SUPPLEMENTARY MATERIAL

Activation of PXR-Cytochrome P450s axis: A Possible Reason for the Enhanced Accelerated Blood Clearance Phenomenon of PEGylated Liposomes *in vivo*

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PXR-CYPs axis involved in the ABC phenomenon

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Supplementary methods

Preparation for blood and tissues of interest samples

The samples preparation for blood and tissues of interest (liver and spleen) are the same as our previous studies. In brief, under isoflurane anesthesia, approximate 0.3 mL intraorbital blood samples were harvested in pre-heparinized tubes before (blank blood) and after intravenous administration of the preplanned time points (0.167, 0.5, 1, 2, 4, 6, 8, 10, and 12 h). After centrifuging the samples at 3000 rpm for 10 min, and 100 μL plasma fractions were obtained and kept at -80°C until further liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Liver and spleen were rapidly removed and collected after the last blood sample was obtained at 12 h, and one piece of liver and spleen was excised for biodistribution analysis. A piece of liver was fixed with 4% paraformaldehyde for immunochemical staining. The rest of liver was then rinsed in ice-cold 0.9% NaCl, and a small piece of hepatic tissue was placed into a RNase-free Eppendorf tube for the measurement of mRNA. The remaining liver and spleen tissues were quickly frozen in liquid nitrogen and kept at -80°C until additional analysis.

The DTX levels in blood and tissue samples were measured using a previously developed detection method. Briefly, 3-fold volume of methyl tert-butyl ether with 25 ng/mL paclitaxel (internal standard, IS) was added in plasma or tissue homogenates followed by swirling for 3 min. The supernatant was separated by centrifuging for 5 min at high speed (12,000 rpm) and low temperature (4°C). Subsequently, the samples evaporated to dryness under nitrogen and reconstituted with methanol for LC-MS/MS analysis.

An Agilent 1260 series HPLC system (Agilent Technologies Inc., Shanghai, China) coupled

with a G6460H MS/MS spectrometer (Agilent Technologies Inc., Waldbronn, Germany) was applied to chromatographic analysis. 5 μL sample solution were injected into LC vial. Analytes were separated on a DIKMA® Leapsil C_{18} column (3.0×50, 2.7 μm) with a run time of 4.0 min at a constant flow rate of 0.4 mL/min maintained at 30°C.

The LC mobile phase for the detection of DTX was a mixture of methanol and aqueous phase 1 mM ammonium formate solution (Merck, Darmstadt, Germany), adjusted to pH 6.5 with formic acid (45:55, v/v, Merck, Darmstadt, Germany) with an injection volume of 5 μL. Quantitation was operated in the positive electrospray ionization mode (ESI^+). In the multiple reaction monitoring (MRM) mode, two optimal MRM transitions per analyte were monitored for DTX (m/z 830.4→549.3 with a collision energy (CE) of 15 V and a fragment voltage of 135 V) and for paclitaxel (IS) (m/z 876.8→308.5 with a CE of 18 V and a fragment voltage of 115 V), respectively. Under the LC-MS/MS conditions described above, DTX and IS were eluted at 1.986 min and 1.785 min, respectively. No significant effect of endogenous compounds on the analytes in the typical chromatograms. The recovery of drug in plasma and tissue samples were >92% and the standard curves with *r* value of more than 0.995 (weighting factor of $1/x^2$).

RT-qPCR analysis

Total RNA extraction and cDNA synthesis as well as RT-qPCR were followed as earlier reports (Rio et al., 2010; Doak and Za ŕ, 2012). Total RNA was isolated from liver samples with TRIzol reagent (Life Technologies Inc., Carlsbad, USA) following the manufacturer's manual. The quality and quantity of the RNA were performed on a SoftMax® Pro 5 Multiscan Spectrum (Molecular Devices, Silicon Valley, USA) with the value of absorbance of each RNA sample (A_{260} nm/A_{280} nm) was 1.8 and 2.0. A StepOne Plus PCR System (Applied Biosystems, CA, USA) with

SYBR Green PCR kit (QIAGEN, Frankfurt, Germany) was used to run PCR samples as reported in detail previously, using the primers (Sangon Biotech Co., Ltd., Shanghai, China) given in Table S1. Running conditions was as follows: initial denaturation (95°C, 10 min) for one cycle, followed by denaturation (95°C, 15 s) for 40 cycles and annealing (60 ℃, 60 s). Last, specificity of the product was confirmed by melting curve analysis. Relative quantification analysis of target gene was calculated using the CT ($2^{-\Delta\Delta CT}$) method and normalized relative to stable reference gene β-actin (a housekeeping gene) (Livak and Schmittgen, 2001). Each result of RT-qPCR reaction was reproducible and reliable in three times.

Supplementary Table S1 Primer sequences for SYBR green-based qPCR used in this study

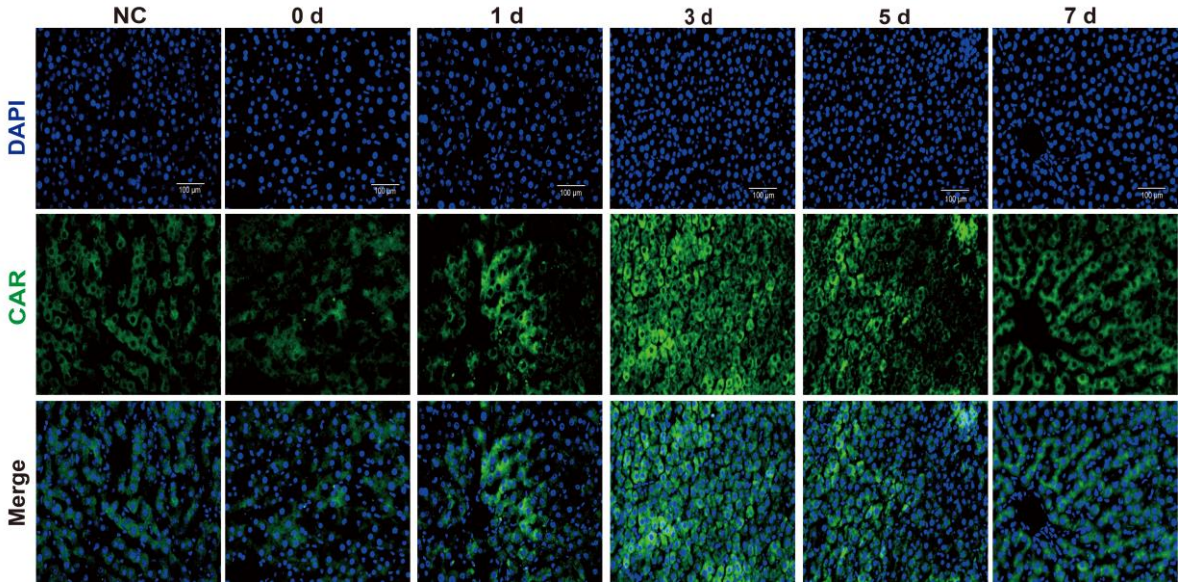
Target gene	Forward primer	Reverse primer
CYP1A2	5'-CATCTTTGGAGCTGGATTTG-3'	5'-CCATTCAGGAGGTGTCC-3'
CYP2C6	5'-TCAGCAGGAAAACGGATGTG-3'	5'-AATCGTGGTCAGGAATAA AAA TAACTC-3'
CYP3A1	5'-TGCCATCACGGACACAGA-3'	5'-ATCTCTTCCACTCCTCATCCTTAG-3'
PXR	5'-GAGGTCTTCAAATCTGCCGTGTA-3'	5'-CGGTGGAGCCTCAATCTTTTC-3'
CAR	5'-GTGTCTAAATGTTGGCATGAGGA-3'	5'-GTGATGGCTGAACAGGTAGGC-3'
β-actin	5'-GCCCAGAGCAAGACAGGTAT-3'	5'-GGCCATCTCCTGCTCGAAGT-3'

Western blotting analysis

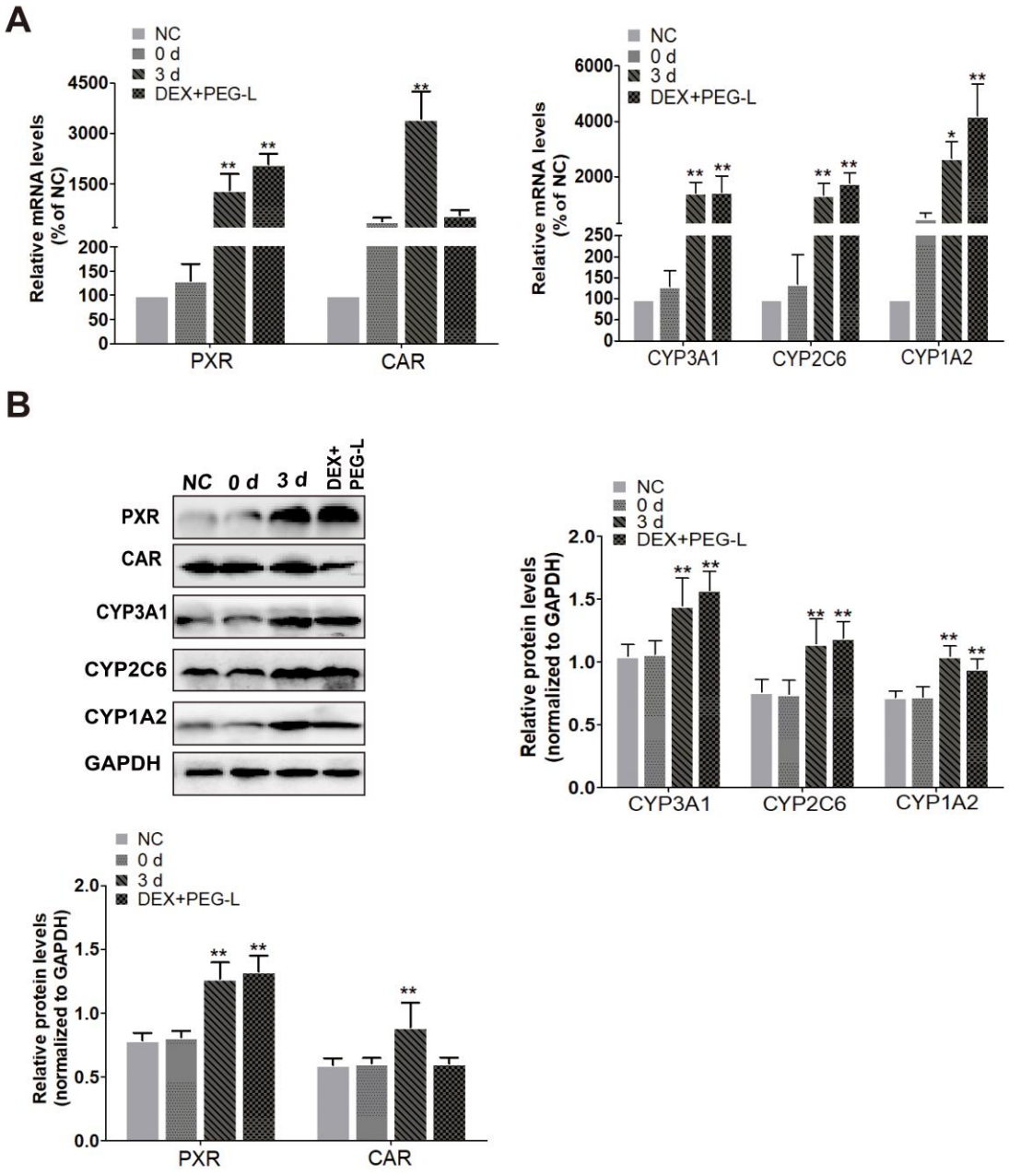
To determine total protein expression, rat liver tissues (~50 mg) were homogenized and lysed with 1mL RIPA lysis buffer for 30 min on ice. Protein concentration was detected by BCA protein assay method. After denaturation with 5×SDS-PAGE loading buffer at 95°C for 10 min, total protein (20~30 μg) from lysates was separated by 12% SDS-PAGE and transferred onto an NC

membrane. After blocking with 5% non-fat milk in Tris buffered saline containing 0.05% Tween-20 (TBS-T), blots were incubated with appropriate dilutions of the primary anti-CYP3A1, anti-CYP2C6, anti-CYP1A2, anti-PXR, anti-CAR and anti-GAPDH (as internal reference) antibodies at 4°C for 12 h. Correspondingly, horseradish peroxidase-conjugated secondary anti-mouse or -rabbit antibodies (in 10 mL 0.05% TBS-T with 1:10000 dilution) were added to the membrane and incubated at room temperature for 2 h. After washing with 0.05% TBS-T, protein bands were visualized using the ECL detection reagents according to the defined operation and the relative intensity of protein expression was normalized to GAPDH. The experiments for determination of all protein expression were performed in triplicates and the results were reproducible. Alpha Chemiluminescent gel imaging system FluorChem FC3 (ProteinSimple, silicon valley, USA) was using for scanning protein bands, which were quantified by an ImageJ software (version 1.45S, NIH, USA).

Supplementary Figures:

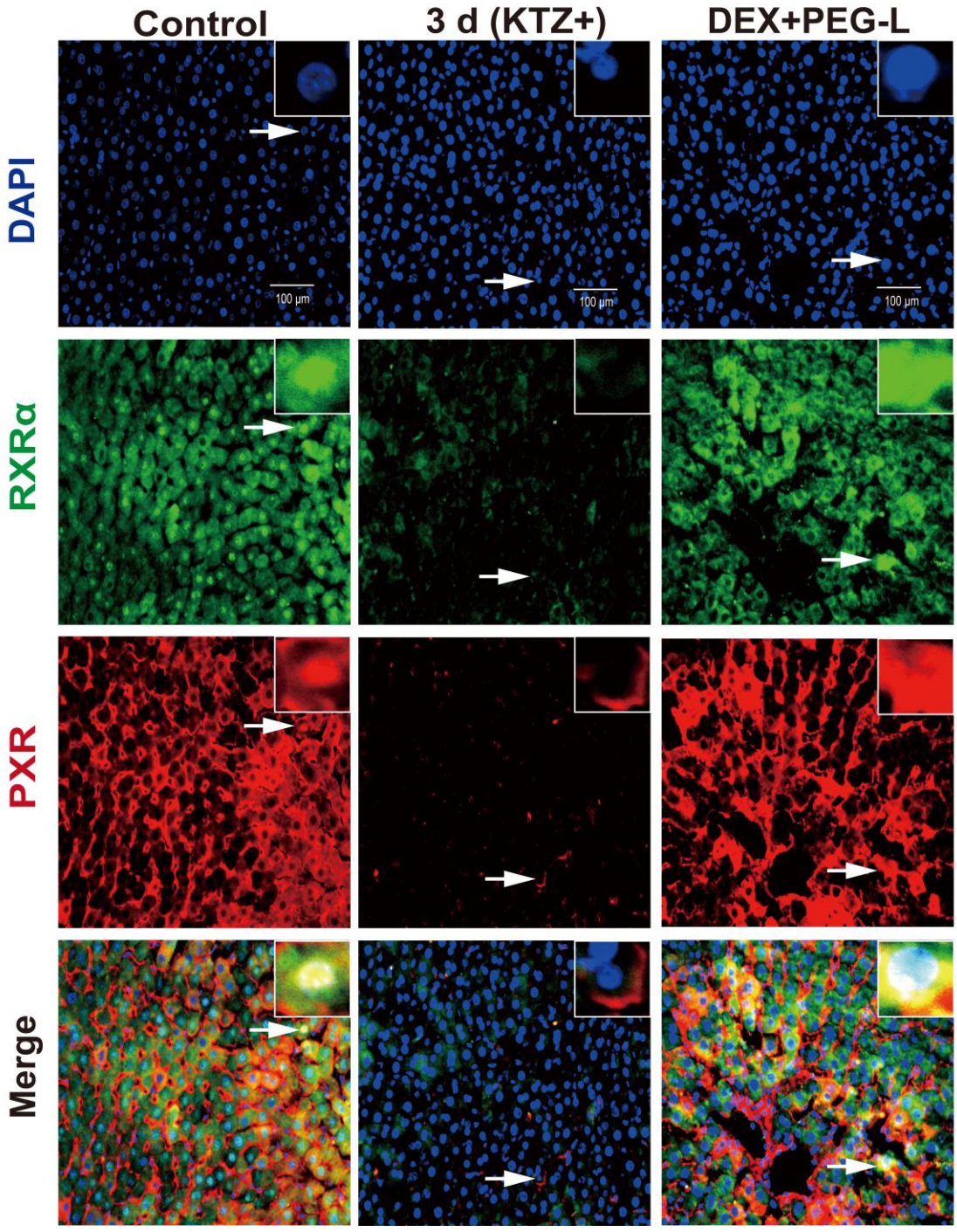


Supplementary Fig. S1. Immunofluorescence staining showed the location of hepatic CAR protein in hepatocytes (n=3). CAR shown in green and nuclei were stained with DAPI (blue). 0 d, 1 d, 3 d, 5 d, and 7 d indicate rats received repeated injection of PEG-L with different time intervals including 0, 1, 3, 5, and 7 days. Normal control group (NC) was injected with a single injection of equal volume of PBS. Scale bar: 100μm. PEG-L, PEGylated liposomes.



Supplementary Fig. S2. Effect of the presence of PXR inducer on the expression of hepatic PXR, CAR and CYPs. (A) RT-qPCR analysis the relative mRNA transcript levels of hepatic PXR, CAR and CYPs. Using β-actin as an endogenous reference gene, the values were normalized to the control group and fold change was calculated by $2^{-\Delta\Delta CT}$ method. (B) The relative protein expression of hepatic PXR, CAR and CYPs were analyzed by western blotting. Bar graphs show quantitative evaluation of PXR, CAR and CYPs bands by densitometry from triplicate

independent experiments. Each band density was evaluated by ImageJ software and these data were calculated as percentage compared to GAPDH. 0 d and 3 d indicate a single injection and repeated injections of PEG-L in rats with 3 days interval, respectively. DEX+ PEG-L presents intraperitoneally pretreated with DEX for 3 consecutive days combined with a subsequent injection of PEG-DTX-L (0.05 μmol/kg). Normal control group (NC) was injected with a single injection of equal volume PBS. Data are presented as the mean ± SD (n=5 ~ 6). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 0 d group.



Supplementary Fig. S3. Response of the nuclear translocation of hepatic PXR and RXRα proteins in rats exposed to the coadministration of DEX with an injection of PEG-DTX-L (n=3). PXR and RXRα are shown in red and green, respectively, and nuclei were stained with DAPI (blue). Control rats received repeated injection of PEG-L with 3-day interval; group 3 d (KTZ+) indicates the samples obtained from our previous studies that rats were pretreated with oral

administration 100 mg/kg/day of KTZ for 7 consecutive days combined with repeated injection of PEG-L with 3-day interval; group DEX+ PEG-L has been mentioned in Table 1. Scale bar: 100μm. PEG-L, PEGylated liposomes; DEX, dexamethasone; KTZ, ketoconazole.

References

Doak S and Za ŕ Z (2012) Real-time reverse-transcription polymerase chain reaction: technical considerations for gene expression analysis. Methods Mol Biol 817:251-270.

Livak K and Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408..

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