Supplementary Information

Metabolomics reveals the efficacy of caspase inhibition for saikosaponin d-induced hepatotoxicity

Qian-qian Zhang1,2, Wan-qi Huang1,2, Yi-qiao Gao1,2, Zhao-di Han1,2, Wei Zhang3, Zun-jian Zhang1,2*, Feng-guo Xu1,2*

1 Key Laboratory of Drug Quality Control and Pharmacovigilance (Ministry of Education), China Pharmaceutical University, China
2 State Key Laboratory of Natural Medicine, China Pharmaceutical University, Nanjing, China
3 State Key Laboratory for Quality Research in Chinese Medicines, Macau University of Science and Technology, Taipa, Macau, Macau

* Correspondence:
Zun-jian Zhang
Email: zunianzhangcpu@hotmail.com Feng-guo Xu
Email: fengguoxu@gmail.com
Metabolomics Analysis

Sample preparation

10 μL of serum was extracted by 100 μL methanol (5 μg/mL of heptadecanoic acid in methanol), vortex for 15 min, then followed by centrifugation (14000 g×10 min, 4°C) for two times, 80 μL of the supernatant was derivatized using a two-step procedure. 25 μL of MOX (10 μg/mL in pyridine) was added to the vial and shook for 90 min at 37°C, evaporated and followed by 120 μL MSTFA: ethyl acetate (1:4, V/V) at 37°C for 120 min. The derivatized sample was for GC-MS analysis.

For LC-MS analysis, 140 μL of acetonitrile (5 μg/mL of glibenclamide in acetonitrile) was added to 20 μL of serum for protein precipitation, vortex for 5 min, the mixture was centrifuged at 16000 g for 10 min twice. The supernatant was for LC-MS analysis.

GC-MS analysis

The samples were performed on GC-MS-QP2010 Ultra (Shimadzu Co, Kyoto, Japan) equipped with a fused silica capillary column (Rtx-5MS:30 m×0.25 mm i.d., film thickness 0.25 μm, Thames Restek, UK). The helium carrier gas flow rate was set at 1.0 mL/min. The GC oven was initially held at 70°C for 3 min, and increased to 320°C at a rate of 10 °C/min, where it was held for 2 min. 1 μL of derivatized samples was injected at a split ratio of 1:50. The temperature of the injector and ion source was set at 250°C and 200°C, respectively. The MS was operated in electron impact mode (70 eV) with a mass range of m/z 45 to 600.

LC-MS analysis

The samples were performed on Ultra Fast Liquid Chromatography (UFLC) system (Shimadzu Co, Kyoto, Japan) equipped with an electrospray ionization source operating in both positive and negative ion mode. The column held at 40°C for separation was Phenomenex Kinetex C18 (100 mm×2.1 mm, 2.6 μm) (Phenomenex, Torrance, CA, USA). The mobile phase was a mixture of (A) H2O with 0.1% formic acid and (B) acetonitrile, with a programmed gradient as follows: linear gradient from 5% B to 95% B, 0–20 min; maintained with 95% B in 3 min, and returned to 5% B for column equilibration for 7 min. The injection volume was 5 μL and the flow rate was set at 0.4 mL/min. The scan ranges were of m/z 100-1000; the detector voltage was 1.7 kV; the ESI voltage was 4.5 kV in positive ion mode and -3.5 kV in negative ion mode; the ion source and heat block were maintained at 200°C; the nebulizing gas flow rate was 1.5 L/min; the drying gas flow was 0.1 MPa, and the ion accumulation time was 30 ms.

Data preprocessing and analysis

Each chromatogram obtained was processed by profiling solution version 1.1 (Shimadzu Co, Kyoto, Japan) for peak deconvolution and alignment. Following background-peak-filtering, 80% rule, limitation of QCs, normalization by the total ion intensity. After data preprocessing, a new data matrix containing sample names, variable names, and normalized ion intensities was imported to SIMCA-P software (version 13.0, Umetrics, Sweden) for the performance of PCA and OPLS-DA. A number of variables with
VIP value > 1.0 were identified from OPLS-DA as being responsible for the differences between treatment and control groups. Furthermore, the nonparametric Mann–Whitney U test was performed to determine the significance of each metabolite using PASW Statistics 18 (SPSS Inc., Chicago, USA).

Identification of metabolites

Compound identification in GC-MS was performed by comparing the mass fragments and intensities with NIST MS search 2.0 software (NIST, Gaithersburg, MD). Metabolites with a similarity of more than 70% were finally verified by available reference compounds. Then those metabolites were further confirmed by comparing with the standards that available in our lab. The identification of biomarkers in LC-MS followed a comprehensive strategy involving the determination of the accurate m/z, retention time, and typical MS/MS fragment and pattern. Discriminative markers were then compared with the mass-to-charge ratio (m/z), formulae and the MS/MS fragmentation of metabolites proposed by literatures and online databases, including HMDB, METLIN, Mass Bank and LIPID MAPS. Then, the putative identifications were verified by comparing the MS^n fragmentation patterns and retention time with those of authentic standard compounds that available in our lab.

Quantification of bile acids in serum

A total of 200 μL methanol and 10 μL cortisone acetate (IS, 100 μg/ mL) were added to 50 μL serum for protein precipitation, vortex for 5 min, the mixture was centrifuged at 16000 g for 10 min twice. The supernatant was for LC-MS/MS analysis. The mobile phase consisted of (A) acetonitrile and (B) 0.1% formic acid, with a programmed gradient as follows: started with 25% A for 20 min; linearly increased to 40% A in 65 min and brought back to 25% A in 5 min followed by 10 min of column equilibration. The parameters were set as follows: ESI (-), spray voltage, 3.8 kV; capillary temperature, 380°C; scan width for multiple-reaction monitoring (MRM), 0.1 m/z. Nitrogen was used as sheath (40 arb) and auxiliary (25 arb) gas. Argon was used as the collision gas (1.0 mTorr).
Figure S1: Experimental design. Animals either received vehicle alone, SSd alone, or SSd and z-VAD-fmk. group M and group MF were received intraperitoneal injection of 25 mg/kg of SSd at time 28 h. group MF was given z-VAD-fmk by intraperitoneal injections of 10 mg/kg at 0 h and 24 h. The weight loss and food intake of animals were monitored daily. The mice were finally sacrificed on 52 h. Serum and liver were collected for preparation and analysis.
Figure S2: Representative photographs of histological examination. (A) Normal liver of group C, (B) liver of group M and (C) liver of group MF. Bar=20 μm.
Figure S3: PCA and OPLS-DA score plots of group C and M. Score plots of PCA (A, B and C) and OPLS-DA (D, E and F) models of serum metabolite profiles after SSd (M) exposure detected by (A and D) GC-MS analysis, $R^2_X=0.886$, $Q^2=0.747$; $R^2_X=0.761$, $R^2_Y=0.926$, $Q^2=0.867$; (B and E) LC-MS(+) analysis, $R^2_X=0.578$, $Q^2=0.309$; $R^2_X=0.521$, $R^2_Y=0.995$, $Q^2=0.969$; (C and F) LC-MS(-) analysis, $R^2_X=0.632$, $Q^2=0.345$; $R^2_X=0.6$, $R^2_Y=0.971$, $Q^2=0.907$. (QC, quality control).
Figure S4: PCA and OPLS-DA score plots of the three groups. Score plots of PCA (A, B and C) and OPLS-DA (D, E and F) models of serum metabolite profiles after SSD (M) and z-VAD-fmk (MF) exposure detected by (A and D) GC-MS analysis, $R^2_X=0.891$, $Q^2=0.758$; $R^2_X=0.704$, $R^2_Y=0.686$, $Q^2=0.574$; (B and E) LC-MS(+) analysis, $R^2_X=0.535$, $Q^2=0.326$; $R^2_X=0.642$, $R^2_Y=0.991$, $Q^2=0.757$; (C and F) LC-MS(-) analysis, $R^2_X=0.771$, $Q^2=0.557$; $R^2_X=0.592$, $R^2_Y=0.799$, $Q^2=0.722$. (QC, quality control).
Figure S5: RDV of the three groups. RDV from (A) GC-MS and (B) LC-MS of different groups. Values are expressed as the mean ± S.D. Group M vs. C, *P < 0.05, **P < 0.01; Group MF vs. M, #P < 0.05, ##P < 0.01.
Table S1 ALT, AST, relative body weight (RBW) and relative food intake (RFI) monitored in all groups.

| Groups | ALT mean±SD | AST mean±SD | RBW (H52/H0%)±SD | RFI (H52/H0%) |
|--------|-------------|-------------|------------------|---------------|
| C      | 53.6±21.7   | 90.3±14.7   | 116.9±2.7        | 120.4         |
| M      | 244.0±64.3**| 1055±322.3**| 95.1±2.8**       | 23.8          |
| MF     | 121.4±32.5##| 589.5±221.0##| 97.5±2.6        | 31.5          |

Note: RBW and RFI on hour 52 were calculated relative to animals' weight and food intake at the beginning of the experiment (hour 0), respectively. Group M vs. C, *P <0.05, **P <0.01; Group MF vs. M, #P <0.05, ##P <0.01.
| No | Metabolite                   | m/z   | Rt(min) | Similarity | VIP      | p-value |
|----|------------------------------|-------|---------|------------|----------|---------|
|    | Carbohydrate and conjugates |       |         |            |          |         |
| 1  | Carbohydrate 1               | 217.10| 16.797  | 90         | 4.95365  | 0.002   |
| 2  | Carbohydrate 2               | 217.10| 17.365  | 90         | 6.01114  | 0.003   |
| 3  | Carbohydrate 3               | 204.10| 17.894  | 94         | 4.44312  | 0.002   |
| 4  | Carbohydrate 4               | 205.10| 17.983  | 91         | 6.33812  | 0.002   |
| 5  | Carbohydrate 5               | 204.10| 18.016  | 89         | 3.85493  | 0.013   |
| 6  | Carbohydrate 6               | 204.10| 18.771  | 97         | 3.98056  | 0.002   |
|    | Amino acids and derivatives  |       |         |            |          |         |
| 7  | Glycine                      | 102.11| 5.770   | 92         | 1.41392  | 0.013   |
| 8  | Alanine                      | 116.13| 7.140   | 94         | 1.12178  | 0.035   |
| 9  | Leucine                      | 102.11| 9.840   | 96         | 1.36435  | 0.048   |
| 10 | Isoleucine                   | 147.11| 10.184  | 89         | 1.38753  | 0.009   |
| 11 | Threonine                    | 101.10| 11.571  | 96         | 2.35533  | 0.004   |
| 12 | Methionine                   | 100.10| 13.361  | 85         | 1.13129  | 0.018   |
| 13 | Phenylalanine                | 73.06 | 14.749  | 94         | 1.08176  | 0.025   |
|    | Others                       |       |         |            | 3.32777  | 0.002   |
| 14 | Cholesterol                  | 129.11| 28.659  | 92         | 3.32777  | 0.002   |
| 15 | Urea                         | 147.13| 9.301   | 92         | 9.47279  | 0.002   |
| 16 | Citric acid                  | 273.14| 17.019  | 87         | 1.29856  | 0.048   |
| 17 | Pentanedioic acid            | 147.11| 14.058  | 89         | 1.33423  | 0.002   |
| No | Metabolite                | m/z   | Rt(min) | Adduct ions | MS/MS fragment | VIP     | p-value   |
|----|---------------------------|-------|---------|-------------|----------------|---------|-----------|
|    | Amino acids and derivatives |       |         |             |                |         |           |
| 1  | Histidine                 | 156.042 | 0.571  | [M+H]^+     | 140.0676       | 1.41024 | 0.003     |
| 2  | Methionine                | 150.0576 | 0.667   | [M+H]^+     | /              | 1.32408 | 0.002     |
| 3  | Phenylalanine             | 166.0864 | 0.797   | [M+H]^+     | 120.0834       | 2.17863 | 0.004     |
| 4  | Acetylcarnitine           | 204.1223 | 0.655   | [M+H]^+     | 145.0473       | 5.42315 | 0.003     |
| 5  | Propionylcarnitine        | 218.1374 | 0.667   | [M+H]^+     | 85.0270        | 1.09131 | 0.018     |
| 6  | Linoleylcarnitine         | 424.3426 | 13.312  | [M+H]^+     | 189.1493       | 1.44322 | 0.013     |
| 7  | Stearoylcarnitine         | 428.3729 | 15.343  | [M+H]^+     | 267.2595, 369.2955 | 1.50689 | 0.002     |
|    | Carnitines                |         |         |             |                |         |           |
|    | LPC(14:0)                 | 468.3078 | 11.811  | [M+H]^+     | 450.3002, 184.0794 | 1.23   | 0.006     |
| 8  | LPC(20:5)                 | 542.3240 | 12.082  | [M+H]^+     | 524.3098, 184.0745 | 2.47081 | 0.002     |
| 9  | LPC(16:1)                 | 494.3232 | 12.287  | [M+H]^+     | 476.3113, 184.0706 | 2.3756 | 0.002     |
| 10 | LPC(22:6)                 | 568.3383 | 12.816  | [M+H]^+     | 550.3271, 184.0732 | 1.52763 | 0.004     |
| 11 | LPC(18:2)                 | 564.3110 | 12.818  | [M+HCOO]^+  | 504.2924       | 2.94175 | 0.002     |
| 12 | LPC(20:4)                 | 544.3384 | 12.843  | [M+H]^+     | 526.3261, 184.0768 | 2.31527 | 0.002     |
| 13 | LPC(22:5)                 | 614.3217 | 13.111  | [M+HCOO]^+  | 554.3051, 184.0768 | 1.06415 | 0.002     |
| 14 | LPC(20:3)                 | 590.3245 | 13.326  | [M+HCOO]^+  | 530.3070       | 1.66917 | 0.002     |
| 15 | LPC(16:0)                 | 540.3121 | 13.385  | [M+HCOO]^+  | 480.2939       | 2.98152 | 0.006     |
| 16 | LPC(18:1)                 | 566.3270 | 13.81   | [M+HCOO]^+  | 506.3063       | 4.08927 | 0.002     |
| 17 | LPC(18:0)                 | 568.3425 | 15.098  | [M+HCOO]^+  | 508.3215       | 2.55761 | 0.003     |
| 18 | LPE(20:5)                 | 500.2762 | 11.905  | [M+H]^+     | 482.2679, 359.2543 | 1.11124 | 0.018     |
| 19 | LPE(18:2)                 | 478.2922 | 12.579  | [M+H]^+     | 460.2779, 337.2703 | 2.82207 | 0.002     |
| 20 | LPE(20:4)                 | 502.2925 | 12.603  | [M+H]^+     | 484.2824, 361.2769 | 1.98615 | 0.013     |
| 21 | LPE(22:6)                 | 526.2914 | 12.62   | [M+H]^+     | 508.2802, 385.2738 | 4.87399 | 0.002     |
| 22 | LPE(22:5)                 | 528.2990 | 12.633  | [M+H]^+     | 510.2810       | 1.12961 | 0.006     |
| 23 | LPE(16:0)                 | 454.2920 | 13.096  | [M+H]^+     | 436.2847       | 1.46036 | 0.006     |
| 24 | LPE(18:1)                 | 480.3437 | 13.943  | [M+H]^+     | 462.3220       | 1.1997  | 0.002     |
| 25 | LPE(18:0)                 | 482.3235 | 14.642  | [M+H]^+     | 464.3108       | 1.28074 | 0.018     |
| 26 | Sphingosine               | 300.2901 | 12.11   | [M+H]^+     | 282.2843       | 1.19202 | 0.003     |
|   | Compound       | m/z       | Retention | Charge State | Mass (amu)         | Error (ppm) | Purity (%) |
|---|----------------|-----------|-----------|--------------|--------------------|-------------|------------|
| 28 | HDoHE          | 343.2182  | 13.777    | [M-H]^-      | 281.2176, 205.1181, 233.1149 | 1.22925     | 0.003      |
| 29 | HETE           | 319.2169  | 13.852    | [M-H]^-      | 301.2108, 179.1046, 257.2185 | 3.45625     | 0.002      |
| 30 | TMCA           | 514.2675  | 7.046     | [M-H]^-      | 515.2702, 516.2687   | 2.3472      | 0.002      |
| 31 | TCA            | 514.2669  | 7.966     | [M-H]^-      | 515.2703, 516.2674   | 4.58256     | 0.002      |
| 32 | γ-Aminobutyric acid | 104.1061  | 0.584     | [M+H]^+     | /                  | 1.5784      | 0.018      |

Bile acids and derivatives

Others