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Metabolomic Study of Biochemical Changes Related to Toxicity Induced by Bupleurotoxin Using LC-MS Coupled with a Pattern Recognition Approach

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ABSTRACT

The purpose of this study was to detect changes in urine of mice and to clarify the toxicity induced by bupleurotoxin (BETX) using liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS). A procedure for urine analysis using pattern recognition was proposed to evaluate the toxicity induced by BETX in male BALB/c mice. BETX at 2.5 mg/kg was administrated intraperitoneally (i.p.), and urine samples for the metabolomic study were collected from control and BETX experimental groups. Changes in the concentrations of some urine metabolites were detected exclusively in the experimental group. All results suggested that exposure to BETX might cause a disturbance in fatty acid metabolism and the oxidative stress system. These results may not only clarify the underlying mechanism of diverse intoxication effects of BETX but also provide the guidance in preclinical toxicity screening for new drugs.
Keywords: bupleurotoxin, metabolomic profiling, toxicity, biomarker, LC-Q-TOF-MS

INTRODUCTION

Polyacetylenes and related compounds are widely distributed in the families of Apiaceae, Araliaceae, and Asteraceae and have been found sporadically in 21 other families[1–5]. More than 1,400 polyacetylenes have been isolated from different plants. These compounds are highly toxic to mammalian cells, bacteria, and fungi, and they also have neurotoxic, anti-inflammatory and anti-platelet-aggregatory effects[6–11]. Until now, the neurotoxicity of these compounds has not been systematically studied. Due to the wide distribution and the significant pharmacological activity of these compounds, we have conducted extensive research for these compounds in the past few years[12–14]. In order to study the neurotoxicity of these compounds we selected Bupleurotoxin (BETX) (Figure 1B) as the neurotoxicity-inducing compound, which was a toxic compound of polyacetylenes isolated from the roots of *B. longiradiatum* (Figure 1A), a poisonous plant that has been misused as a substitute for the traditional Chinese medicinal herb Chaihu, *B. scorzonerifolium* (Apiaceae). The substitution of *B. longiradiatum* for Chaihu has caused several cases of human poisoning, and at least three people have died after administration of this plant. Their symptoms included twitching, severe opisthotonus, nausea and vomiting[15, 16]. In previous study, we found that BETX induced cerebral lesion in the hippocampus of the brain by inhibiting GABA receptor in mice[12]. An isomer of BETX, oenanthotoxin (OETX) (Figure 1C), extracted from plants of the genus *Oenanthe*, was studied by Louvel and colleagues, who found that OETX-induced epilepsy were accompanied by changes in [K+]0, and [Ca2+]0. The finding suggested that OETX, like other epileptogenic drugs, initiated the epileptiform activity by Ca2+-dependent mechanisms[17, 18]. Recent studies have further demonstrated that OETX blocked GABAA receptors through a complex mechanism[19, 20, 21].

Compared with these traditional toxicity experiments that focused on a single or a few tissue types, a metabolomic study can provide complementary information about biomolecules. Metabolites usually have clear functions and participate in well-characterised pathways. Furthermore, metabolite levels respond to differences in gene and protein expression and represent a more holistic response[22]. Metabolomics has been used to evaluate the biological effects, type of tissue damage and/or the mechanism of toxicity for various drugs and toxins[23, 24]. It was also uniquely suited to the discovery of new potential biomarkers of toxicity[23–33].

In this toxicity study, our objective was to discover the end products response to BETX in the urine. The metabolic changes induced by BETX in mice and the relevant biochemical pathways were studied using a metabolomic approach based on a LC system combined with electrospray ionisation time-of-flight mass spectrometry (ESI-TOF-MS).

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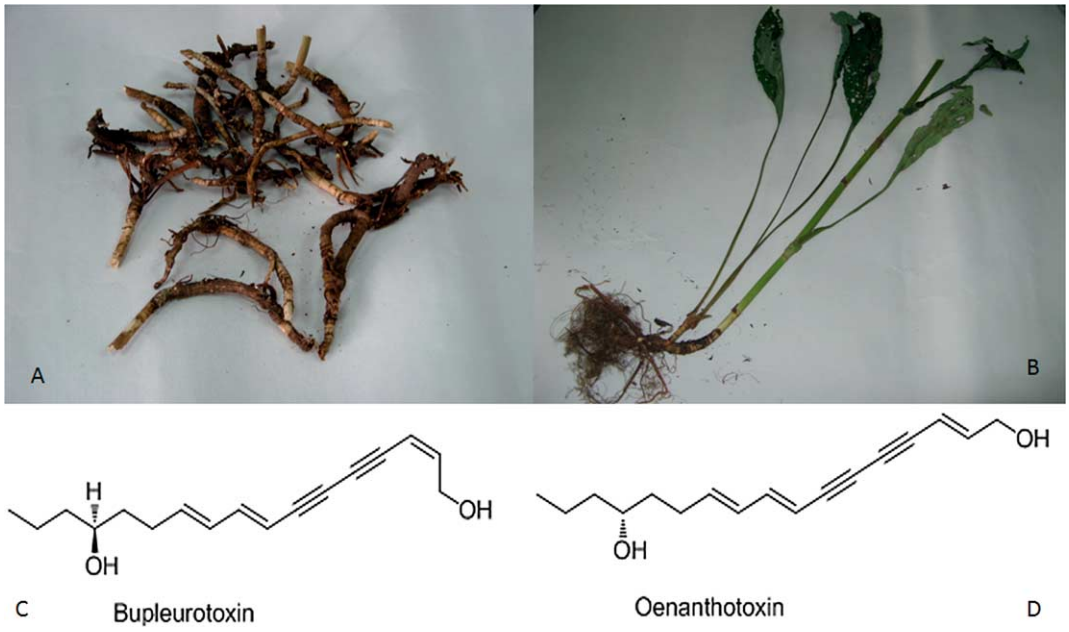

Figure 1. Pictures of the B. longiradiatum and structures of BETX and OETX. (A) Roots of Plant of *B. longiradiatum*; (B) Whole plant of *B. longiradiatum*; (C) Structure of BETX; (D) Structure of OETX.

MATERIALS AND METHODS

Chemicals and reagents

The roots of *B. longiradiatum* were collected from Heilongjiang Province in China and identified by specialist in September 2011. Analytical grade chemicals and reagents were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). LC-MS grade acetonitrile and methanol were purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA). Mass Spectroscopic grade formic acid was purchased from Fluka (Buchs, Switzerland). Ultrapure water was obtained in a Milli-Q system from Millipore (Bedford, MA, USA). Dimethyl sulfoxide (DMSO) and other chemical standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction of BETX

The air-dried and powdered sample of *B. longiradiatum* was extracted with CH_2Cl_2. The extract was eluted by column chromatography on silica gel using gradient mixtures of hexane-EtOAc (100–0%). BETX was produced through repeated chromatographic separation as described in the previous study[12, 15].

Structural characterization

Bupleurotoxin (2Z,8E,10E)-Heptadecatriene-4,6-diyne-1,14-diol colourless oil; $[\alpha]^{17}_{D}$+16.3° (c 0.04, MeOH); UV (MeOH) λ_{max} 334, 314, 294, 277, 265, 249 nm; IR (KBr) ν_{max} 1743, 2167, 1644 cm^{-1}; EIMS 70 eV m/z 258 $[M]^+$, 240, 222, 197, 169, 153, 141, 128, 115, 91, 77; HRESI-MS m/z 274.1920$[M]^+$ (calculated for $C_{17}H_{22}O_2$, 274.1908). Compound was identified as bupleurotoxin on the basis of its 1D-NMR spectroscopic data and by comparison with the literature data (including UV, IR, optical rotation)[15].

Investigation of acute toxicity of BETX

For toxic studies, stock solution of the BETX was prepared in physiological saline (final concentration of DMSO was less than 0.1%) at concentration of 12 mg/mL and then diluted with physiological saline. BALB/c mice, half males and half females, with body weights of 20 ± 2 g, were treated the BETX solution by i.p. administrated after fasting, with a dosing volume of 10 mL/kg body weight. The starting dose (n = 5) was 12 mg/kg which was decreased according to the results. The control animals (n = 5) received 0.9% normal saline alone. The death status of animals was consecutively observed for 14 days after administration, see Table 1.

The experiment was carried out in accordance with the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee of the Second Military Medical University.

Mice urine sample collection

Thirty male BALB/c mice (20 ± 2 g) were purchased from the Slac Laboratory Animal Co., LTD (Shanghai, China). The mice were housed in stainless steel metabolic cages with free access to food and tap water under standard conditions of

Table 1. Acute toxicity in vivo of BETX[a]

Dose(mg/kg)	BETX
Control	0/5
0.75	0/5
1.5	1/5
3	2/5
6	4/5
12	5/5

[a]Result are expressed as the number of deaths per group of 5 mice 3 days post injection

humidity (50 ± 10%) and temperature (25 ± 2 °C) under a 12 h light-dark cycle. The animals were acclimatised to the facilities for 7 days. All animals were handled with humane care throughout the experiment.

After acclimatisation, the mice were randomly divided into two groups with 10 mice in a control group, and 20 mice in an experimental group. They were then i.p. administrated with either 2.5 mg/kg bodyweight BETX (n = 20) or 0.9% normal saline alone (control, n = 10) once a day for consecutive 7 days.

The experiment was carried out in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of Shanghai, China. The study protocol was approved by the Animal Care and Use Committee of Second Military Medical University.

A 1.5 mL aliquot of the urine sample of was collected from each mouse over ice packs in the metabolic cages for 24 h on the 7th day. The urine samples were then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and stored at −80 °C. Prior to the analysis, urine samples were thawed and centrifuged at 12,000 rpm for 10 min. A 500 μL supernatant was collected, diluted with Milli-Q water at a ratio of 1:3 and vortex mixed for LC/MS analysis. And then a 5 μL aliquot of the diluted urine sample was injected into the column.

LC-Q-TOF -MS conditions

The LC-Q-TOF-MS analysis was performed on an Agilent-1200 LC system which was coupled with an electro spray ionisation (ESI) source and an Agilent-6520 Q-TOF mass spectrometer (Agilent Technologies, CA, USA). Chromatographic separation was performed on an Eclipse plus C18 column (1.8 μm, 3.0 mm × 100 mm, Agilent) at a temperature of 45 °C. The flow rate was 0.25 mL/min, and the mobile phase was a mixture of ultrapure water with 0.1% formic acid (A) and acetonitrile (B). The following gradient program was used: 0–2 min, 2% B; 2–4.5 min, 2–50% B; 4.5–14 min, 50–100% B; 14–15 min, 100–100% B; 15–19 min, 100%-2%, followed by a re-equilibration step of 3 min. The sample injection volume was 5 μL.

The drying gas (nitrogen) flow rate was set to 8 L/min at a temperature of 350 °C. The capillary voltage was set at 4100 V; The TOF-MS data was acquired from m/z 80–1000. The MS/MS data was acquired in the targeted MS/MS mode with collision energies of 10, 20 and 40 eV to identify potential biomarkers.

Sequence analysis

The pooled QC sample was analyzed randomly through the analytical sequence. The sequence of samples run as following: 1 QC urine sample, 6 unknown urine samples, 1 QC sample as our previous studies[12, 34, 35].

Data extraction

The raw LC-Q-TOF-MS data for all samples (excluding the data from blank samples) were first processed using the Agilent Mass Hunter Qualitative Analysis and Mass profiler Software (Agilent Technologies, CA, USA). A peak table was then created, including information on the retention time, m/z and ion intensity of all ions. The filter parameters were used as follows: restrict retention time, 0–19 min; restrict m/z, 80–1000 amu; peak relative height, ≥ 1.5%; mass tolerance, 0.05 Da; retention time windows, 0.1 min. Finally overall 1861retention time-exact mass pairs were determined in each sample profile. All processed data of each chromatogram were normalized and Pareto scaled, prior to multivariate statistical analysis. SPSS 13.0 for Windows was used for the statistical analysis. The data were analysed using the Wilcoxon Mann-Whitney Test, with $p < 0.05$ set as the level of statistical significance.

Multivariate analysis

PCA was used first to investigate general interrelation between groups, including clustering and outliers among the samples. And then, PLS-DA was used to maximize the difference of metabolic profiles between control and BETX-treated group. To improve data analysis, a pre-processing filter, termed orthogonal signal correction (OSC), was selected[36]. The OSC filter can selectively remove the variation of data X (the LC-MS data set) having no correlation with Y. OPLS[37] is an extension to the supervised PLS regression method featuring an integrated OSC-filter. In simple terms, OPLS uses information in the Y matrix to decompose the X matrix into blocks of structured variation correlated to and orthogonal to Y, respectively. In our study, OPLS in the software SIMCA-P (Ver 11, Umetrics, Umea, Sweden) was used for the multivariate analysis. The significance was expressed using the one-way analyses of variance (ANOVA) of SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA), followed by a Wilcoxon Mann-Whitney Test P values less than 0.05 were considered significant. To better visualize the contribution of ions in the separation of the classes, the S-plot was used. The S-plot combines the contribution (w^*c) and reliability/correlation (p(corr)) from the OSC-PLS model and helps to identify differential markers between classes[38]. With a significance level of 0.05, a p(corr) of 0.5 was adopted as an arbitrary cut-off value to select the variables. Potential markers of interest were extracted from the combining S- and VIP- plots (VIP>2) that were constructed from the OPLS analysis, and markers were chosen based on their contribution to the variation and correlation within the data set.

With the completion of the OPLS analysis, we also try computational systems analysis with MetaboAnalyst data annotation approach (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp) to distinguish between control and BETX-treated subjects. The heatmap, implemented in MetaboAnalyst tool commonly used for unsupervised clustering, were constructed based on the potential candidates of importance, which were extracted with OPLS analysis.

Potential biomarkers validation

To determine whether these metabolic markers which were identified using our metabolomics platform, we performed a preliminary validation using a second set of BETX-treated animals (n = 15) and control subjects (n = 10), which were

blindly selected. Urine was collected from animals at approximately 1 week after injection. Urine was extracted using methods identical to those used for the first group. Urine extract (5 μL) was injected, with a 19 min gradient from 2% to 98% solvent B, using the same column and mobile phases that were used for profiling. Peaks were automatically integrated using instrument software.

RESULTS AND DISCUSSION

Assessment of the repeatability and stability of the LC-Q-TOF-MS method

Five common extracted ion chromatograms (EICs) with different chemical polarities and m/z values were selected to assess the repeatability and stability of the method. The relative standard derivations (RSDs) of these peaks were less than 15% for peak areas and less than 0.1% for retention times in the whole sequence. And the QC samples were tight clustered in the score plot. These results indicated the LC-Q-TOF-MS method was stable and repeatable for large-scale sample analysis in our study.

Potential biomarkers

OSC-PLS is a method to screen significant molecule that contribute to sample classifications and removing non-correlated variations contained within the spectra. Features in an OPLS score plot indicate that the control and experimental groups are clearly distinguishable (each dot in the plot represents a sample) (Figure 2A). The S-plot shows the distribution of potential biomarkers (Figure 2B). The farther away a triangle (a retention time-m/z pair) is from the origin, the more influence it should have on the separation of groups. In our study, OPLS was used to classify potential biomarkers. Because there were many significant variables in the OPLS loading, the top 100 ions that were significantly down-regulated between control and experimental groups were first selected according to their (w * c) values. From these, overall 11 metabolites with VIP values larger than 2.0 and P values less than 0.05 and fold changes larger than 1.5 were selected and chosen for further analysis of their MS/MS structure. We obtained information about the predicted potential biomarkers that could correspond to the significant m/z values by searching the biofluid metabolites database (http://metlin.scripps.edu) and HMDB. The differential RT-m/z pairs were then identified between the two groups by matching the tandem MS fragmentation patterns to reference standards and the database. The heatmaps, commonly used for unsupervised clustering, were constructed based on the potential candidates of importance, which were extracted with OPLS analysis. The parallel heatmap visualization (Figure 3) using Ward's method in computational systems analysis for the BETX-treated animals and controls showed distinct segregation.

Metabolic Pathway and Function Analysis

Among the twelve identified potential biomarkers, two were down-regulated, and the other ten were up-regulated in the urine of mice treated by BETX (Figure 2B). The related pathway of each biomarker was recorded by searching the KEGG PATHWAY Database (http://www.genome.jp/kegg/). The 11 potential biomarkers distributed in ten pathways, including the pathways of fatty acid metabolism, branched-chain amino acid metabolism, and PGE2 biosynthesis (Table 2). More detailed analysis of the most relevant pathways and

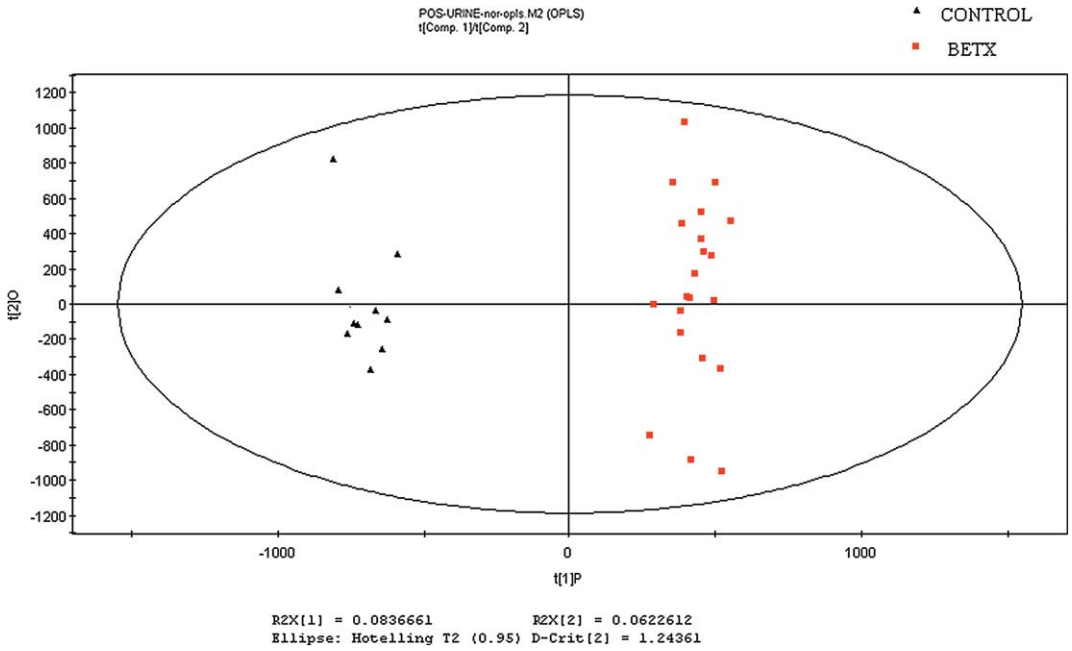

Figure 2. OPLS score and S-plot of control and BETX treated groups. (A) OPLS score. (B) OPLS S-plot (R2X [1] = 0.146, R2Y = 0.984 Q2 = 0.723, A = 1 + 1). Each point represents an individual sample. (Left, ▲) samples of control group; (Right, ■) samples of BETX group. The S-plot showed significant potential biomarkers of the toxicity induced by BETX.

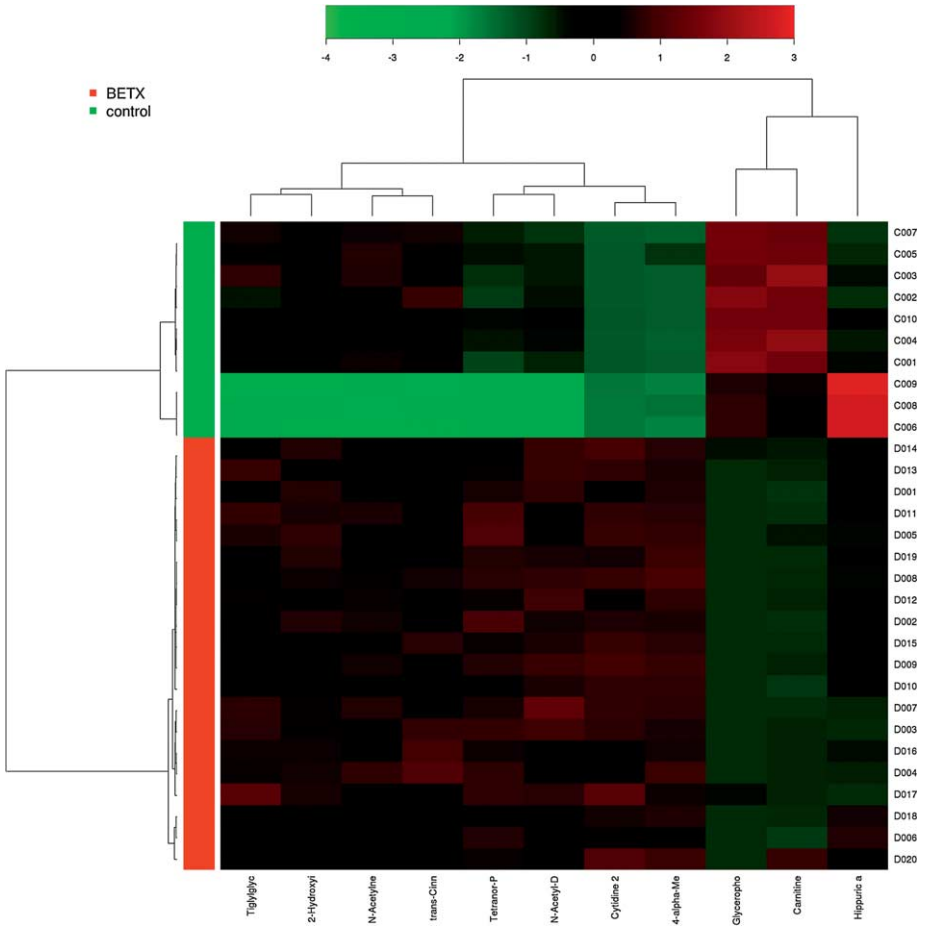

Figure 3. Heat map visualization constructed based on the differential metabolites of importance for the urine of the toxicity of BETX. Variable differences marked on the bottom corresponding to Table 2 are revealed between the control and BETX group. Rows: samples; Columns: differential metabolites; Color key indicates metabolite expression value, green: Lowest, red: highest.

Table 2. Differential metabolites identified in control and BETX-treated mice

Retention time (min)	Exact mass	Identified metabolites	Formula	ID	Tend[a]	Related pathway
1.7	161.1052	Carnitine	$C_7H_{15}NO_3$	HMDB00062	↓	Fatty acid metabolism
1.8	309.1060	N-Acetylneuraminic Acid	$C_{11}H_{19}NO_9$	HMDB00230	↑	Amino Sugar metabolism
2.4	102.0886	Acetoacetic acid	$C_4H_6O_3$	HMDB00060	↑	Tyrosine metabolism
2.6	301.0563	N-Acetyl-D-mannosamine 6-phosphate	$C_8H_{16}NO_9P$	HMDB01121	↑	Nucleotide sugar metabolism
3.6	257.1028	Glycerophosphocholine	$C_8H_{20}NO_6P$	HMDB00086	↓	Betaine metabolism
6.3	323.0519	Cytidine 2'-phosphate	$C_9H_{14}N_3O_8P$	HMDB11692	↑	Cytidine nucleotide metabolism
6.9	157.0739	Tiglylglycine	$C_7H_{11}NO_3$	HMDB00959	↑	Fatty acid metabolism
7.0	179.0582	Hippuric acid	$C_9H_9NO_3$	HMDB00714	↑	Phenylalanine metabolism
7.1	326.1365	Tetranor-PGEM	$C_{16}H_{22}O_7$	LMFA03010032	↑	PGE2 biosynthesis
7.3	132.0786	2-Hydroxyisocaproic acid	$C_6H_{12}O_3$	HMDB01624	↑	Branched-chain amino acid metabolism
7.6	148.0524	Cinnamic acid	$C_9H_8O_2$	HMDB00567	↑	Phenylalanine metabolism

[a]↑ and ↓ represent the compound is up- or down-regulated in the BETX group compared with the control group.

networks of the toxicity of BETX were performed by Metaboanalyst, a web-based tool that uses the high-quality KEGG (www.genome.jp/kegg/) pathway database as the backend knowledgebase. Consequently, potential targets of metabolic pathway analysis with Metaboanalyst revealed that the metabolites that were identified are responsible for D-glutamine and D-glutamate metabolism, Synthesis and degradation of ketone bodies, Fatty acid metabolism, Glycerophospholipid metabolism, Tyrosine metabolism.

Potential biomarkers validation

To determine whether these metabolic markers which were identified using our metabolomics platform, we performed a preliminary validation using a second set of BETX-treated

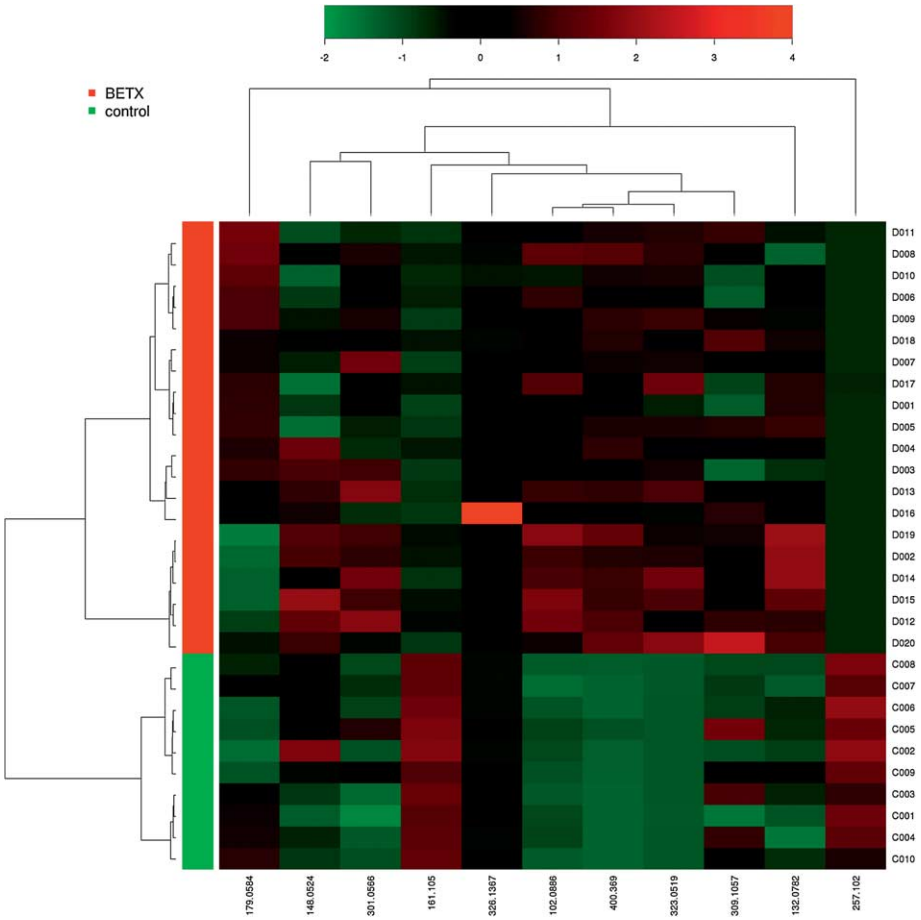

Figure 4. Unsupervised hierarchical clustering and potential of the markers of interest that distinguish BETX group from control samples.

animals and control subjects. Our results indicated that the model had a high sensitivity and specificity for the discrimination between the control and BETX-treated, as shown in Figure 4.

Changes in metabolite levels and biological significance

In order to explicate the mechanism of the toxicity of BETX, a comprehensive and holistic approach based on metabolomics was performed to reveal urine metabolic changes and identify urinary potential biomarkers of toxicity induced by BETX. The list of endogenous metabolites designated as potential biomarkers is identical to the list derived from the loading plot in Figure 2B. Among these metabolites, carnitine, tiglylglycine, 2-hydroxycaproic acid, and tetranor-PGEM, which were potentially linked to oxidative stress, mitochondrial dysfunction, and inflammation due to the toxicity of BETX. To our knowledge, this was the first detection of metabolic changes and the relevant biochemical pathways induced by BETX in mice. This study therefore provides crucial information on the BETX toxicity in vivo.

Several theories have been put forward to clarify the mechanisms underlying the neurotoxicity of fatty acids. Previous studies have found that fatty acids could inhibit the activity of the Na-K-ATPase to disrupt the function of neural membranes[39]. Besides that fatty acids can disturb mitochondrial metabolism by inhibiting the mitochondrial transport. Fatty acids provide highly efficient energy storage, storing much more energy by weight than carbohydrates such as glucose. Fatty acids travel through the blood to other tissues such as muscle, where they are oxidised to provide energy through the mitochondrial beta-oxidation pathway. Inherited defects in mitochondrial fatty-acid beta-oxidation have become increasingly recognised as an important class of disease[40]. Significant increase in tiglylglycine and decrease in carnitine were detected in the BETX-treated group. This indicated the effect of BETX on both fatty acid metabolism and the cell's ability to provide energy through the mitochondrial beta-oxidation pathway.

Tiglylglycine was a minor metabolite of fatty acids. In certain cases, measurements of these metabolites in body fluids can be used to diagnose disorders associated with mitochondrial fatty acid beta-oxidation[41, 42]. An elevated level of tiglylglycine was present in the urine of patients with beta-keto thiolase deficiency or propionate metabolism disorders[43]. Carnitine was an essential compound that played an indispensable role in fatty acid metabolism by facilitating the transport of activated long-chain fatty acids across the inner mitochondrial membrane[44, 45]. Inborn errors in carnitine metabolism can lead to brain deterioration, such as Reye's syndrome, gradually worsening muscle weakness,

Duchenne-like muscular dystrophy and extreme muscle weakness with fat accumulation in muscles. In the present study, the decreased levels of carnitine in the BETX treatment group suggested that the beta-oxidation pathway might be inhibited so that carnitine could not carry the activated long-chain fatty acids across the inner mitochondrial membrane.

2-Hydroxycaproic acid was a branched-chain alpha-keto acid that has been reported in normal human blood and normal amniotic fluid. It has been found that 2-hydroxycaproic acid was associated with the appearance of neurological symptoms and was most likely the primary neurotoxic metabolite in maple syrup urine disease[46, 47]. Another report observed that the 2-hydroxycaproic acid accumulated in homogenates of rat brain resulting from this neurological disease stimulation[48]. Lipid peroxidation was an important event induced by oxidative stress. It has been related to the pathogenesis of some neuron-diseases, such as demyelination neurodegenerative disorders, dementia, epileptic seizures and others[49, 50]. In our study, a significant increase of 2-hydroxycaproic acid in the BETX-treated group might indicate a change in lipid peroxidation in the BETX-treated group. In our study, 11 identified potential biomarkers in the urine provided new insights into the toxicity of BETX in vivo. These findings suggested that oxidative stress, mitochondrial dysfunction and neuron inflammation were the underlying mechanisms of BETX-induced toxicity. However, in the future further study on the pathways of these markers is required to determine the mechanisms of toxicity.

CONCLUSIONS

In conclusion, we performed metabolomic study of urine extracts from BETX-treated and control groups. Using an OPLS analysis based on liquid chromatography-mass spectrometry, we observed a distinct separation in the clustering of the urine samples and proposed 11 potential biomarkers related to the toxicity induced by BETX. These potential biomarkers can be used as early toxicity screening marker for predicting neurotoxic of drugs, and the current study provided the guidance in preclinical toxicity screening for new drugs.

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