Supplementary Information for **The journal of Pharmacology and Experimental Therapeutics**:

*Titile***: Acetylcholinesterase inhibitors for Alzheimer's disease treatment ameliorate acetaminophen-induced liver injury in mice via central cholinergic system regulation**

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Supplementary Methods

Chemials and drugs

The standard products chlorzoxazone (CZX), 6-hydroxychlorzoxazone (HCZX) and phenacetin (used as the internal standard) were bought from Sigma Aldrich Co. (Shanghai, China). Methanol and acetonitrile were of HPLC grade, and other reagents were of AR grade unless indicated.

Storage solution preparation

Stock solutions of CZX (2 mmol/L), HCZX (2 mmol/L) and phenacetin (1 mmol/L) were prepared in methanol and stored at -20^{o}C. Working solutions were freshly prepared in doubly distilled water.

Co-incubation of CZX with mice hepatic microsomal

The co-incubation system contained 5 mM MgCl2, 1 mM SAM, and 6.8 mg mice hepatic microsomal protein in 50 mM phosphate buffer solution (pH 7.4). After pre-incubation at 37℃ for 5 min, CZX was added into the mixture at a final concentration of 0.25 mM and subjected to further incubation for 15 min at 37℃. Then the reaction was terminated by adding 5 M HCl, and the mixture was extracted with 3 volumes of acetonitrile. After vortex-mixing for 5 min and centrifugation at 8, 000 g for 10 min, the supernatant was separated and evaporated to dryness for subsequent HPLC assay.

Chromatographic conditions

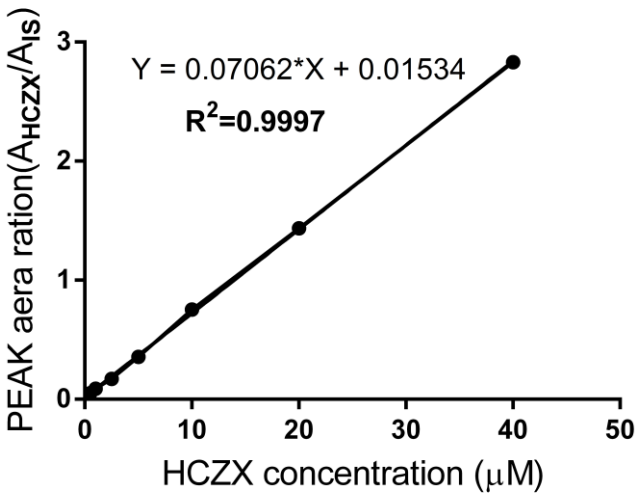
The HPLC system consisted of a LC-20A series, a reverse-phase C18 column (250 mm × 4.6 mm; 5 μm partical size) and a UV detector. The column temperature was

maintained at 35℃. The mobile phase composed of mixture of acetonitrile and 0.05% formic acid was delivered at a flow rate of 1 mL/min in isocratic elution mode with a volume ratio of 33:67 for incubation systems. Injection volume was set at 20 μL, and HPLC detection was conducted at 282 nm for 0-8 min, and then shifted to 264 nm afterwards.

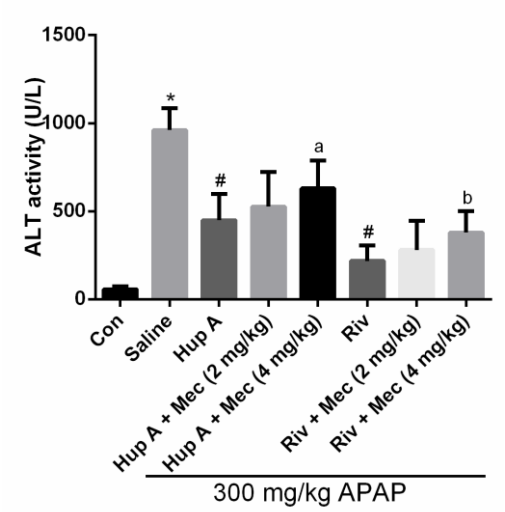
Calibration curves and validation study

To prepare standard curves, appropriate amounts of CZX and HCZX were added to the incubation systems to yield following concentration of each: 0.5, 1, 2.5, 5, 10, 20, 40 μM. These samples were immediately extracted by 3 ml acetonitrile according to the procedure described above and run in duplicate. Quantification was performed by calculating the peak-area ratio of each compound to the IS. Three standard solutions containing 1, 10, 40 μM of both CZX and HCZX were prepared to test the accuracy and precision of the assay. The lower limit of detection was defined as a single-to-noise ratio of 4:1. The potential interference from endogenous sources was examined with blank microsomal.

Supplementary Figures



Supplementary Figure S1. The calibration curves of HCZX detected by HPLC



Supplementary Figure S2. The serum ALT activity of mice co-treated with AChEI and various doses of mecamylamine. Serum were obtained when the mice were sacrificed 8 h after APAP treatment. Then the ALT activity was assayed by commercial kit. Data are expressed as mean ± SD (n = 10). (*$p < 0.01$ vs Control; $^{\#}p < 0.01$ vs APAP; $^{a}p < 0.05$ or $^{b}p < 0.05$ vs Hup A or Riv)

Supplementary Table

Supplementary Table S1. Intra- and inter-day validation of HCZX at different concentration in microsomal

Concentration (μM)	Inter-day (RSD %)	Intra-day (RSD %)
1	5.4	6.3
10	3.9	4.2
40	2.6	3.9