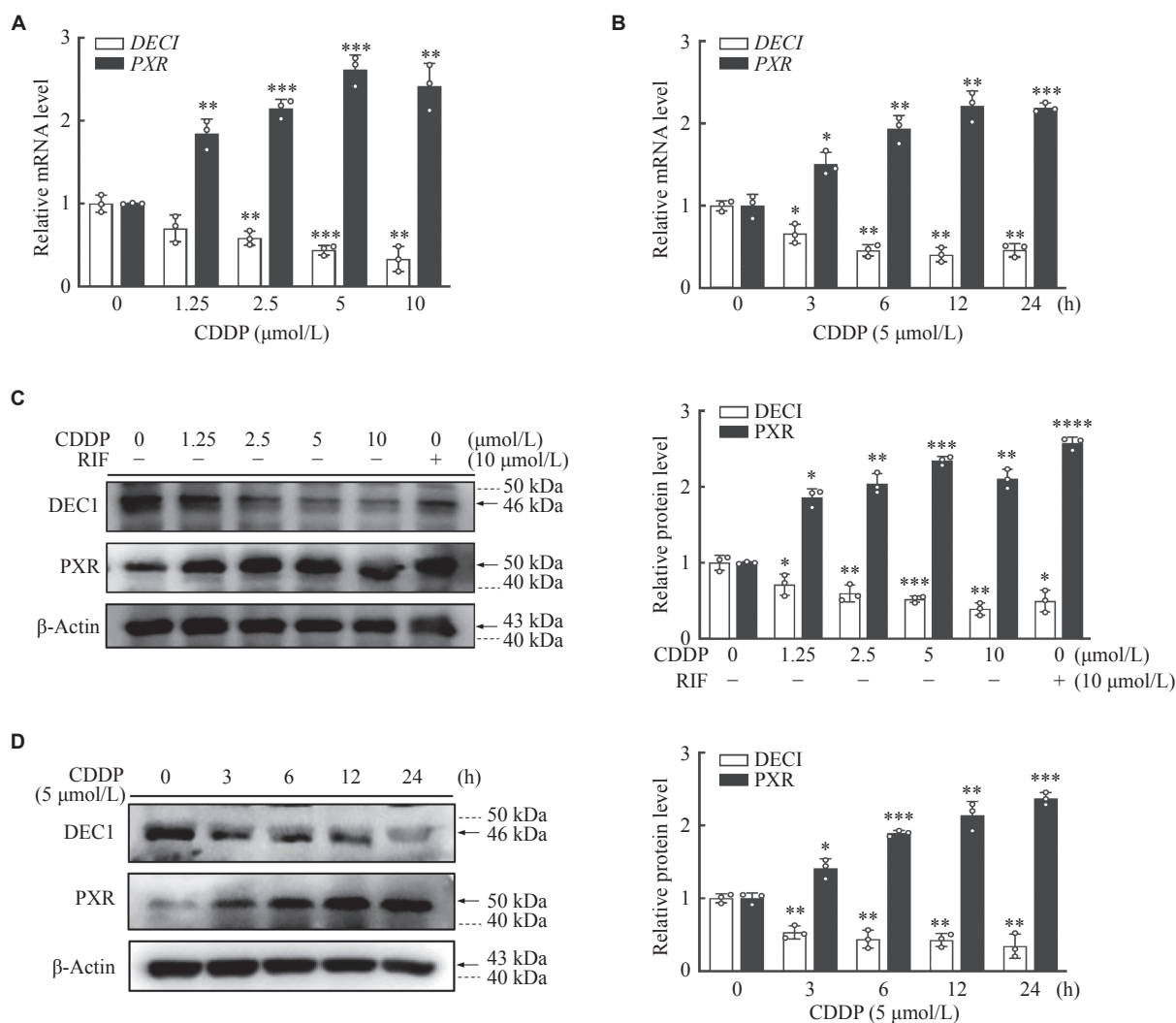
To determine if primary mouse hepatocytes respond to CDDP similarly to human hepatoma cells in terms of the altered expression of carboxylesterases, primary mouse hepatocytes were treated with CDDP (0, 2.5, 5, 10, and 20  $\mu\text{mol/L}$ ) or phenobarbital (1 mmol/L, as a positive control), and cell lysates were prepared and analyzed for the hydrolysis of PNPA and the expression of carboxylesterases. Consistent with the results from HepG2 cells, the hydrolysis of PNPA markedly increased in primary mouse hepatocytes treated with CDDP in dose- and time-dependent manners (**Supplementary Fig. 3A** and **3B**, available online). Likewise, the protein levels of CES1D and CES1E were comparably increased in dose- and time-dependent manners (**Supplementary Fig. 3C** and **3D**). It should be noted that CES1D, and CES1E were detected by antibodies against human CES1 and CES2, respectively<sup>[25]</sup>. Consistent with that in human hepatoma cells, CDDP decreased STRA13 expression and increased PXR expression in dose- and time-dependent manners in primary mouse hepatocytes (**Supplementary Fig. 3C** and **3D**). The data imply that the responses to CDDP in both human and mouse hepatocytes were similar.

#### **CDDP increased carboxylesterases expression and activities along with increased PXR and decreased STRA13 in the liver and intestine of mice**

To confirm that CDDP can stimulate the carboxylesterases *in vivo*, mice were intraperitoneally injected with CDDP [0, 2.5 or 5 mg/(kg·day)], and S9 fractions of the liver and intestine were prepared and analyzed for carboxylesterases, *i.e.*, CYP3A11, PXR, STRA13 protein levels, as well as the hydrolysis activities. As shown in **Fig. 3**, CDDP increased the expressions of CES1D, CES1E, and CYP3A11 in the liver and intestine in a dose-dependent manner, with an increase ranging from two-fold to three-fold (**Fig. 3A–3D**). Likewise, comparable increases in hydrolysis activity of the liver and intestine induced by CDDP were detected in mice (**Fig. 3E** and **3F**). Consistent with that in HepG2 cells and primary mouse hepatocytes, CDDP decreased the STRA13 expression and increased mouse PXR expression (**Fig. 3A–3D**). These results indicate that CDDP induced carboxylesterases expression and their activities along with the increased PXR but decreased DEC1 expressions *in vivo*.

#### **Involvement of PXR in the increase of carboxylesterases induced by CDDP**

Next, to test whether PXR plays a role in the increases of CES1, CES2, and CYP3A4 expressions



**Fig. 2** Inverse regulation of PXR and DEC1 expression by CDDP in HepG2 cells. HepG2 cells were treated with various concentrations (0, 1.25, 2.5, 5, and 10 μmol/L) of CDDP or RIF (10 μmol/L, as a positive control) for 24 h or with the same concentration (5 μmol/L) of CDDP for 0, 3, 6, 12, and 24 h. Relative mRNA and protein levels of DEC1 and PXR were detected by qRT-PCR (A and B) and Western blotting (C and D), respectively. *Gapdh* was used as a reference gene for qRT-PCR and  $\beta$ -actin was used as a loading control for Western blotting. The data are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). The significance determined by one-way analysis of variance, followed by Tukey's post hoc test and the paired comparisons were analyzed by Student's *t*-test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$  vs. the control group (PBS or 0 h). Abbreviations: CDDP, *cis*-diamminedichloroplatinum; DEC1, differentiated embryonic chondrocyte-expressed gene 1; PXR, pregnane X receptor; RIF, rifampicin.

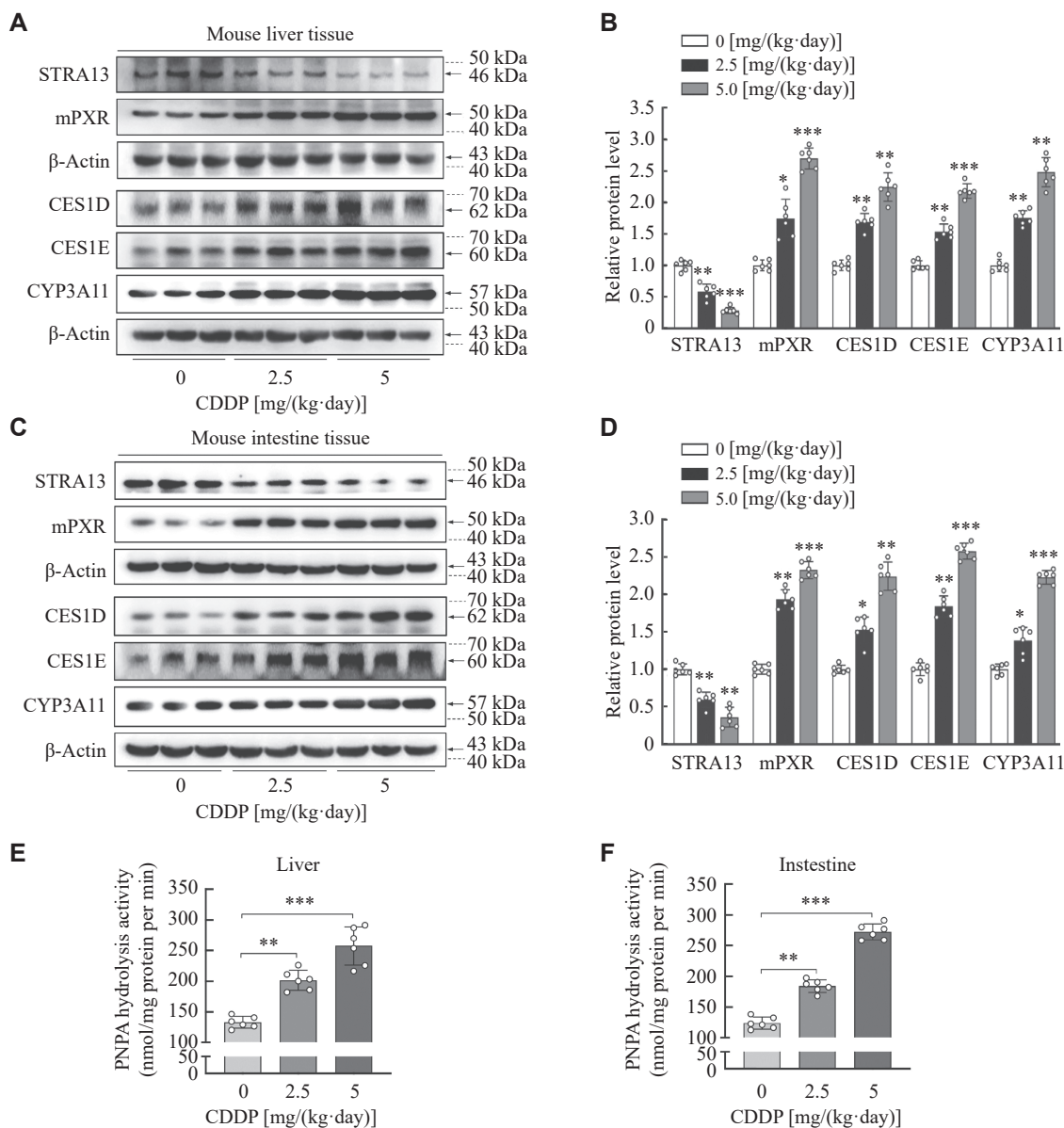
induced by CDDP, we performed knockdown and overexpression of PXR experiments. As shown in **Fig. 4**, CDDP increased the expressions of CES1, CES2, and CYP3A4 significantly in the vector group; whereas knockdown of *PXR* almost abolished these increases induced by CDDP with the knockdown *PXR* efficiency of more than 80% (**Fig. 4A** and **4B**). Conversely, the overexpression of *PXR* alone increased the expressions of CES1, CES2, and CYP3A4 significantly, compared with that in the transfected vector; moreover, it increased the expressions of CES1, CES2, and CYP3A4 much more than that in the transfected vector induced by CDDP (**Fig. 4C** and **4D**). It should be noted that neither the knockdown nor the overexpression of *PXR* changed

the expression of DEC1 and the response to CDDP (**Fig. 4A–4D**). These data imply that *PXR* was involved in the increase of carboxylesterases induced by CDDP, but not regulated DEC1 expression.

#### Involvement of DEC1 in the induction of *PXR* and its targets by CDDP

Next, to test the role of DEC1 in the *PXR* and target genes induced by CDDP, we did the overexpression and knockdown of *DEC1* experiments. As shown in **Fig. 5**, the overexpression of DEC1 alone decreased the expressions of *PXR* and targets, such as CES1, CES2, and CYP3A4, significantly, compared with that in the transfected vector; whereas the overexpression of DEC1 almost abolished the increased *PXR* and





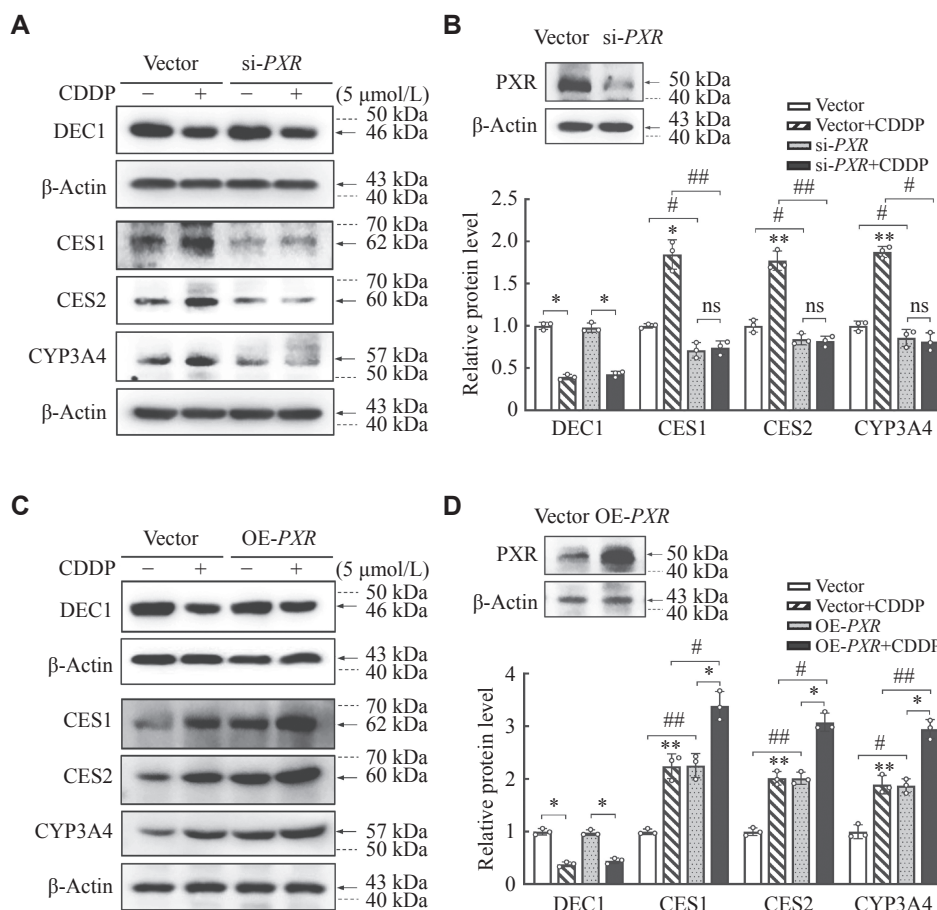
**Fig. 3** CDDP increased carboxylesterases expression and their activities along with increased PXR and decreased STRA13 in the liver and intestine of mice. Mice were intraperitoneally injected with CDDP [0, 2.5, or 5 mg/(kg·day)] for 3 days ( $n = 5$  per group). Mice in the control group received the same volume of normal saline. A–D: STRA13, mouse PXR (mPXR), CES1D, CES1E, and CYP3A11 protein levels in the liver (A and B) and intestine (C and D) of mice were detected by Western blotting.  $\beta$ -Actin was used as a loading control. E and F: S9 fractions of the mice's liver and intestine were prepared and analyzed for overall hydrolysis activity with standard substrate PNPA. The data are expressed as mean  $\pm$  standard deviation ( $n = 6$ ). The significance was determined by one-way analysis of variance, followed by Tukey's post hoc test and the paired comparisons were analyzed by Student's  $t$ -test. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. the control group (0 mg/kg or 0 h). Abbreviations: CDDP, *cis*-diamminedichloroplatinum; STRA13 (DEC1), stimulated with retinoic acid 13; mPXR, mouse pregnane X receptor; CES1D, carboxylesterase 1d; CES1E, carboxylesterase 1e; CYP3A11, cytochrome P450 3A11; PNPA, p-nitrophenylacetic acid.

target expression induced by CDDP in the vector (**Fig. 5A** and **5B**). Oppositely, the knockdown of *DEC1* alone increased the expressions of PXR and targets CES1, CES2, and CYP3A4 significantly, compared with that in the transfected vector; however, the knockdown of DEC1 abolished these increases, compared with that in the transfected vector induced by CDDP (**Fig. 5C** and **Fig. 5D**). These results

suggested that CDDP increased CES1, CES2, and CYP4A4 through activating PXR mediated by decreasing DEC1 expression.

To validate the PXR and its target involvement of DEC1 *in vivo*, we examined the PXR and its targets, such as CES1, CES2 and CYP3A11, in the liver and intestine of the *Dec1*<sup>-/-</sup> and *Dec1*<sup>+/+</sup> mice (24 weeks old). Compared with *Dec1*<sup>+/+</sup> mice, *Dec1*<sup>-/-</sup> mice had





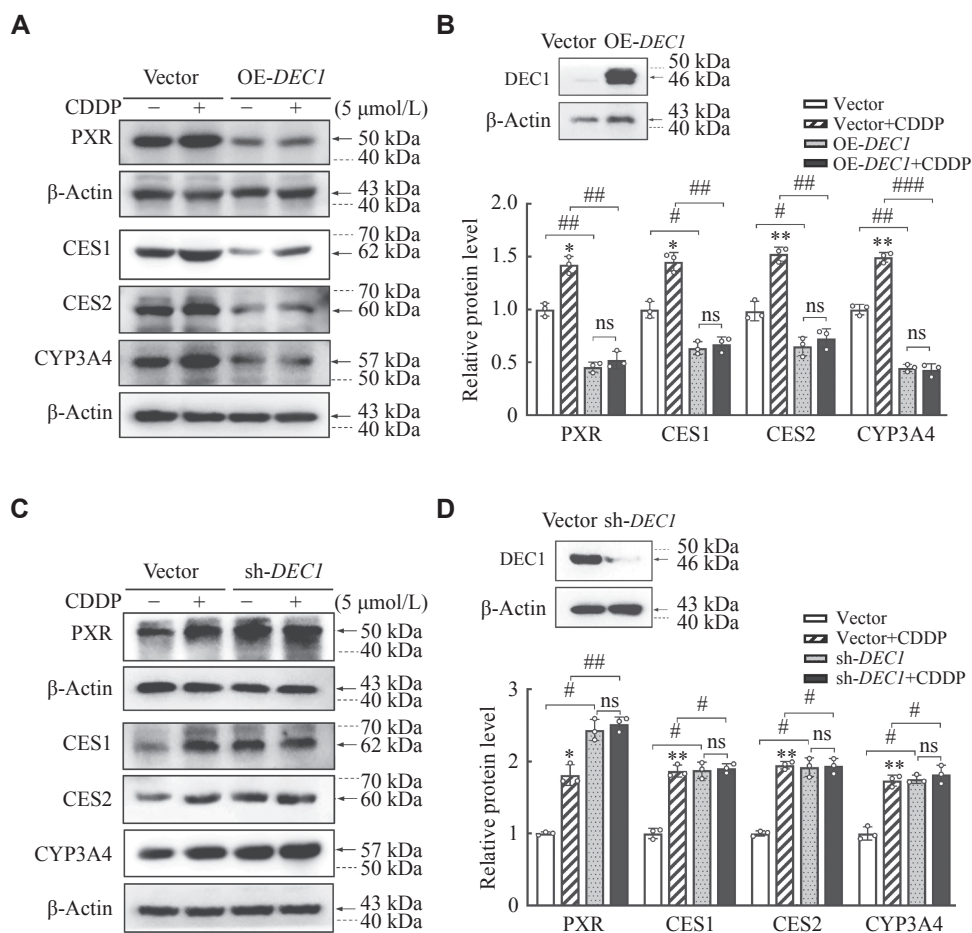
**Fig. 4** Involvement of PXR in the increase of carboxylesterases induced by CDDP in HepG2 cells. A and B: HepG2 cells were transfected with si-PXR construct or an equal amount of corresponding vector, cultured for 48 h, and then treated with CDDP (5  $\mu\text{mol/L}$ ) or DMSO (0.1%, v/v) for 24 h. Protein levels of DEC1, CES1, CES2, and CYP3A4, as well as the knockdown efficiency of PXR, were detected by Western blotting. C and D: HepG2 cells were transfected with OE-PXR construct or an equal amount of the corresponding vector, cultured for 24 h, and received the treatment as mentioned above. Protein levels of DEC1, CES1, CES2, and CYP3A4, as well as the overexpression efficiency of PXR, were detected by Western blotting.  $\beta$ -Actin was used as a loading control. The data are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). The significance was determined by two-way analysis of variance, followed by Tukey's post hoc test and the paired comparisons were analyzed by Student's  $t$ -test. \* $P < 0.05$  and \*\* $P < 0.01$ , # $P < 0.05$ , ## $P < 0.01$ , and ns $P > 0.05$ , as comparisons shown in the figures. Abbreviations: CDDP, *cis*-diamminedichloroplatinum; DEC1, differentiated embryonic chondrocyte-expressed gene 1; PXR, pregnane X receptor; CES1, carboxylesterase 1; CES2, carboxylesterase 2; CYP3A4, cytochrome P450 3A4.

the increased expression of PXR and its targets, such as CES1D, CES1E, and CYP3A11 at both mRNA (Fig. 6A and 6B) and protein levels in the liver (Fig. 6C and 6D) and intestine (Fig. 6E and 6F) as well as the overall hydrolysis activity (Fig. 6G and 6H). The increases of PXR and its targets were more than two-fold in *Dec1*<sup>-/-</sup> mice, compared with those in *Dec1*<sup>+/+</sup> mice, indicating that DEC1 was involved in the regulation of PXR and its targets *in vivo*.

#### Transcriptional involvement in DEC1 suppression by CDDP

The decrease of *DEC1* mRNA by CDDP suggests two possibilities: (1) CDDP suppresses the transcription and/or (2) increases the degradation of mRNA. To test the first possibility, a transcriptional inhibition assay was performed with CDDP in the

presence or absence of the transcription inhibitor Actinomycin D (Act D). HepG2 cells were treated with CDDP (5  $\mu\text{mol/L}$ ) alone or together with Act D (2  $\mu\text{mol/L}$ ) for 0, 20, 40, 60, 80, 100, and 120 min. The total RNA was isolated and analyzed for the *DEC1* mRNA level. As shown in Fig. 7, the slopes of the *DEC1* mRNA attenuated curve did not change when either being treated with CDDP alone or together with Act D (Fig. 7A), suggesting that CDDP decreased DEC1 expression not through the increase of degradation but through transcription suppression. This possibility was also tested with DEC1 promoter reporters. The DEC1 reporters contained 1.6-kb and 1.1-kb upstream sequences, respectively (kindly provided by Dr. Yan's Lab). HepG2 cells were transfected with a promoter reporter (DEC1-1.3-kb-Luc or DEC1-1.1-kb-Luc) and the Renilla plasmid, and the transfected cells were treated with CDDP or



**Fig. 5** Involvement of DEC1 in the induction of PXR and its targets by CDDP in HepG2 cells. A and B: HepG2 cells were transfected with OE-DEC1 construct or an equal amount of corresponding vector, cultured for 24 h, and then treated with CDDP (5  $\mu\text{mol/L}$ ) or DMSO (0.1%, v/v) for 24 h. Protein levels of PXR, CES1, CES2, and CYP3A4, as well as the overexpression efficiency of DEC1, were detected by Western blotting. C and D: HepG2 cells were infected with lentivirus (LV-shDEC1 or LV-Con) and screened with a medium containing puromycin. Purified DEC1 knockdown cells were treated as mentioned above.  $\beta$ -Actin was used as a loading control. Protein levels of PXR, CES1, CES2, and CYP3A4, as well as the knockdown efficiency of DEC1, were detected by Western blotting. The data are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). The significance was performed by two-way analysis of variance, followed by Tukey's post hoc test, and the paired comparisons were analyzed by Student's *t*-test. \* $P < 0.05$  and \*\* $P < 0.01$ , # $P < 0.05$ , ## $P < 0.01$ , and ns $P > 0.05$ , as comparisons shown in the figures. Abbreviations: CDDP, *cis*-diamminedichloroplatinum; DEC1, differentiated embryonic chondrocyte-expressed gene 1; PXR, pregnane X receptor; CES1, carboxylesterase 1; CES2, carboxylesterase 2; CYP3A4, cytochrome P450 3A4.

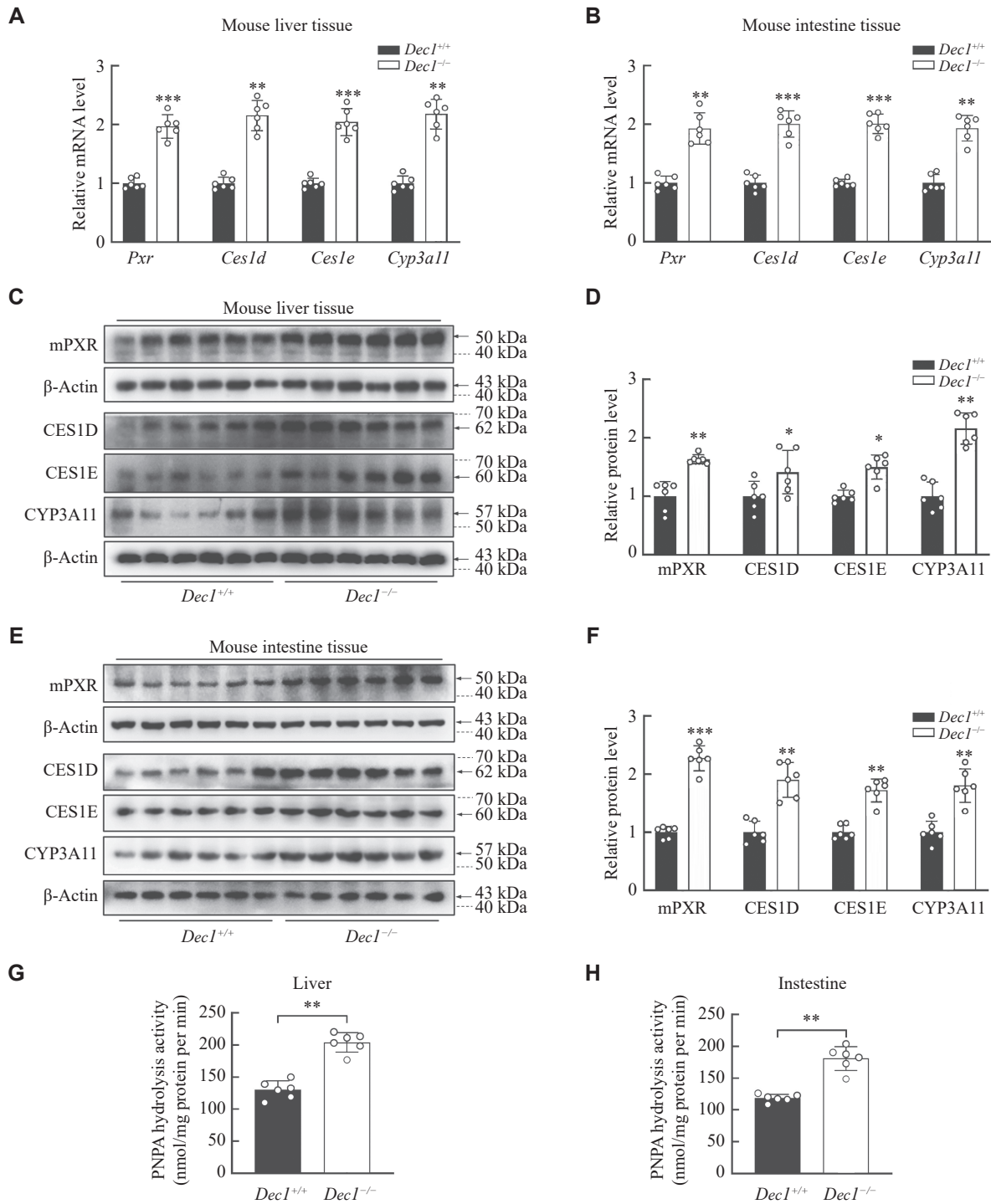
PBS. After a 48-h incubation, cells were lysed and the luciferase activities were determined. As shown in **Fig. 7B**, treatment of CDDP significantly decreased the activity of DEC1 reporters. The transcription inhibition of DEC1-1.3-kb-Luc was 55%, which was comparable to the mRNA level detected by qRT-PCR (**Fig. 2A** and **2B**). Likewise, the transcription inhibition of DEC1-1.1-kb-Luc (33%) was less than that in the mRNA level. These data suggested the transcriptional involvement in DEC1 inhibition by CDDP.

#### Enhanced cytotoxicity effect of CPT11 by CDDP through increasing PXR expression mediated by inhibiting DEC1 expression

To explore whether CDDP and CPT11 have a synergistic effect, HepG2 cells were seeded in 96-well plates at a density of 5 000 cells/well overnight and treated

with CPT11 (0, 1, 5, 10, 20, 40, and 80  $\mu\text{mol/L}$ ) alone, together with CDDP (5  $\mu\text{mol/L}$ ) for 24 h, or treated with CDDP (5  $\mu\text{mol/L}$ ) for 2 h first, and then added CPT11 (0, 1, 5, 10, 20, 40, and 80  $\mu\text{mol/L}$ ) for 22 h. The cell viability was determined by MTT. As shown in **Fig. 8A**,  $\text{IC}_{50}$  of CPT11 alone, and together with CDDP (5  $\mu\text{mol/L}$ ) were 25.99 ( $\pm$  2.73)  $\mu\text{mol/L}$  and 17.15 ( $\pm$  1.66)  $\mu\text{mol/L}$ , respectively. Whereas,  $\text{IC}_{50}$  of CPT11 being treated with CDDP first and then added CPT11 was 11.26 ( $\pm$  1.37)  $\mu\text{mol/L}$  (**Fig. 8A**, right). These results imply the combination effect of CPT11 with CDDP, especially being treated with CDDP first, and then added CPT11. It was noted that CDDP (5  $\mu\text{mol/L}$ ) was no cytotoxicity (**Supplementary Fig. 2B**).

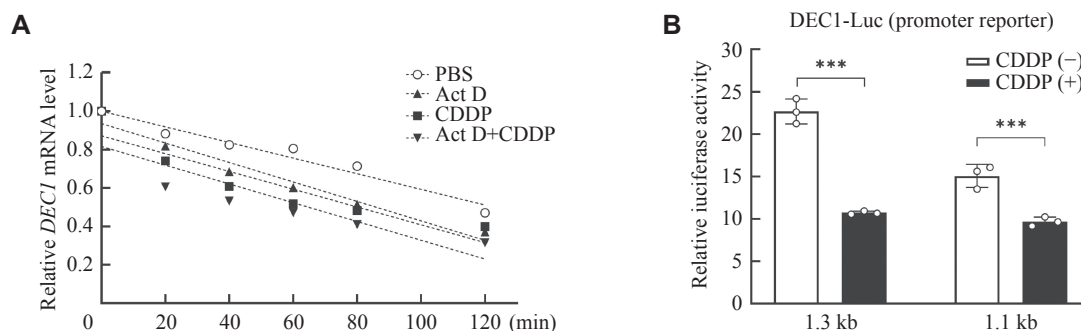
Next, we inquired into the roles of DEC1 and PXR in the synergistic effect between CDDP and CPT11.



**Fig. 6** *Dec1* deficiency showed an increase of PXR and its targets in the liver and intestine of mice. A–F: The mRNA and protein levels of mouse PXR (mPXR) and its targets, such as CES1D, CES1E, and CYP3A11 in the liver and intestine of *Dec1*<sup>+/+</sup> and *Dec1*<sup>-/-</sup> mice were detected by qRT-PCR (A and B) and Western blotting (C–F), respectively. *Gapdh* was used as a reference gene for qRT-PCR and  $\beta$ -actin was used as a loading control for Western blotting. G and H: The overall hydrolysis activities of the liver and intestine S9 fraction obtained from the above mice were determined with standard substrate PNPA. The data are expressed as mean  $\pm$  standard deviation ( $n = 6$ ). The significance was performed by one-way analysis of variance, followed by Tukey's post hoc test, and the paired comparisons were analyzed by Student's *t*-test. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , compared with the *Dec1*<sup>+/+</sup> group. Abbreviations: *Dec1*, differentiated embryonic chondrocyte-expressed gene 1; mPXR, mouse pregnane X receptor; *Ces1d*, carboxylesterase 1d; *Ces1e*, carboxylesterase 1e; CYP3A11, Cytochrome P450 3A11.

The transfected cells (Vector, sh-*DECI*, OE-*DECI*), (Vector, si-*PXR*, OE-*PXR*), or (Vector, OE-*DECI*+si-

*PXR*, sh-*DECI*+OE-*PXR*) were seeded in 96-well plates at a density of 5 000 cells/well overnight and



**Fig. 7 CDDP suppressed DEC1 transcriptionally.** A: HepG2 cells were treated with CDDP (5  $\mu\text{mol/L}$ ) alone or together with transcription inhibitor Act D (2  $\mu\text{mol/L}$ ) for 0, 20, 40, 60, 80, 100, and 120 min. The total RNA was isolated and analyzed for the *DEC1* mRNA level by qRT-PCR. *GAPDH* was used as a reference gene. B: HepG2 cells were transfected with a promoter reporter construct pGL3-DEC1-1.3kb-Luc or pGL3-DEC1-1.1kb-Luc and Renilla plasmid and treated with CDDP or PBS for 48 h. Cells were lysed and the luciferase activities were determined. The data are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). The significance was determined by one-way analysis of variance, followed by Tukey's post hoc test and the paired comparisons were analyzed by Student's *t*-test. \*\*\* $P < 0.001$ . Abbreviations: CDDP, *cis*-diamminedichloroplatinum; Act D, actinomycin D; DEC1, differentiated embryonic chondrocyte-expressed gene 1.

treated with CDDP (5  $\mu\text{mol/L}$ ) for 2 h first, then added CPT11 (0, 1, 5, 10, 20, 40, 80  $\mu\text{mol/L}$ ) for 22 h. The cell viability was determined by MTT. As shown in **Fig. 8**, the knockdown or overexpression of *DEC1* could enhance ( $\text{IC}_{50}$  of CPT11 was from  $[11.92 \pm 0.72]$   $\mu\text{mol/L}$  to  $[7.64 \pm 0.94]$   $\mu\text{mol/L}$ ), or alleviate ( $\text{IC}_{50}$  of CPT11 was from  $[11.92 \pm 0.72]$   $\mu\text{mol/L}$  to  $[18.64 \pm 1.08]$   $\mu\text{mol/L}$ ) the synergistic effect of CPT11 and CDDP significantly (**Fig. 8B**, right). Likewise, the knockdown or overexpression of *PXR* could alleviate ( $\text{IC}_{50}$  of CPT11 was from  $[11.99 \pm 0.74]$   $\mu\text{mol/L}$  to  $[18.64 \pm 1.08]$   $\mu\text{mol/L}$ ), or enhance ( $\text{IC}_{50}$  of CPT11 was from  $[11.99 \pm 0.74]$   $\mu\text{mol/L}$  to  $[6.54 \pm 0.80]$   $\mu\text{mol/L}$ ) the synergistic effect of CPT11-CDDP significantly (**Fig. 8C**, right). The knockdown of *DEC1* plus the overexpression of *PXR* could not enhance the synergistic effect of CPT11 and CDDP significantly, compared with that in the knockdown of *DEC1* or with that in the overexpression of *PXR* alone ( $[5.01 \pm 0.74]$   $\mu\text{mol/L}$  vs.  $[7.64 \pm 0.94]$   $\mu\text{mol/L}$  or  $[6.54 \pm 0.80]$   $\mu\text{mol/L}$ ) (**Fig. 8D**, right). Also, the knockdown of *PXR* plus the overexpression of *DEC1* could not decrease the synergistic effect of CPT11 and CDDP significantly, compared with that in the knockdown of *PXR* or with that in the overexpression of *DEC1* alone ( $[18.88 \pm 1.32]$   $\mu\text{mol/L}$  vs.  $[18.64 \pm 1.08]$   $\mu\text{mol/L}$  or  $[18.36 \pm 0.77]$   $\mu\text{mol/L}$ ) (**Fig. 8D**, right). Actually, the knockdown efficiency of *PXR* and *DEC1* was more than 70% (**Supplementary Fig. 4A**, available online) and their overexpression efficiency was over three-fold (**Supplementary Fig. 4B**, available online). Likewise, we got similar results in SW480 cells, which are summarized in **Supplementary Fig. 2C** and **2D**, **4C** and **4D**, and **5**. These data suggested that the combination of CDDP and CPT11 had a synergistic effect, especially, in the concurrent sequential use of CDDP and CPT11.

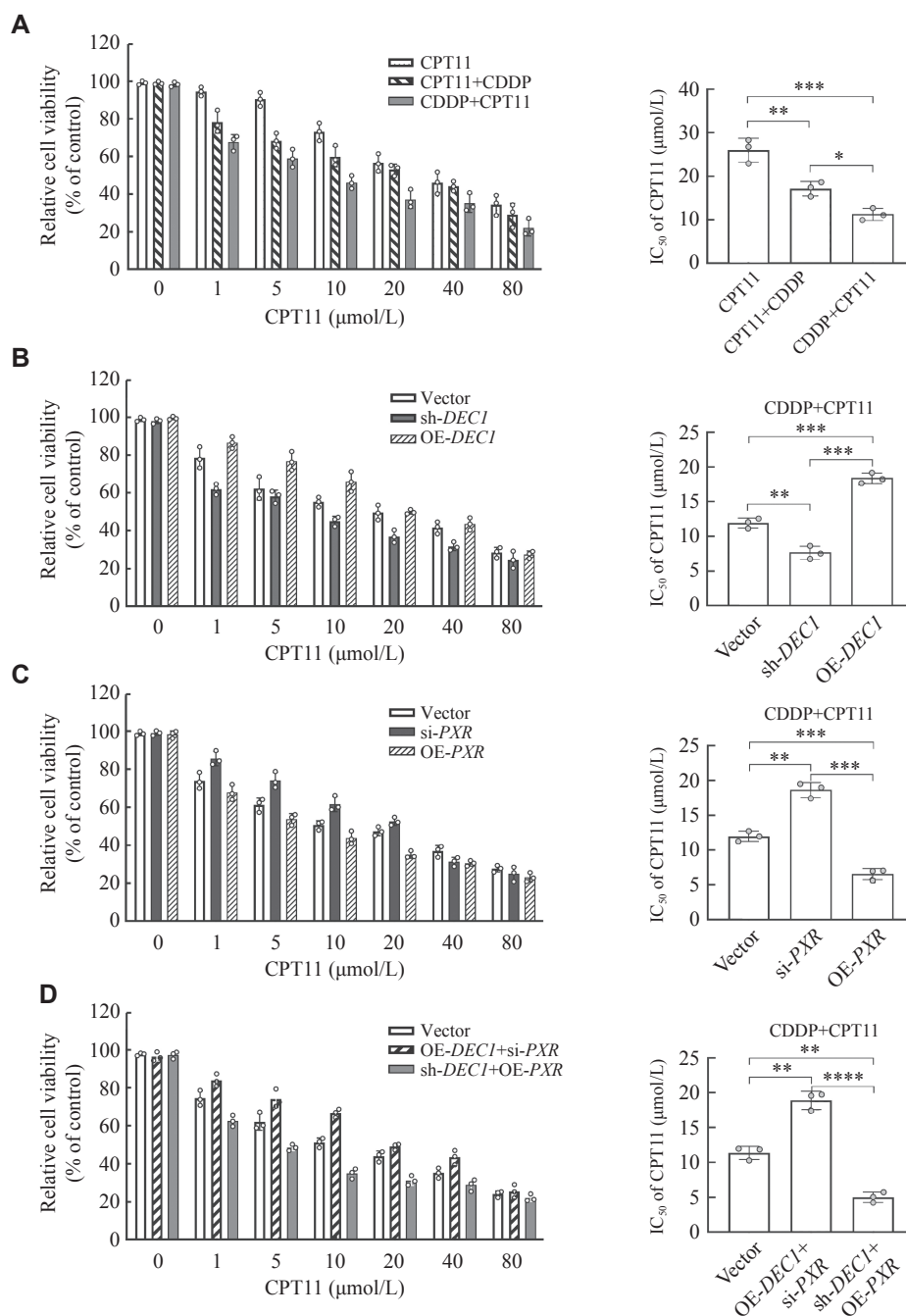
*DEC1* and *PXR* were involved in the synergistic effect of CDDP and CPT11.

## Discussion

CDDP, widely used to treat a multitude of human cancers, such as cancers of lung, ovarian, breast, bladder, testicle, and brain, either alone or in combination with other drugs<sup>[26]</sup>, exerts its anti-tumor activity by covalently binding to DNA-forming adducts and therefore by triggering apoptosis<sup>[27]</sup>. CDDP is also used in combination with other antitumor drugs, such as CPT11<sup>[28]</sup> and doxorubicin<sup>[29]</sup>. Therefore, it is critically important to elucidate the effect of CDDP on the drug metabolic enzymes that influence the drug-drug interaction. In the present study, we, for the first time, report that CDDP is an efficacious inducer of carboxylesterases. In HepG2 cells and primary mouse hepatocytes, CDDP markedly increases the expressions of CES1 (CES1D) and CES2 (CES1E) and hydrolysis activity as well as CYP3A4 (CYP3A11). Along with these changes, CDDP increases *PXR* expression, a major xenobiotic nuclear receptor that regulates CYP3A4 and carboxylesterases<sup>[24,30]</sup>, and decreases *DEC1* expression. These *in vitro* data are confirmed by animal experiments (*in vivo*).

That the increase of CES1, CES2, and CYP3A4 by CDDP are abolished or proportionally increased when the expression of *PXR* is knocked down or overexpressed, and that the knockdown or overexpression of *PXR* alone decreases or increases CES1, CES2, and CYP3A4 expressions, it is assumed that the increases of CES1, CES2, and CYP3A4 by CDDP are caused by the increase of *PXR*. These conclusions are supported by other studies<sup>[13–14]</sup>. But it seems to be in contrast with the reported effects in the





**Fig. 8** Enhanced effect of CPT11 by CDDP through increasing PXR expression mediated by inhibiting DEC1 expression in HepG2 cells. A: HepG2 cells were seeded into 96-well plates at a density of 5 000 cells per well overnight. The cells were subsequently treated with the indicated concentrations of CPT11 alone or together with CDDP (5 μmol/L) for 24 h, or the cells were treated with CDDP (5 μmol/L) for 2 h first, and then treated with the indicated concentrations of CPT11 (including CDDP) for another 22 h. B–D: HepG2 cells with *DEC1* knockdown or overexpression (transfected with vector, sh-*DEC1*, or OE-*DEC1*) (B), *PXR* knockdown or overexpression (transfected with vector, si-*PXR*, or OE-*PXR*) (C), *PXR* knockdown plus *DEC1* overexpression or *DEC1* knockdown plus *PXR* overexpression (transfected with vector, OE-*DEC1* + si-*PXR*, or sh-*DEC1* + OE-*PXR*) (D) were seeded into 96-well plates at a density of 5 000 cells per well overnight. The cells were subsequently treated with CDDP (5 μmol/L) for 2 h, followed by treatment with the indicated concentrations of CPT11 (including CDDP) for another 22 h. Cell viability (relative to the control group) was determined by the MTT assay, and the IC<sub>50</sub> of CPT11 was calculated. The data are expressed as mean ± standard deviation ( $n = 3$ ). The significance was determined by one-way (A) or two-way (B–D) analysis of variance, followed by Tukey's post hoc test and the paired comparisons were analyzed by Student's *t*-test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . Abbreviations: CDDP, *cis*-diamminedichloroplatinum; CPT11, irinotecan; DEC1, differentiated embryonic chondrocyte-expressed gene 1; PXR, pregnane X receptor.

kidney, where CDDP inhibits PXR expression in humans and mice<sup>[31]</sup>. The reason is that the dose of

CDDP in making acute kidney injury is too large [20 mg/(kg·day) vs. 2.5 or 5 mg/(kg·day)]. It should

be noted that the knockdown or overexpression of PXR do not change the DEC1 expression and neither do they change the decrease of DEC1 by CDDP. Thus, DEC1 is not regulated by PXR.

Next, we found that the overexpression or knockdown of DEC1 alone decreased or increased PXR expression, and abolished or alleviated the increases of PXR and its targets by CDDP. Thus, DEC1 is upstream of PXR and regulates PXR and its targets. These *in vitro* data are supported by the fact that the increase of mPXR and its targets in the liver and intestine was over twofold in *Dec1*<sup>-/-</sup> mice, compared with those in *Dec1*<sup>+/+</sup> mice, indicating that DEC1 was involved in the regulation of PXR and its targets *in vivo*.

The present study has provided some evidence to support that CDDP suppresses DEC1 transcriptionally. (1) The slopes of *DEC1* mRNA attenuated curve do not change either when treated with CDDP alone or together with Act D, implying that CDDP does not influence the degradation of *DEC1* mRNA and (2) CDDP significantly decreased the activity of DEC1 promoter reporters. Taken together, CDDP increases the expressions of CES1, CES2 and CYP3A4 through increasing PXR expression mediated by suppressing DEC1 transcriptionally. These data are consistent with the results of the decrease of PXR by IL-6<sup>[17]</sup> and fluoxetine<sup>[30]</sup> mediated by the increase of DEC1. Thus, exogenous or endogenous substances (chemicals or cytokines) which influenced the expression of DEC1 affect PXR and target genes. Noda K *et al* have reported that CPT11 plus CDDP is an effective treatment for metastatic small-cell lung cancer, in a phase II clinical trial<sup>[28]</sup>. Increasing clinical studies show that CPT11 plus CDDP is commonly used as community standard regimens for many advanced cancers<sup>[32-33]</sup>. Clinically, with the recommended concentration of CPT11 (350 mg/m<sup>2</sup>), the maximum plasma concentration is 3 150 ng/mL (5.37 μmol/L)<sup>[34]</sup>, which is comparable to the concentration of CPT11.

The present study, for the first time, provides pieces of evidence that CDDP increases the activity of carboxylesterases, including CES1 and CES2: (1) CDDP markedly increases the expressions of CES1 (CES1D) and CES2 (CES1E) in HepG2 cells, mouse hepatocytes and mouse liver; (2) CDDP markedly increases the overall activity of hydrolysis; and (3) CDDP can increase the toxicity of oseltamivir and decrease the toxicity of clopidogrel, suggesting that it increases CES1 activity, whereas CDDP can increase the toxicity of CPT11, suggesting that it increases CES2 activity. More importantly, the synergy chemotherapeutic action of CDDP plus CPT11 is

because of CDDP increasing the expression and activity of CES2, and it is through this mechanism that CPT11 is hydrolyzed to produce the active SN-38. IC<sub>50</sub> of CPT11 in combination with CDDP is significantly lower than that in the use of CPT11 alone in two cell lines. Interestingly, the synergistic effect of CDDP and CPT11 is closely related to the order of the two drugs. Using CDDP first and then CPT11 is more conducive to obtaining synergy than concurrently using both. The reason is probably that the more CES2 is induced, the more CPT11 (SN-38) is activated, and the more synergistic effect is obtained. Pharmacologically, the involvement of PXR in CDDP-mediated induction implies that this chemotherapeutic agent causes more extensive drug-drug interactions in the clinic.

## Fundings

The work was supported by the National Natural Science Foundation of China (Grant Nos. 82073934 and 81872937) to J.Y.

## Acknowledgments

We appreciate Dr. Yan from University of Cincinnati in USA to provide us CES2 antibody, the DEC1, PXR and other plasmids.

## References

- [1] Ghosh S. Cisplatin: the first metal based anticancer drug[J]. *Bioorg Chem*, 2019, 88: 102925.
- [2] Tesniere A, Schlemmer F, Boige V, et al. Immunogenic death of colon cancer cells treated with oxaliplatin[J]. *Oncogene*, 2010, 29(4): 482–491.
- [3] Muttar A, Ahmed I, Hameed H. The optimum inhibitory effects of Alpha Interferon and Cisplatin in colon cancer, a comparative *in vitro* study[J]. *J Med Life*, 2022, 15(2): 269–277.
- [4] Kim SJ, Park C, Lee JN, et al. Erdosteine protects HEI-OC1 auditory cells from cisplatin toxicity through suppression of inflammatory cytokines and induction of Nrf2 target proteins[J]. *Toxicol Appl Pharmacol*, 2015, 288(2): 192–202.
- [5] Pezeshki Z, Khosravi A, Nekuei M, et al. Time course of cisplatin-induced nephrotoxicity and hepatotoxicity[J]. *J Nephropathol*, 2017, 6(3): 163–167.
- [6] Rambeau A, Cherifi F, Nevier Z, et al. Platinum-induced nausea and vomiting in patients treated for head and neck cancer[J]. *Bull Cancer*, 2021, 108(5): 449–454.
- [7] Quintanilha JCF, de Sousa VM, Visacri MB, et al. Involvement of cytochrome P450 in cisplatin treatment: implications for toxicity[J]. *Cancer Chemother Pharmacol*,



- 2017, 80(2): 223–233.
- [8] Goldstein I, Rivlin N, Shoshana OY, et al. Chemotherapeutic agents induce the expression and activity of their clearing enzyme CYP3A4 by activating p53[J]. *Carcinogenesis*, 2013, 34(1): 190–198.
- [9] Drozdziak M, Busch D, Lapczuk J, et al. Protein abundance of clinically relevant drug-metabolizing enzymes in the human liver and intestine: a comparative analysis in paired tissue specimens[J]. *Clin Pharmacol Ther*, 2018, 104(3): 515–524.
- [10] Poso A, Honkakoski P. Ligand recognition by drug-activated nuclear receptors PXR and CAR: structural, site-directed mutagenesis and molecular modeling studies[J]. *Mini Rev Med Chem*, 2006, 6(8): 937–943.
- [11] Di Consiglio E, Darney K, Buratti FM, et al. Human variability in carboxylesterases and carboxylesterase-related uncertainty factors for chemical risk assessment[J]. *Toxicol Lett*, 2021, 350: 162–170.
- [12] Mao Z, Li Y, Peng Y, et al. Lipopolysaccharide down-regulates carboxylesterases 1 and 2 and reduces hydrolysis activity in vitro and in vivo via p38MAPK-NF- $\kappa$ B pathway[J]. *Toxicol Lett*, 2011, 201(3): 213–220.
- [13] Masuyama H, Nakatsukasa H, Takamoto N, et al. Down-regulation of pregnane X receptor contributes to cell growth inhibition and apoptosis by anticancer agents in endometrial cancer cells[J]. *Mol Pharmacol*, 2007, 72(4): 1045–1053.
- [14] Masuyama H, Nakamura K, Nobumoto E, et al. Inhibition of pregnane X receptor pathway contributes to the cell growth inhibition and apoptosis of anticancer agents in ovarian cancer cells[J]. *Int J Oncol*, 2016, 49(3): 1211–1220.
- [15] Li S, Peng D, Yin Z, et al. Effect of DEC1 on the proliferation, adhesion, invasion and epithelial-mesenchymal transition of osteosarcoma cells[J]. *Exp Ther Med*, 2020, 19(3): 2360–2366.
- [16] Marczak MM, Yan B. Circadian rhythmicity: a functional connection between differentiated embryonic chondrocyte-1 (DEC1) and small heterodimer partner (SHP)[J]. *Arch Biochem Biophys*, 2017, 631: 11–18.
- [17] Ning R, Zhan Y, He S, et al. Interleukin-6 induces DEC1, promotes DEC1 interaction with RXR $\alpha$  and suppresses the expression of PXR, CAR and their target genes[J]. *Front Pharmacol*, 2017, 8: 866.
- [18] Yang J, Luan X, Gui H, et al. Byakangelicin induces cytochrome P450 3A4 expression via transactivation of pregnane X receptors in human hepatocytes[J]. *Br J Pharmacol*, 2011, 162(2): 441–451.
- [19] Wu L, Hafiz MZ, Guan Y, et al. 17 $\beta$ -estradiol suppresses carboxylesterases by activating c-Jun/AP-1 pathway in primary human and mouse hepatocytes[J]. *Eur J Pharmacol*, 2018, 819: 98–107.
- [20] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited[J]. *FASEB J*, 2008, 22(3): 659–661.
- [21] Le F, Yang L, Han Y, et al. TPL inhibits the invasion and migration of drug-resistant ovarian cancer by targeting the PI3K/AKT/NF- $\kappa$ B-signaling pathway to inhibit the polarization of M2 TAMs[J]. *Front Oncol*, 2021, 11: 704001.
- [22] Miyazaki K, Miyazaki M, Guo Y, et al. The role of the basic helix-loop-helix transcription factor *Dec1* in the regulatory T cells[J]. *J Immunol*, 2010, 185(12): 7330–7339.
- [23] Shi D, Yang J, Yang D, et al. Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and the activation is inhibited by antiplatelet agent clopidogrel[J]. *J Pharmacol Exp Ther*, 2006, 319(3): 1477–1484.
- [24] Yang J, Yan B. Photochemotherapeutic agent 8-methoxypsoralen induces cytochrome P450 3A4 and carboxylesterase HCE2: evidence on an involvement of the pregnane X receptor[J]. *Toxicol Sci*, 2007, 95(1): 13–22.
- [25] Xiao D, Chen Y, Yang D, et al. Age-related inducibility of carboxylesterases by the antiepileptic agent phenobarbital and implications in drug metabolism and lipid accumulation[J]. *Biochem Pharmacol*, 2012, 84(2): 232–239.
- [26] Dasari S, Njiki S, Mbemi A, et al. Pharmacological effects of cisplatin combination with natural products in cancer chemotherapy[J]. *Int J Mol Sci*, 2022, 23(3): 1532.
- [27] Gonzalez VM, Fuertes MA, Alonso C, et al. Is cisplatin-induced cell death always produced by apoptosis?[J]. *Mol Pharmacol*, 2001, 59(4): 657–663.
- [28] Noda K, Nishiwaki Y, Kawahara M, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer[J]. *N Engl J Med*, 2002, 346(2): 85–91.
- [29] Lyass O, Hubert A, Gabizon AA. Phase I study of doxorubicin plus cisplatin combination chemotherapy in patients with advanced malignancies[J]. *Clin Cancer Res*, 2001, 7(10): 3040–3046.
- [30] Shang W, Liu J, Chen R, et al. Fluoxetine reduces CES1, CES2, and CYP3A4 expression through decreasing PXR and increasing DEC1 in HepG2 cells[J]. *Xenobiotica*, 2016, 46(5): 393–405.
- [31] Yu X, Xu M, Meng X, et al. Nuclear receptor PXR targets AKR1B7 to protect mitochondrial metabolism and renal function in AKI[J]. *Sci Transl Med*, 2020, 12(543): eaay7591.
- [32] Morizane C, Machida N, Honma Y, et al. Effectiveness of etoposide and cisplatin vs irinotecan and cisplatin therapy for patients with advanced neuroendocrine carcinoma of the digestive system: the TOPIC-NEC phase 3 randomized clinical trial[J]. *JAMA Oncol*, 2022, 8(10): 1447–1455.
- [33] Okuma Y, Hosomi Y, Takagi Y, et al. Cisplatin and irinotecan combination chemotherapy for advanced thymic carcinoma: evaluation of efficacy and toxicity[J]. *Lung Cancer*, 2011, 74(3): 492–496.
- [34] Herviou P, Richard D, Roche L, et al. Determination of irinotecan and SN38 in human plasma by TurboFlow™ liquid chromatography-tandem mass spectrometry[J]. *J Pharm Biomed Anal*, 2016, 118: 284–291.