Supporting Information for: Metabolism and bioactivation of flurochloridone, a novel selective herbicide, in vivo and in vitro

Jingmin Shi,[†,‡,⊥] Cen Xie,[‡,⊥] Hongbing Liu,[§] Kristopher W. Krausz,[‡] Carole A. Bewley,[§] Suhui Zhang,[†] Liming Tang,[†,*] Zhijun Zhou,[∥] and Frank J. Gonzalez[‡,*]

[†]Pharmacology and Toxicology, Department/Center for Drug Safety Evaluation, Shanghai Institute for Food and Drug Control, Shanghai 201203, PR China
[‡]Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, United States
[§]Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, United States
[∥]School of Public Health, Fudan University, Shanghai 200032, PR China
[⊥]Co-first author

*Correspondence authors: Emails: gonzalef@mail.nih.gov (F.J.G.) and tangliming@smda.gov.cn (L.T.)

Pages: 15
Tables: 5
Figures: 6

SUPPLEMENTAL EXPERIMENTAL SECTION

***In vivo* Metabolism.** Male 6- to 8-week-old C57BL/6 mice were selected for metabolite profiling. FLC dissolved in corn oil was administered by oral gavage at a dose of 5 or 50 mg/kg BW. Control mice were treated with corn oil alone. Each group consist of 4 mice. Mice were fasted overnight and refed 2 h after dosing. Urine and feces samples were collected for 24 h using metabolic cages (Metabowls, TechPlast Sales Inc. New York, USA). Blood samples were collected by retro-orbital bleeding at 4 and 24 h, respectively. After centrifugation for 10 min at 14 000×g, serum was obtained for metabolites analysis. Gallbladder samples were collected after euthanized. After centrifugation for 10 min at 14 000×g, bile samples were collected. For toxicity study, mice were fed on control diet or 0.5% FLC-containing diet for 10 days. Serum and liver samples were collected for toxicity evaluation.

Purification and Characterization of the GSH Conjugate of Flurochloridone. A scaled-up mouse liver microsomal incubation (10 mL) with 5 mg/mL FLC was performed under similar experimental conditions as described above. After a 4-h incubation, the reaction was terminated by adding equal volume of ice-cold acetonitrile and then centrifuged at 14 000×g for 10 min. The resulting supernatants were separated by preparative HPLC (flow rate of 12 mL/min, eluting with a 60 min gradient from 0–50% acetonitrile in H_2O). The fraction containing the GSH conjugate was collected, halved and evaporated to dryness under reduced pressure to yield a yellow solid product (11.0 mg, 90.8% purity). The NMR spectra were recorded at 298 K on a 500 MHz NMR spectrometer equipped with a triple resonance cryoprobe and z gradient (Bruker Biospin, Switzerland). Chemical shifts (δ) were reported in parts per million (ppm) relative to the solvent. Preparative HPLC was performed on an Agilent 1100 and a Waters SymmetryPrep C18 column (19 × 300 mm, S-7 μm, 12 nm). All solvents used were of HPLC grade (Sigma Aldrich).

***In vivo* Sample Preparation.** Urine samples were prepared by mixing 25 μL urine with 175 μL 50% aqueous acetonitrile. Serum samples were prepared by mixing 20 μL serum with 180 μL 50% aqueous acetonitrile. Bile samples were prepared by mixing 2 μL bile with 198 μL 50% aqueous acetonitrile. Feces homogenates in a 20-fold volume of 50% aqueous acetonitrile were prepared. After centrifugation at 14 000× g for 20 min, a 5 μL aliquot of the supernatants was injected into a Waters UPLC-ESI-QTOFMS system (Waters Corporation, Milford, MA). injected into a UPLC-ESI-QTOFMS.

UHPLC-ESI-QTOFMS and UHPLC-ESI-TQMS Analysis. Metabolite profiling and identification were performed on an Acquity UHPLC/Premier Q-TOF MS (Waters Corp.) with an electrospray ionization source. Separation was achieved on an Acquity C18 BEH UPLC column (50 mm × 2.1 mm i.d., 1.7 mm; Waters Corp.). The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The following gradient condition was used: using 2% B in 0.5 min, increasing B up to 20% over the next 3.5

min, then increasing B up to 95% for another 4 min, then increasing B up to 99% for another 1 min, bringing back to 2% at 0.1 min and holding at 2% until the end. The flow rate of the mobile phase was set 0.4 $mL \cdot min^{-1}$. Column temperature was maintained at 40°C throughout the run. Data were collected in both positive ion mode, which was operated in full-scan mode at 50–850 m/z. Nitrogen was used as both cone gas (50 $L \cdot h^{-1}$) and desolvation gas (600 $L \cdot h^{-1}$). Source temperature and desolvation temperature were set at 150°C and 400°C, respectively. The capillary voltage and cone voltage were 3000 and 58 V, respectively. The structures of metabolites were elucidated by tandem MS fragmentography with collision energies ranging from 15 to 40 eV. To determine the GSH adduct produced by recombinant enzymes and liver extracts and FLC in mice serum, bile, urine, and feces, an Acquity UPLC system coupled with a XEVO triple-quadrupole tandem mass spectrometer (Waters Corporation) was used. Multiple reaction monitoring (MRM) was employed, and the MRM transition 583→454 was used for GSH adduct and 312→292 was for FLC.

SUPPLEMENTAL TABLES

Table S1. UHPLC-ESI-QTOFMS data for flurochloridone metabolites detected in mice at 5 mg/kg

No.	Metabolic pathway	*R*t (min)	Measured $[M+H]^+$	Formula	Error (ppm)	Matrices
M0	Parent	6.62	312.017	$C_{12}H_{10}Cl_2F_3NO$	-0.8	U, F, S
M1	Reductive dechlorination	6.31	278.057	$C_{12}H_{11}ClF_3NO$	3.2	U, F
M2	Oxidative dechlorination of 3-Cl	5.67	294.051	$C_{12}H_{11}ClF_3NO_2$	-0.1	U, F
M3	Oxidative dechlorination of 6-Cl	5.78	294.052	$C_{12}H_{11}ClF_3NO_2$	4.6	U, B, F
M4	Oxidative dechlorination of 6-Cl to carboxylic acid	5.84	308.029	$C_{12}H_9ClF_3NO_3$	-5.2	U, B
M5-1	Hydroxylation	6.06	328.011	$C_{12}H_{10}Cl_2F_3NO_2$	-1.4	U, F
M5-2	Hydroxylation	6.24	328.011	$C_{12}H_{10}Cl_2F_3NO_2$	-2.4	F
M6	Substitution of chlorine with cysteine	5.35	397.060	$C_{15}H_{16}ClF_3N_2O_3S$	-0.6	U, F, B
M7-1	Substitution of chlorine with cysteine and hydroxylation	4.64	413.055	$C_{15}H_{16}ClF_3N_2O_4S$	-0.8	U
M7-2	Substitution of chlorine with cysteine and hydroxylation	4.80	413.056	$C_{15}H_{16}ClF_3N_2O_4S$	1.6	U
M7-3	Substitution of chlorine with cysteine and hydroxylation	4.97	413.057	$C_{15}H_{16}ClF_3N_2O_4S$	4.0	U
M7-4	Substitution of chlorine with cysteine and hydroxylation	5.04	413.056	$C_{15}H_{16}ClF_3N_2O_4S$	2.8	U
M8	Substitution of chlorine with *N*-acetylcysteine	5.94	439.071	$C_{17}H_{18}ClF_3N_2O_4S$	0.3	U, B, F, S
M9	Substitution of chlorine with glycerylcysteine	5.30	454.083	$C_{17}H_{19}ClF_3N_3O_4S$	4.2	U, F
M10-1	Substitution of chlorine with *N*-acetylcysteine and hydroxylation	4.05	455.066	$C_{17}H_{18}ClF_3N_2O_5S$	0.7	U
M10-2	Substitution of chlorine with *N*-acetylcysteine and hydroxylation	5.43	455.066	$C_{17}H_{18}ClF_3N_2O_5S$	0.2	U
M10-3	Substitution of chlorine with *N*-acetylcysteine and hydroxylation	5.54	455.063	$C_{17}H_{18}ClF_3N_2O_5S$	-4.9	U
M10-4	Substitution of chlorine with *N*-acetylcysteine and hydroxylation	5.59	455.065	$C_{17}H_{18}ClF_3N_2O_5S$	-2.0	U
M11-3	Oxidative dechlorination of 6-Cl and glucuronidation	5.32	470.084	$C_{18}H_{19}ClF_3NO_8$	1.2	U, B
M12	*N*-acetylcysteine conjugation	5.40	473.032	$C_{17}H_{17}Cl_2F_3N_2O_4S$	0.2	B
M13	Substitution of chlorine with glutathione	5.34	583.128	$C_{22}H_{26}ClF_3N_4O_7S$	6.2	B
M14-1	Substitution of chlorine with glutathione and hydroxylation	4.80	599.119	$C_{22}H_{26}ClF_3N_4O_8S$	0.2	B
M14-2	Substitution of chlorine with glutathione and hydroxylation	4.85	599.121	$C_{22}H_{26}ClF_3N_4O_8S$	3.3	B
M14-3	Substitution of chlorine with glutathione and hydroxylation	4.94	599.122	$C_{22}H_{26}ClF_3N_4O_8S$	4.7	B
M14-4	Substitution of chlorine with glutathione and hydroxylation	5.03	599.120	$C_{22}H_{26}ClF_3N_4O_8S$	1.2	B

Table S2. MS/MS fragments and proposed structures of flurochloridone metabolites

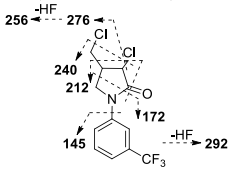
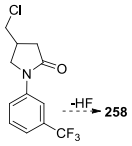
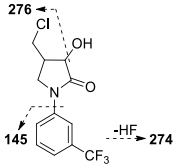
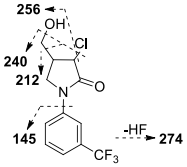
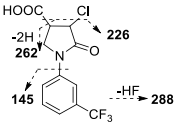
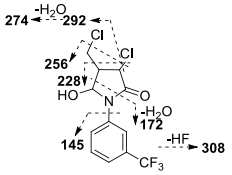
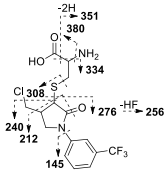
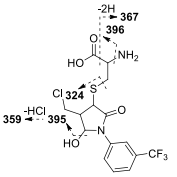
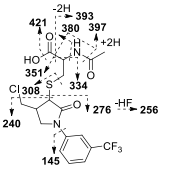
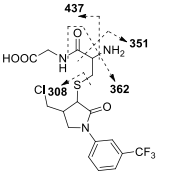
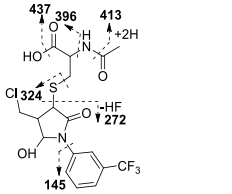
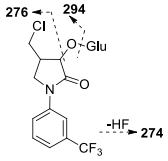
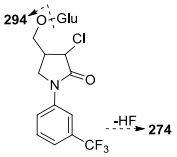
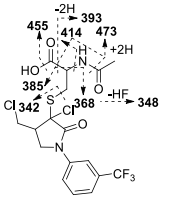
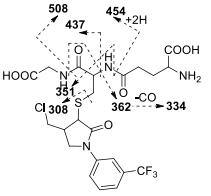
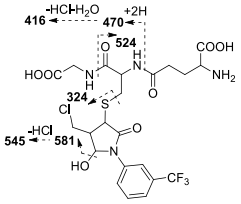
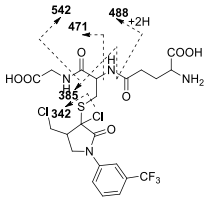
M0 (Flurochloridone)  292.010, 276.040, 256.037, 240.060, 212.071, 198.053, 172.033, 145.031	M1  258.056	M2  276.047, 274.044, 145.023	M3  274.041, 256.054, 240.043, 228.067, 145.028	M4  288.024, 262.026, 226.042, 145.026
M5-1, M5-2  308.010, 292.035, 274.030, 256.060, 228.054, 172.037, 145.028	M6  380.028, 351.051, 334.023, 308.010, 276.034, 256.032, 240.061, 212.060, 145.026	M7-1 ~ M7-4  396.023, 395.054, 367.046, 359.066, 324.009	M8  421.055, 397.059, 393.061, 380.033, 351.056, 334.023, 308.015, 276.036, 256.035, 240.056, 145.026	M9  437.0557, 362.0155, 351.0525, 308.0099
M10-1 ~ M10-4  437.061, 413.046, 396.031, 324.005, 272.033	M11-1, M11-2  294.052, 276.039, 274.040	M11-3  294.050, 274.043	M12  455.041, 431.015, 413.990, 385.005, 367.985, 347.979, 341.975	M13  565.115, 508.088, 454.079, 437.052, 362.020, 351.050, 334.025, 308.010
M14-1 ~ M14-4  581.104, 545.127, 524.088, 470.085, 416.083, 324.003	M15  542.053, 488.046, 471.016, 385.016, 341.974			

Table S3. Serum flurochloridone concentrations and urinary and feces excretion

Matrices	Time interval (h)	Unit	Dose (mg/kg)	
			5	50
Serum	4	μM	0.941 ± 0.220	8.02 ± 2.81
	24		0.053 ± 0.025	0.285 ± 0.076
Urine	0 – 24	% of the dose	0.122 ± 0.080	0.134 ± 0.033
Feces	0 – 24	% of the dose	1.21 ± 0.120	1.40 ± 0.198

Table S4. UPLC-ESI-QTOFMS data for flurochloridone metabolites detected in microsomes

No.	Metabolic pathway	*R*t (min)	Measured $[M+H]^+$	Formula	Error (ppm)
M0	Parent	6.63	312.017	$C_{12}H_{10}C_{l2}F_3NO$	0.1
M3	Oxidative dechlorination of 6-Cl	5.78	294.050	$C_{12}H_{11}ClF_3NO_2$	-4.6
M4	Oxidative dechlorination of 6-Cl to carboxylic acid	5.84	308.032	$C_{12}H_9ClF_3NO_3$	5.8
M5-1	Hydroxylation	6.05	328.011	$C_{12}H_{10}Cl_2F_3NO_2$	-1.7
M5-2	Hydroxylation	6.25	328.014	$C_{12}H_{10}Cl_2F_3NO_2$	5.0
M13	Subtitution of chlorine with glutathione	5.32	583.127	$C_{22}H_{26}ClF_3N_4O_7S$	5.2
M14-1	Subtitution of chlorine with glutathione and hydroxylation	4.80	599.120	$C_{22}H_{26}ClF_3N_4O_8S$	1.3
M14-4	Subtitution of chlorine with glutathione and hydroxylation	5.03	599.116	$C_{22}H_{26}ClF_3N_4O_8S$	-5.0

Table S5. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for flurochloridone GSH conjugate in CD_3OD

No.	δ_H (mult. *J* in Hz)	δ_C
2		174.2
3	3.82 (d, 8.4)	49.5/50.5
4	2.73 (m)/2.78 (m)	41.7/42.0
5	4.10 (t, 9.0)	51.0/50.9
	3.85 (dd, 10.7, 4.3)	
6	3.92 (m)	45.9/45.8
	3.89 (m)	
1’		140.9
2’	8.14 (s)	118.2
3’		132.5, 132.2, 132.0, 131.7
4’	7.51 (d, 8.0)	122.6
5’	7.61 (dd, 8.0, 8.0)	131.0
6’	7.83 (d, 8.5)	124.6
$-CF_3$		162.2, 161.9, 161.7, 161.4
1’’		171.6
2’’	4.05 (t, 6.4)	53.4
3’’	2.26 (m)	27.1/27.0
	2.19 (m)	
4’’	2.61 (t, 6.9)	32.4
5’’		174.3
7’’	4.73 (dd, 9.0, 5.8)	54.1/54.6
8’’		172.9
10’’	3.97 (m, 2H)	41.8
11’’		172.6
12’’	3.37 (dd, 13.9, 5.7)	34.6/34.4
	2.98 (dd, 13.9, 8.4)	
	/3.26 (dd, 13.9, 7.5)	
	/3.16 (dd, 13.9, 5.7)	

SUPPLEMENTAL FIGURES

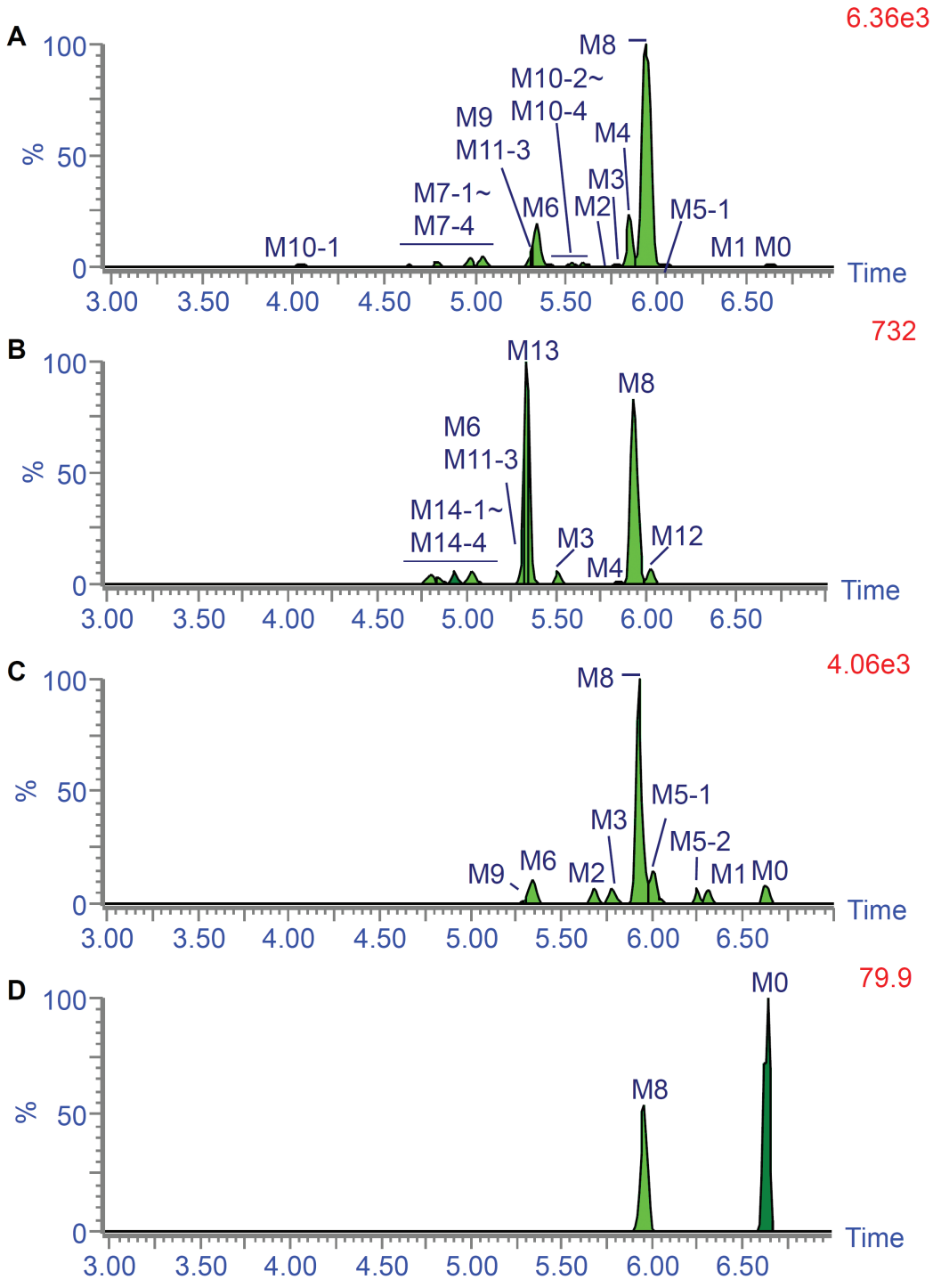


Figure S1. Metabolic profiles of flurochloridone in urine (A), bile (B), and feces (C) collected at 0 to 24 h postdose, and serum samples (D) collected at 4 hour postdose. Mice received a single oral administration of 5 $mg \cdot kg^{-1}$ flurochloridone.

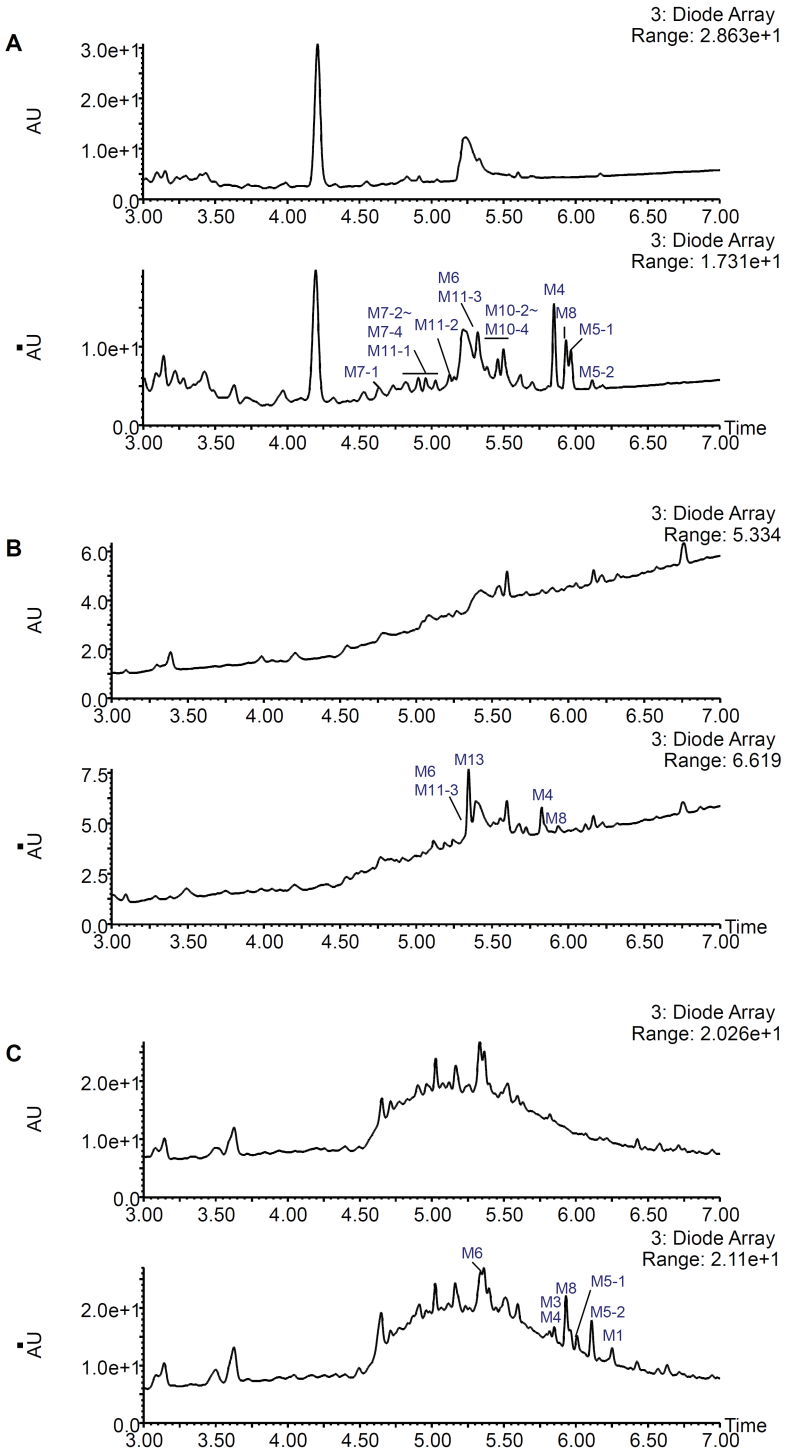


Figure S2. UHPLC-UV metabolic profiles of flurochloridone in urine (A), bile (B), and feces (C) collected at 0 to 24 h postdose. Mice received a single oral administration of 50 mg·kg^{-1} flurochloridone. Upper trace, sample before dose. Lower trace, dosed sample.

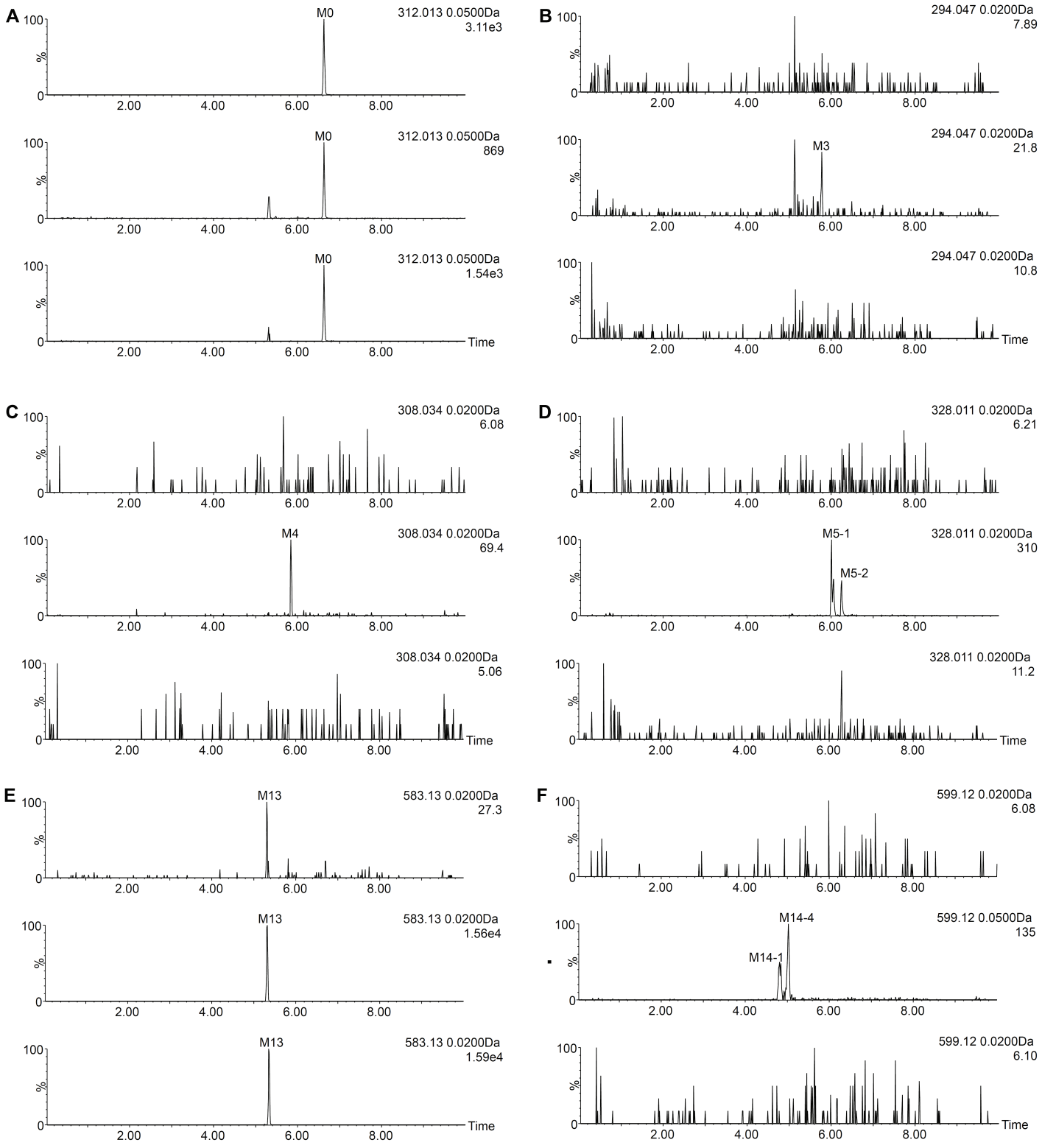
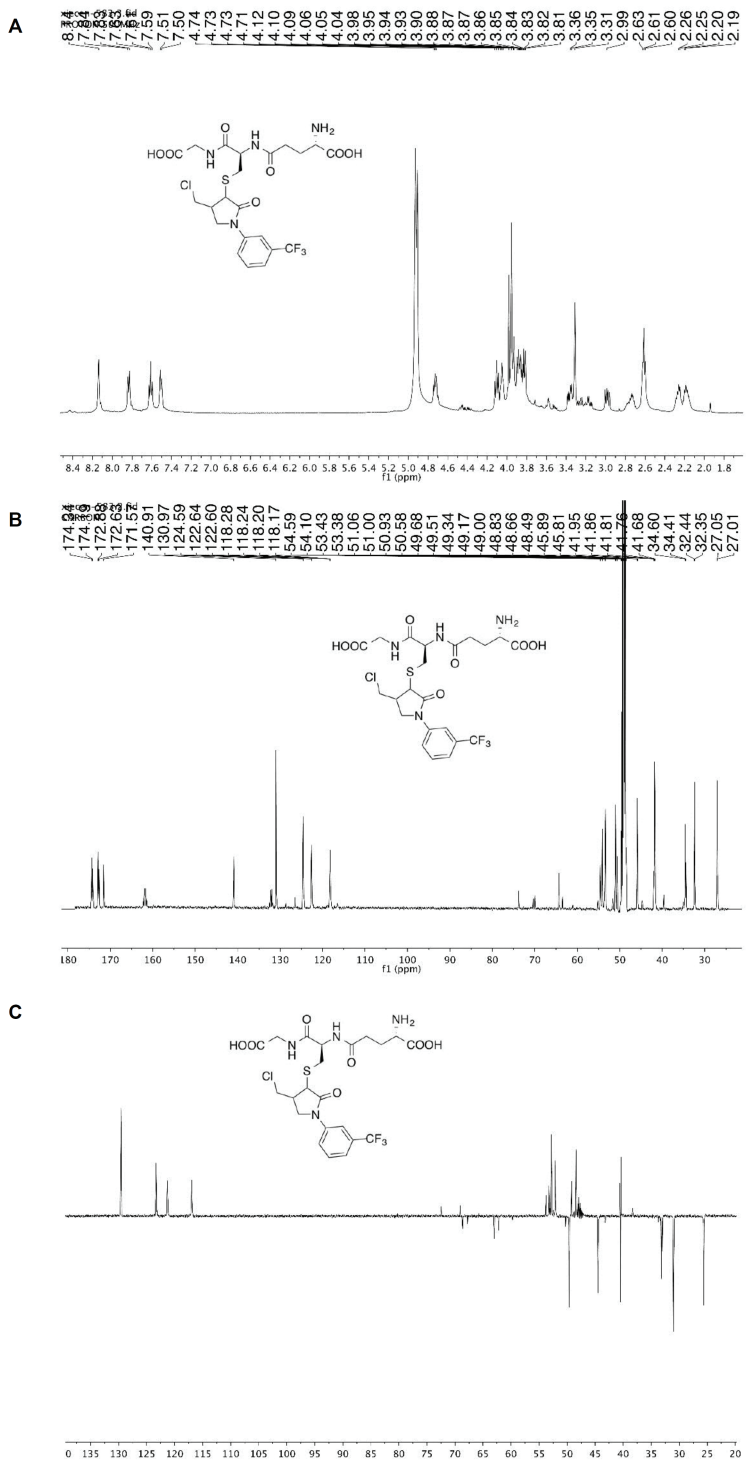


Figure S3. Extracted ion chromatograms of flurochloridone and its metabolites in HLM incubations: buffer (A), with NADPH and GSH (B), and with GSH but without NADPH (C).


A
B
C
HOOC
NH2
COOH
Cl
CF3
f1 (ppm)

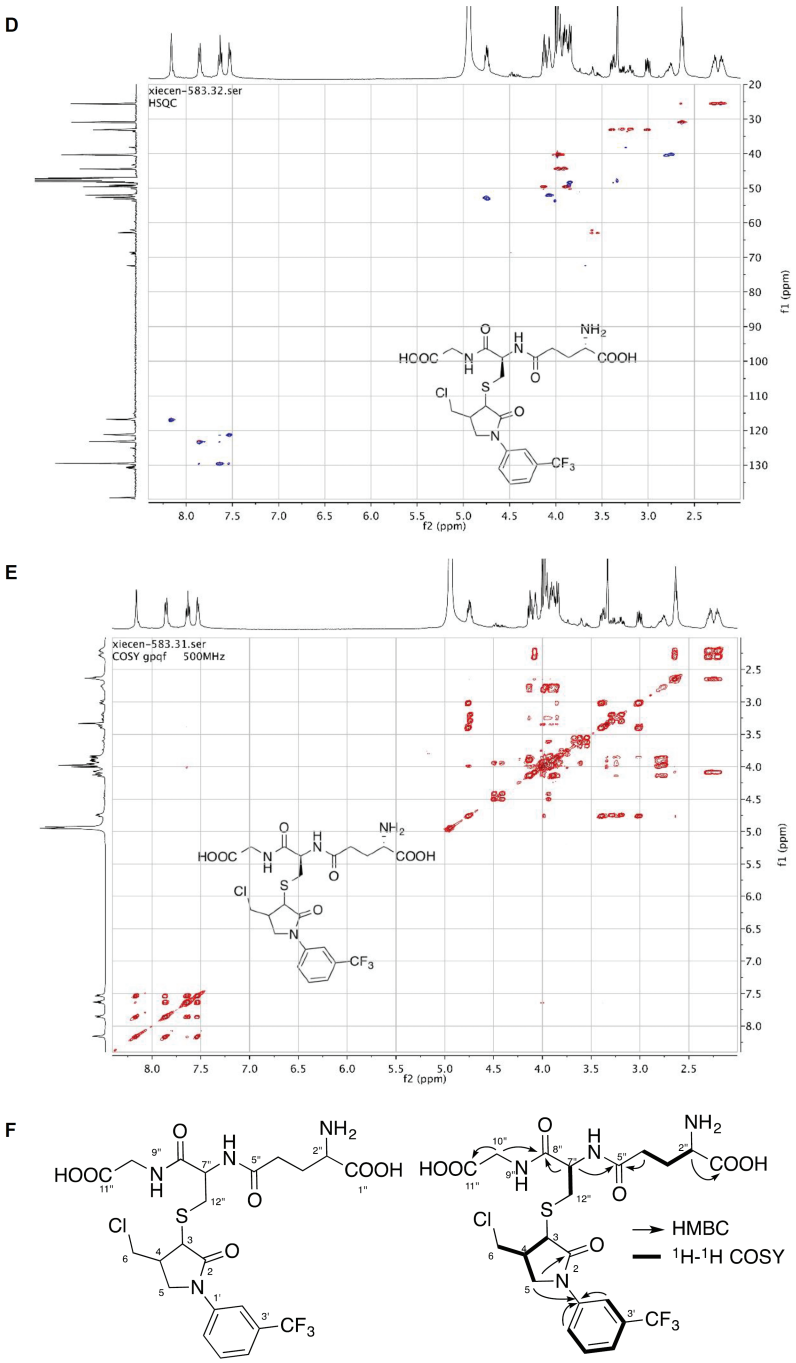


Figure S4. (A) ^{1}H NMR spectrum. (B) ^{13}C NMR spectrum. (C) DEPT spectrum. (D) HSQC spectrum. (E) COSY spectrum. (F) Structure and key ^{1}H–^{1}H COSY (−) and HMBC (→) correlations of flurochloridone GSH conjugate.

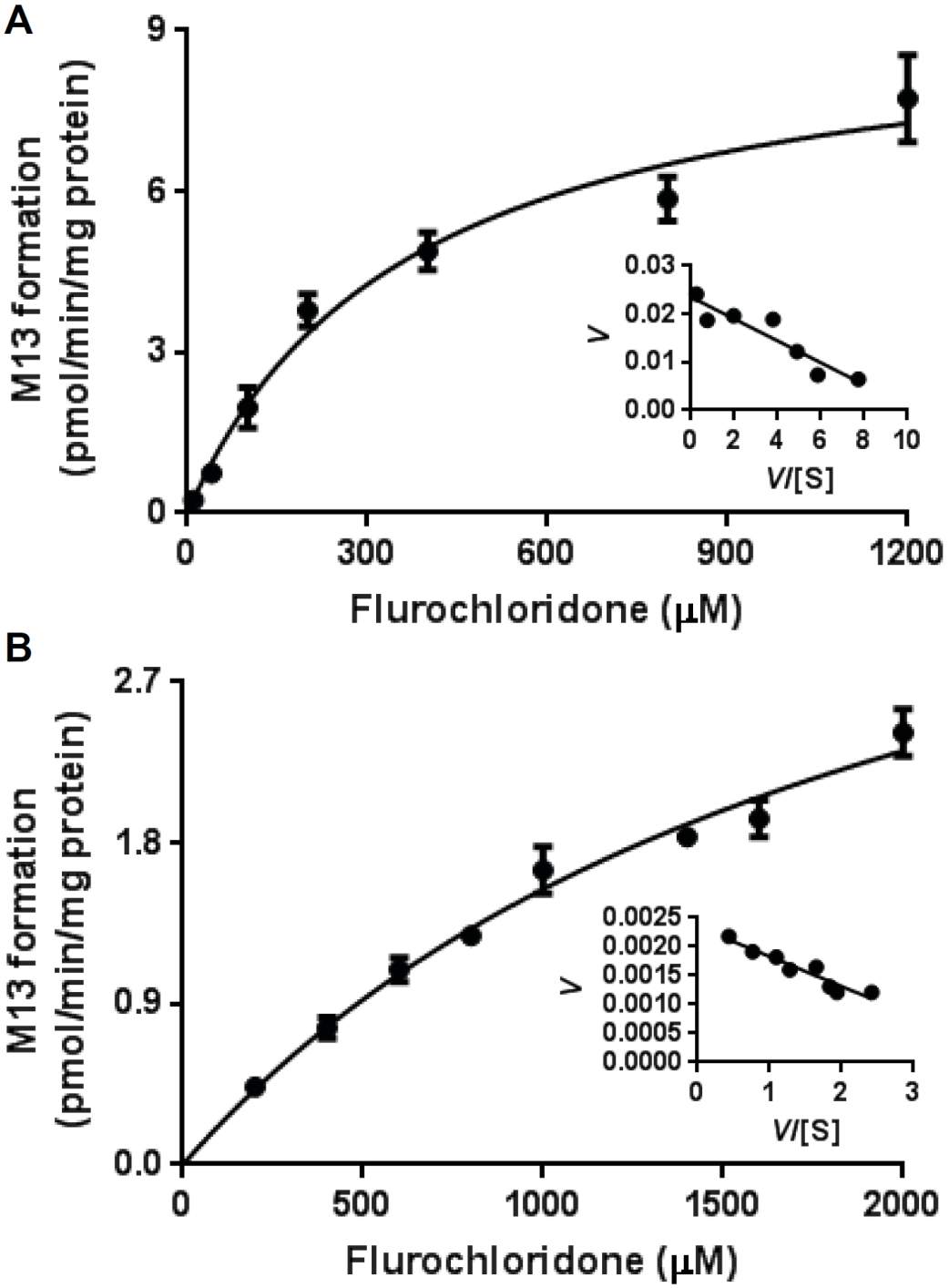


Figure S5. Enzyme kinetics of GSH conjugation by MLM (A) and MLC (B) fitted by Michaelis-Menten kinetic model. Eadie-Hofstee plots are shown as insets. Data were obtained from three replicates.

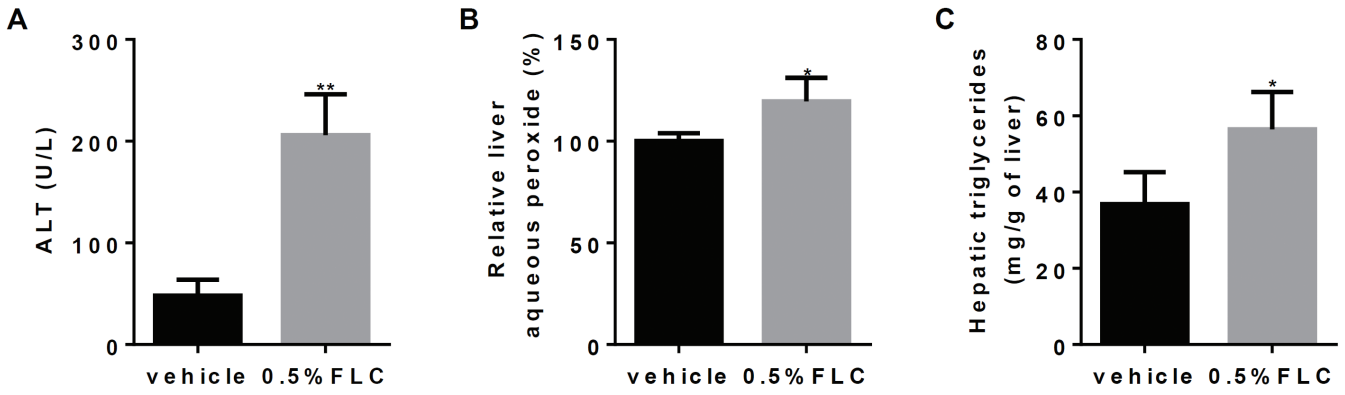


Figure S6. Toxic response in mice fed on the diet containing 0.5% FLC or control diet for 10 days. (A) Liver ALT. (B) Liver aqueous peroxide. (C) Liver triglycerides. Data are presented as mean ± SD; n = 4/group. *P < 0.05, and **P < 0.01 versus vehicle group, by two-tailed Student's t-test.