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Serum Metabolomics Reveals Betaine and Phosphatidylcholine as Potential Biomarkers for the Toxic Responses of Processed Aconitum Carmichaeli Debx

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Abstract

We had recently reported that the processed Aconitum carmichaelii debx (Bai-Fu-Pian in Chinese, BFP) elicits differential toxic responses in rats under various health conditions. The present study aimed to determine the graded toxicity of BFP so as to derive a safe therapeutic rationale in clinical practice. The sensitive and reliable biomarkers of toxicity were also identified, with the corresponding metabolic pathways being unveiled. Thirty male Sprague-Dawley rats were divided into five groups (n = 6) and received oral administration of BFP extract (0.32, 0.64, 1.28 or 2.56 g/kg per day) or equal volume of drinking water (control) for 15 days. The metabolomic profiles of rat serum were analyzed by liquid chromatography quadruple time-of-flight mass spectrometry (LC-Q-TOF-MS). Linear regression analysis and Ingenuity pathway analysis (IPA) were used to elucidate the differentiated altered metabolites and associated network relationships. Results from biochemical and histopathological examinations have revealed that BFP would induce eminent toxicity in the heart, liver and kidneys at the dose of 2.56 g/kg per day. Betaine up-regulation and phosphatidylcholine down-regulation were detected in the serum samples of drug-treated groups in a dose-dependent manner. In summary, betaine and phosphatidylcholine could be regarded as sensitive biomarkers for the toxic responses of BFP. Perturbations of RhoA signaling, choline metabolism and free radical scavenging were found to be partly responsible for the toxic effects of the herbal drug. Based on the metabolomics findings, we could establish a safe therapeutic range in the clinical use of BFP, with a promising predictive power of possible drug toxicity.

Introduction

The daughter or lateral root of Aconitum carmichaelii Debx (Fuzi) is a valuable Traditional Chinese medicine (TCM) that has been commonly used for over 2000 years. It has a wide range of pharmacological functions in the treatment of diseases such as rheumatic fever, painful joints, bronchial asthma, gastroenteritis, collapse, syncope, diarrhea, edema, various tumors and endocrine disorders.1 Due to the known toxicity of Fuzi, only processed Fuzi is allowed to be used clinically.2 Although conventional processing procedures can largely reduce the toxic effects of the herb,1 there have been multiple reports of processed Fuzi poisoning in China and in other parts of the world.1 The primary target organs of processed Fuzi toxicity include the heart and the central nervous system, possibly due to stimulation of the vagus nerve that results in atrioventricular block and symptomatic bradycardia, and subsequently arrhythmia, all related to interference of the sodium channels.3 The toxic alkaloid ingredient of the processed Fuzi including aconitine, mesaconitine and hyaconitine are responsible for the systemic toxicity. Bai-Fu-Pian (BFP), a processed Fuzi, has been most commonly used in TCM clinics and was therefore tested in the present study.

Despite the known toxicity of some medicinal herbs, failures of prediction and lack of knowledge on the underlying toxicological mechanisms remains a major obstacle in their rational clinical applications.5 6 Hence, systematic characterization of BFP toxicity is necessary to evaluate its safety use. A medicinal herb is a mixture of multiple chemical ingredients with multiple potential targets and only in a holistic way its effect mechanism can be clarified clearly.7 9 Since conventional analytical techniques such as histological and
biochemical tests may not be able to detect the complex groups
of plant metabolites in Fuzi and its processed products.10,11 it is
difficult to estimate the toxic manifestations of BFP early in
time and in a comprehensive manner. With the advancement of
analytical technology, metabolomics could provide detailed
evidence for in-depth study on the efficacy as well as toxicity
of drugs.12 It involves the study of the small biochemicals
present in a biological sample, bringing enormous opportunities
for improved detection of toxicity, biomarker discovery and
identification of perturbed pathways due to intervention in a
holistic context.13 The disease biomarkers represent a diverse
group of low-molecular-weight structures including lipids,
amino acids, peptides, nucleic acids, organic acids, vitamins,
thiols and carbohydrates. Metabolomics enables toxicity
detection at an earlier time than traditional clinical chemical
and histopathological methods, and provide a better
understanding of the toxic mechanism that may facilitate the
prediction of TCM toxicity.14

Previous studies have shown that metabolomics could be
used to evaluate the toxicological and pharmacological actions
of aconite products. By using urinary metabolomics based
ingenuity pathway analysis (IPA) with pattern recognition
methods, it was revealed that Chuanwu, the mother root of
Aconitum carmichaelii Debx., could lead to serious heart and
liver toxicity. Significant alterations of 17 metabolites were
identified and validated as phenotypic biomarkers of Chuanwu
toxicity. In addition, there were time- and dose-dependent
biochemical perturbations being induced by Chuanwu.15

Another study also reported that Heishunpian, the processed
lateral root of Aconitum carmichaeli, caused significant
changes in the urinary metabolic profile of rats, with toxicity in
the heart and liver. The study conceded that urinary taurine is
the potential biomarker of the toxic effects of Heishunpian.16

Our earlier study had deciphered the differential toxic
responses of BFP in healthy and hydrocortisone-pretreated rats
based on their serum metabolic profiles.17 Results showed that
BFP could induce severe toxicity in the heart, liver and kidneys,
whereas betaine was found to be the common metabolite being
altered that was in line with the differential toxic responses of
BFP in rats under various health conditions. In the present
study, a metabolomics-based liquid chromatography quadruple
time-of-flight mass spectrometry (LC-Q-TOF-MS) technique
with pattern recognition approach and IPA was employed to
demonstrate the serum metabolic characteristics following
different doses of BFP treatment. We aimed to unveil the
sensitive, reliable biomarkers of BFP toxicity, and to explore
the corresponding metabolic pathways.

Results

Main constituents of the BFP extract

HPLC analysis of the ethanol extract of BFP indicates that
its three major constituents were aconitine (0.0169 mg/g),
mesaconitine (0.5056 mg/g) and hypaconitine (0.0253 mg/g),
respectively.

Identification of blood cell count, biochemical and
histopathological changes

In order to confirm the toxicity of BFP, the number of
WBC, RBC, PLT and HGB level in the whole blood as well as
serum levels of TG, CHO, TP, GLU, T-BIL, GGT, BUN, CRE,
UAC, AST, ALT, CK, LDH in drug-treated animals were
compared with those in control rats. The altered parameters in
the BFP treatment groups are summarized in Fig. 2(a-g). No
change in WBC, RBC, PLT count or HGB level in the whole
blood was recorded. Serum levels of GLU in the CB3 and CB4
groups were significantly decreased while that of CHO was
markedly increased in the CB4 group. Serum levels of CK and
LDH (representing myocardial injury), ALT (representing
hepatic damage), as well as BUN and CRE (representing renal
damage) were all significantly elevated in the CB4 group. All
the changes of GLU, LDH, ALT and BUN are dose-dependent.

Histopathology of tissues (i.e. heart, kidneys, liver)
exposed to BFP was examined to further investigate drug
toxicity. Results show that the heart, liver and kidney tissue
samples from the CB4 group had shown pathological changes
when compared with those in the control group (Fig. 3).
Inflammatory infiltration, edema and rupture of the
cardiomyocytes were observed in heart tissues, while vascular
dilatation and congestion in the interstitial area of the renal
collecting duct were observed. The pathological changes in
liver tissue include unclear hepatic lobular structures, necrosis
and disappearance of hepatocytes, along with dissolving
cytoplasm and lymphocytic infiltration.

These results indicate that BFP could induce severe organ
toxicity as manifested by hematological and histopathologic
aberrations at its upper dose range.

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

Extracts from six aliquots of a random blood sample were
continuously injected to evaluate the repeatability. Five
common extracted ion chromatograms (EICs) shared by these
injections were selected according to their different chemical
polarities and m/z values. The relative standard derivations
(RSDs) of these peaks were 3.27-14.98% for peak areas and
0.03-0.81% for retention times.

The LC-MS system stability for the large-scale sample
analysis was demonstrated by the test of pooled QC samples.
PCA result shows that QC samples were tight clustered.
Moreover, peak areas, retention times and mass accuracies of
five selected EICs in five QC samples also showed good system
stability. RSDs of the five peaks were 6.12-14.56% for peak areas,
0.03-1.11% for retention times and 0.1x10^-4 to 0.86x10^-4
for mass accuracy. These results indicate that large-scale
sample analysis had hardly any effect on the reliability of data.

Examination of MS spectra and identification of the differential
metabolites

Typical base peak chromatograms (BPCs) of serum
samples were obtained from both healthy control and BFP
treated rats. Eleven metabolites were found to be most
significant among the treatment groups (Table 1). Based on the
metabolic changes in C and CB rats as revealed by BPCs, we
had adopted the multiple pattern recognition methods PCA (Fig.
4) and OPLS (Supplementary Fig. s1- s4).

These approaches facilitate classification of the metabolic
phenotypes and enable us to further identify the differential
metabolites. Score plots from PCA have shown obvious
separation between C, CB1, CB2, CB3 and CB4 groups was
illustrated in Fig. 4. The separation of the groups could be
achieved with the model parameters R2 [1] = 0.415, R2X [2] =
0.203, Q2 = 0.267, A = 5. These parameters indicate that the
model can accurately describe the data.
In order to fully differentiate between the metabolites in the C (healthy control) and the four CB drug-treated groups, OPLS was conducted (Supplementary Fig. S1-s4). OPLS is an efficient method for identifying ions that contributes to the clustering of samples. It also helps to eliminate non-correlated variations contained within spectra. There was a distinct clustering between C and the four CB groups. Q2Y and R2Y in the OPLS models indicate that the class prediction ability of all models was high and that there was an authentic difference between groups. The corresponding S-plot in turn shows the contribution of different variables for the differentiation between C and the four CB groups. Each triangle in the S-plot represents an ion. Ions far away from the origin are potential biomarkers.

As shown in Table 1, among the 4 identified metabolites in the CB1 group of animals, betaine and uridine triphosphate (UTP) were up-regulated, while dimethyl-allylldiphosphate and phosphatidylcholine were down-regulated. Among the 8 identified metabolites in the CB2 group of animals, betaine and N,N-diacytelychitobiose were up-regulated, while the other 6, including dimethyl-allyldiphosphate, phosphatidylcholine, phenylpyruvic acid, 5-hydroxymethyl-3-acetic acid, acetyl-L-carnitine and propionyl carnitine, were down-regulated. Among the 6 identified metabolites in the CB3 group of animals, betaine, UTP, N,N-diacytelychitobiose and selenocystathionine were up-regulated, while dimethylallyl diphasphate and phosphatidylcholine were down-regulated. Among the 5 identified metabolites in CB4 group rats, betaine, N,N-diacytelychitobiose and pregnenolone sulfate were up-regulated, while phosphatidylcholine and propionylcarnitine were down-regulated.

Taken together, up-regulated betaine and down-regulated phosphatidylcholine were common candidate metabolites in every drug-treated group, whereas up-regulated N,N-diacytelychitobiose was the common candidate metabolite in the later 3 higher dose groups when compared with the healthy control. In order to elucidate whether the three metabolites perturbed by BFP have dose-dependent manner and whether there is correlation between candidate metabolites and haematological and biochemical indicators, linear regression analysis and spearman correlation analysis were carried out. Results show that the trajectories of betaine and phosphatidylcholine exhibit a dose-dependent manner (Fig. 5a-c) and the two metabolites are closely correlated with some haematological and biochemical indicators representing heart, liver and kidney injury (Table 2).

Metabolic network and pathway analysis using IPA

In order to further understand the correlation between the candidate biomarkers, bioinformatics analyses were performed by using the IPA software, leading to the identification of biological association networks and canonical pathways (Fig. 6a-d). Fig. 6a shows the merged network of the metabolites being identified in the CB1 group. These metabolites correlate with five important canonical pathways, including RhoA signaling, choline biosynthesis, choline degradation I, trans,trans-farnesyl diphosphate biosynthesis, and free radical scavenging. Associated network functions have been focused on lipid metabolism, free radical scavenging, as well as cellular growth and proliferation. Fig. 6c shows the merged network of the metabolites being identified in the CB3 group. The main canonical pathways being correlated with the identified metabolites include RhoA signaling, choline biosynthesis, choline degradation I, trans,trans-farnesyl diphosphate biosynthesis, glycogen biosynthesis (from UDP-D-glucose), fructose synthesis and selenium glycine metabolism. Associated network functions have been focused on cell-to-cell signaling and interaction, free radical scavenging, as well as carbohydrate metabolism. Fig. 6d shows the merged network of the metabolites being identified in the CB4 group. The main canonical pathways associated with the identified metabolites include RhoA signaling, choline biosynthesis, choline degradation I, fatty acid activation, fructose synthesis and steroids synthesis. Associated network functions have been focused on lipid metabolism and molecular transport.

The above analyses have illustrated that three canonical pathways, RhoA signaling, choline biosynthesis III and choline degradation I, are closely related to the identified differential metabolites in the BFP treatment groups. The two network functions, namely free radical scavenging and lipid metabolism, are common functions that are closely related to the identified metabolites across different dose groups.

Discussion

BFP is commonly prescribed by TCM practitioners. Nevertheless, it has been suggested that the alkaloid contents are responsible for causing toxicity in the heart, liver, and other vital organs when BFP is applied for chronic use, particularly when overdosed. According to the Chinese Pharmacopoeia (2005 edition), the clinical dose range of BFP is 3-15 g, which is equivalent to the dosage from 0.32 to 1.6 g in rats. We had reported that even BFP was used within therapeutic dose range, it may still produce adverse drug reactions. Hence, in order to facilitate a safe clinical application of BFP, it is essential to predict the toxicity of BFP and to further unveil the precise mechanisms of the associated toxic responses. We are the first group to discover the serum biomarkers of BFP toxicity within therapeutic dose range. Up-regulated betaine and down-regulated phosphatidylcholine in the serum have been identified as potential biomarkers for predicting the toxic responses against BFP. The altered lipid metabolism (choline metabolism), RhoA signaling and free radical scavenging should be partly responsible for the systemic toxicity of the herbal drug.

Due to the complexity of TCM working mechanisms, metabolomics is especially suitable in their toxicological study. Small-molecule metabolites play an important role in biological systems and represent attractive candidates to understand the phenotypes of drug toxicities. In particular, highly sensitive and specific biomarkers in biological fluids are very useful for comprehensive study of the efficacy and/or toxicity of Chinese medicinal herbs. Presence of known biomarkers can allow detection of drug toxicity much earlier than conventional tests using clinical chemistry and histopathological assessment, which provide a better understanding of the toxic mechanism so as to facilitate prevention of toxic responses. Therefore, in this study, the metabolomic approach using LC-Q-TOF-MS was employed to determine the subtle metabolic profiles changes, while the metabolic networks and pathways involved had been
analyzed with IPA. As shown in Fig. 7, the 5 identified metabolites that were altered in the CB4 group could be associated with BFP toxicity through perturbation of their inter-relationships. Among these, betaine and phosphatidylcholine were suggested to be potential biomarkers for the toxic responses of BFP, which resulted in the modulation of metabolic regulatory networks involving RhoA signaling, choline metabolism and free radical scavenging.

Betaine is distributed widely in animals. The principal physiologic role of betaine is to act as an osmolyte and methyl donor (in trans-methylation). As an osmolyte, betaine can effectively induce hydration of albumin, and to protect cells, proteins, and enzymes during environmental stress. Being a methyl donor, betaine actively participates in the methionine cycle (Fig. 7). Betaine is synthesized from choline by catabolism via a series of enzymatic reactions that occur mainly in the mitochondria of liver and kidney cells. Elevated total homocysteine concentrations is associated with chronic diseases. Homocysteine is produced as a result of methylation reactions and is removed either by its irreversible conversion to cysteine (by trans-sulfuration) or by re-methylation to form methionine (Fig. 7). There are two separate re-methylation reactions that are catalyzed by betaine. It appears that deterred functions of betaine could lead to the accumulation of homocysteine, which subsequently cause pathological damages to internal organs. Elevated plasma betaine promotes up-regulation of multiple macrophage scavenger receptors that may increase the risk of secondary heart failure and acute myocardial infarction. Besides, betaine could also deteriorate liver functions by perturbing glycine, serine and threonine metabolism while at the same time affect the ability of osmoregulation in various renal cells. Taken together, the toxic responses of BFP in the heart, liver and kidneys in both healthy individuals and patients could be partly due to the elevated betaine level.

Other than the accumulation of homocysteine, another biological property of betaine is probably related to its free radical scavenging action in the heart and liver. Oxidative stress is associated with the osmoregulation dysfunction caused by reactive oxygen species (ROS) through disruption of the enzymes for osmolyte synthesis in renal medulla cells in response to hypertonicity. Oxidative damage plays a critical role in the pathogenesis of human diseases, including cancer, atherosclerosis and heart diseases. Aconitum Chinese herbs and their active ingredients were proven to be effective against inflammation and being capable of scavenging free radicals in rodent models, possibly due to their antioxidative properties by inducing endogenous radical-scavenging enzymes such as catalase and glutathione peroxidase. We cannot fully explain why higher doses of BFP would alternatively evoke toxicity. It could probably be due to the toxic alkaloid constituents of BFP that would alter other pathologic factors despite the high betaine level.

Phosphatidylcholine is an essential phospholipid in mammalian cells and tissues that is synthesized via the choline pathway. During choline synthesis, methylation of phosphatidylethanolamine to phosphatidylcholine will be carried out, which is catalyzed by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT) (Fig. 7). Choline can then be generated from phosphatidylcholine through the action of phospholipases. Following this, choline is oxidized to betaine in the kidneys and liver while being converted to acetylcholine in the nervous system. The ratio of phosphatidylcholine to phosphatidylethanolamine, choline recycling, choline redistribution, choline acquisition and choline depletion all contribute to choline and phosphatidylcholine homeostasis in mice. In the present study, phosphatidylcholine was down-regulated by BFP, resulting in fulminant and subacute hepatic failure. In spite of this, cardiac toxicity induced by aconite (from other toxic plant such as the Aconitum species) has been correlated with metabolic disorders involving polyunsaturated fatty acids. It is therefore of interest to carry out further investigation on phosphatidylcholine to confirm its role as a potential target of BFP toxicity other than betaine.

Previous study had demonstrated that vascular hyporeactivity was associated with aortic inducible nitric oxide (NO) synthase expression and increased serum NO level. Lipopolysaccharide (LPS) can cause NO overproduction through the inducible enzyme. RhoA/Rho-kinase pathway facilitates vasconstriction by a calcium-dependent mechanism. Increased RhoA activity could compensate vascular hyporeactivity, while excessive NO production would in turn inhibit RhoA activity. Furthermore, RhoA promotes cardiomyocyte survival and protects them from ischemia and other oxidative stress. Prolonged activation of RhoA signaling through ROCK has been suggested to be detrimental to inflammatory cells. Besides, RhoA/Rho kinase is involved in the interaction between alpha2-adrenergic receptors and angiotensin II to modify renal vascular resistance by mediating the signaling events downstream of the phospholipase C/protein kinase C/c-src pathway; perturbation of RhoA signaling would therefore contribute to the pathophysiology of renal disorders. In fact, perturbed RhoA signaling could be associated with the potential toxicity of BFP at its toxic dose range. Aconitum and its active ingredients have potent NO inhibitory activities. Among these, the alkaloid higenamine was reported to be beneficial against LPS-induced vascular hyporeactivity and reduced the mortality caused by circulatory failure. It was discovered that the toxicity of Fuzi mainly derives from the diester diterpene, aconitine, hypaconitine and mesaconitine. In a recent quantitative analysis of processed Fuzi decoctions including BFP, it was found that the contents of aconitine and mesaconitine were largely reduced by hydrolysis to monoester alkaloids during the processing, leading to corresponding reduction in toxicity. However, it now appears that at certain high dose of BFP, abundant amount of the non-hydrolyzed toxic diester diterpene alkaloids still possess certain degree of systemic toxicity, compensating the NO-inhibitory potential of the cardioprotective alkaloid higenamine.

In our previously published article, we explained that in TCM clinical practice, BFP should only be used in patients with a particular health condition called “kidney-yang” deficiency pattern. By using a special animal model mimicking the state of “kidney-yang” deficiency, we had demonstrated that the systemic toxicity of BFP even at extremely high dose could be alleviated. In spite of its potential toxicity, BFP has been proven to have superb therapeutic value in treating various diseases such as rheumatic fever, painful joints, etc. Here, by identifying the biomarkers of toxicity, the precise signaling pathway and the metabolic alterations involved after the administration of various doses of BFP, we are capable of predicting the toxic responses of the herbal decoction and to determine a safe therapeutic dosing regimen for its clinical application in all patients. This advancement could expand the treatment option of patients who do not have the “kidney-yang” deficiency body state.
Conclusions

Betaine and phosphatidylcholine have been identified as potential biomarkers for detecting the toxic responses of BFP. Perturbation of RhoA signaling and choline metabolism together with disruption of free radical scavenging all play crucial roles in the toxic mechanism of the herbal drug. The results enable BFP toxicity detection at an earlier time than traditional clinical chemical and histopathological methods and help us for early prevention of BFP toxicity and a safer administration in clinical practice. In addition, our results illustrate that metabolomic analysis offered a promising opportunity to discover sensitive biomarkers of TCM toxicity, which could largely contribute to comprehensively understand the pharmacological and toxic mechanisms of BFP in order to establish a safe therapeutic dosing regimen in clinical practice.

Experimental materials and methods

Chemicals and reagents

LC/MS grade acetonitrile was purchased from Honeywell Burdick and Jackson (MI, U.S.A). Mass spectroscopic grade formic acid was purchased from Fluka (Buchs, Switzerland). Formic acid (spectroscopic grade), leucine enkephalin (spectroscopic grade) and all chemical standards were purchased from Sigma-Aldrich (MO, U.S.A) unless specified otherwise.

Preparation of the ethanol extract of BFP

BFP (Batch no. 081117) was purchased from Yanjing Drug Store (Beijing, China) and authenticated by specialists in pharmacognosy. Powdered BFP (50 g) was extracted with 75% ethanol (600 mL for 3 times) under thermal reflux for 1.5 h. After filtration, the ethanol extract was concentrated under reduced pressure. The resulting residue was dissolved in 0.5% sodium carboxyl methyl cellulose to give an extract with the concentration of 2 g/mL (expressed as the weight of raw materials). We had performed a quality control test on the BFP ethanol extract using high-performance liquid chromatography (HPLC) and AAS-ICP, and found no trace of heavy metals, organic solvents or other contaminants.

Experimental animals

A total of 30 male Sprague-Dawley (SD) rats (230±20 g; license no. SCXK 2009-004) were obtained from the Experimental Animal Center of Beijing Capital University of Medical Sciences (China). They were reared under standard laboratory conditions. Experimental groups were established as follows: [C], healthy control rats; [CB1], [CB2], [CB3] and [CB4], rats with administration of BFP at the dose of 0.32, 0.64, 1.28 and 2.56 g/kg per day, respectively (Fig. 1). All animal experimentations were performed under the Prevention of Cruelty to Animals Act (1986) of China and the NIH Guidelines for Care and Use of Laboratory Animals (U.S.A), and had obtained prior approval by the Animal Ethics Committee of the China Academy of Chinese Medical Sciences under the project “TCM disease syndrome classification research” (date of approval: June 18, 2010).

BFP administration and sample collection/preparation

As shown in Fig. 1, different doses of BFP extract were administrated orally by gavage to rats in the 4 treatment groups once daily for 15 days. The dosages being used in CB1-CB3 rats are equivalent to the clinically relevant human adult dose based on an established formula for human-rat drug conversion. Rats in the control group received an equal volume of the vehicle orally. Whole blood was collected from the abdominal vein of the rats on day 15. Hematological tests such as total white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (HGB) level and platelet (PLT) count were conducted. The remaining blood sample portion was centrifuged at 3,500 x g for 15 min after sedimentation for two hours at 4°C. The collected serum was then transferred to new tubes and stored at -80°C for further analysis. Laboratory biochemical analysis of total glyceral esters (TG), cholesterol (CHO), total protein (TP), glucose (GLU), total bilirubin (TBIL), gamma glutamyltransferase (GGT), urea nitrogen (BUN), creatinine (CRE), uric acid (UAC), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK) and lactate dehydrogenase (LDH) was performed according to the manufacturer’s instructions. A 100 μL serum portion was added to 200 μL of acetonitrile, and the mixture was vortexed for 30 s. After centrifugation at 9560 x g for 10 min at 4°C, the supernatant was stored at -80°C for LC/MS analysis. All experimental rats were sacrificed following blood sampling. Fresh cardiac, hepatic and renal tissues were obtained and fixed in 10% neutral buffered formaldehyde at 4°C for paraffin embedding. Organ samples were sectioned (4 μm) and stained with hematoxylin and eosin.

LC-Q-TOF-MS analysis

The use of high and ultra-high resolution mass analyzers (e.g. Time-of-flight, TOF) is capable to obtain accurate mass measurements for the determination of elemental composition of metabolites, and facilitates tentative identification based on metabolites database (such as the KEGG Pathway Database). By combining this technique with conventional MS/MS will provide useful additional structural information in the identification of altered metabolites. The rapid, sensitive performance and versatility of LC-Q-TOF-MS accelerates drug discovery and medicinal development, by making the screening process and mechanistic research easier.

In this study, LC-Q-TOF-MS analysis was performed by using an Agilent-1200 LC system coupled with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA) and an Agilent-6520 Q-TOF mass spectrometry. Separation of all samples was performed on an Eclipse plus C18 column (1.8 μm, 3.6 mmx100 mm, Agilent) with the column temperature set to 45°C. The mobile phase consisted of ultrapure water with 0.1% formic acid and acetonitrile, with a flow rate of 0.25 mL/min. The following gradient program was used: 2% acetonitrile for 0-1.5 min; 2-100% acetonitrile for 1.5-13 min; washed with 100% acetonitrile for 13-16 min; re-equilibration step for 5 min. The sample injection volume was 5 μL.

Mass detection was operated in the positive ion mode with the following setting: drying gas (N2) flow rate, 8 L/min; gas temperature, 330°C; pressure of nebulizer gas, 35 psig; Vcap, 4000V; fragmentor, 160V; skimmer, 65V; scan range, m/z 80-1000. All analyses were acquired using the instrument mass spray to ensure accuracy and reproducibility. Leucine enkephalin was used as the instrument reference mass (m/z 556.2771) at a concentration of 50 fmol/μL with the flow rate of 40 μL/min. MS/MS analysis was acquired in targeted MS/MS mode with collision energy from 10V to 40V.
Sequence analysis

The pooled QC sample was analyzed at the beginning, the end and randomly throughout the analytical run in order to monitor the stability of sequence analysis. The typical batch sequence of serum samples consisted of consecutive analysis of 1 QC serum sample (at the beginning of the study), followed by 6 unknown serum samples. 1 QC serum sample, before running another 6 unknown serum samples, etc. Meanwhile, samples were analyzed in random order or a normal good practice. An identical sequence was repeated to complete the total set of injections (n = 36, including QCs) being analyzed in less than 1 day per mode.

Data processing and statistical analysis

The LC-MS raw data were processed by Agilent Mass Hunter Qualitative Analysis Software (Agilent Technologies, Palo Alto, CA, USA). The data of each sample were normalized to the total area to correct for the MS response shift between injections due to any possible intra- and inter-day variations. The sum of the ion peak areas within each sample was normalized to 10,000. Principal components analysis (PCA) and orthogonal partial least square (OPLS) were used for metabolite profile analysis. Multivariate analysis was performed by the SIMCA-P version 11 software (Umetrics AB, Umeå, Sweden). The data obtained show a normal distribution. In all cases, analysis of variance, linear regression analysis and spearman correlation analysis were used for comparison between multiple groups, analysis of the dose-dependent behaviour and analysis of the correlation between candidate metabolites and haematological and biochemical indicators, respectively. A value of p < 0.05 was considered to be statistically significant.

IPA analysis

The analyses of the networks, biofunctions and canonical pathways for the candidate metabolites were conducted by Ingenuity Pathway Analysis (IPA) 8.7 software, to explore the typical metabolic perturbations associated with the toxicity of BFP.

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Notes

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Author Contributions: These authors contributed equally to this work.

Abbreviations


References

9. X. Liang, H. Li and S. Li, Molecular bioSystems, 2014.


The numbers in the table represent the correlation coefficient. The significant of spearman correlation analysis:

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<th>Metabolites</th>
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<th>CHO</th>
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<th>BUN</th>
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<td>-0.453</td>
<td>-0.384</td>
<td>-0.527</td>
<td>-0.448</td>
<td>-0.361</td>
</tr>
<tr>
<td>Phenylpyruvic acid</td>
<td>0.119</td>
<td>0.087</td>
<td>-0.089</td>
<td>0.188</td>
<td>0.100</td>
<td>-0.017</td>
<td>0.207</td>
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<tr>
<td>5-Hydroxyindol</td>
<td>0.069</td>
<td>0.032</td>
<td>-0.231</td>
<td>-0.038</td>
<td>-0.052</td>
<td>-0.108</td>
<td>0.175</td>
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<tr>
<td>Acetyl-L-carnitine</td>
<td>-0.011</td>
<td>-0.087</td>
<td>0.176</td>
<td>0.074</td>
<td>0.010</td>
<td>0.196</td>
<td>0.401</td>
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<tr>
<td>Propionylylcarnitine</td>
<td>0.360</td>
<td>-0.111</td>
<td>-0.092</td>
<td>-0.238</td>
<td>-0.193</td>
<td>-0.138</td>
<td>0.231</td>
</tr>
<tr>
<td>N,N-Diacetylchitobiose</td>
<td>-0.183</td>
<td>0.051</td>
<td>0.326</td>
<td>0.201</td>
<td>0.422</td>
<td>0.296</td>
<td>0.200</td>
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<tr>
<td>Selenocystathionine</td>
<td>0.014</td>
<td>-0.092</td>
<td>0.178</td>
<td>-0.003</td>
<td>0.022</td>
<td>0.224</td>
<td>0.377</td>
</tr>
<tr>
<td>Pregnenolone sulfate</td>
<td>-0.053</td>
<td>0.157</td>
<td>-0.097</td>
<td>0.181</td>
<td>0.249</td>
<td>-0.015</td>
<td>0.346</td>
</tr>
</tbody>
</table>

The numbers in the table represent the correlation coefficient. The significant of spearman correlation analysis: * p < 0.05; ** p < 0.01.
**Figure**

**Fig 1.** The experimental design
Fig 2. Effects of BFP on various biochemical parameters in rats. Statistical difference from control: *p < 0.05, **p < 0.01. Linear regression analysis was also performed to estimate dose-dependency: †p < 0.05, ‡p < 0.01. (a) GLU; (b) CHO; (c) CK; (d) LDH; (e) ALT; (f) BUN; (g) CRE.

Fig 3. Photomicrographs of heart, kidney and liver following the administration of BFP at the dose of 2.56 g/kg per day for 15 days. (a) Healthy heart control; (b) histopathological changes in the heart: with inflammatory infiltration, edema and rupture of the cardiomyocytes; (c) healthy renal control; (d) histopathological changes in the kidney: with vascular dilatation and congestion in the interstitial area of collecting duct; (e) healthy liver control; (f) histopathological changes in the liver: with liver cell necrosis, cell disappearance, dissolving cytoplasm and lymphocytic infiltration; hematoxylin and eosin stain, magnification ×200.
**Fig 4.** Multiple pattern recognition of serum metabolites in control and BFP-treated groups. PCA score plot (n = 30, R2X = 0.174, R2Y = 0.490, Q2 = 0.363). Control group (■); CB1 group (▼); CB2 group (●); CB3 group (◆); CB4 group (▲).

**Fig 5.** Effects of BFP on betaine, phosphatidylcholine and N,N-Diacetylchitobiose peak area intensity in rats. Statistical difference from control: *p < 0.05, **p < 0.01. Linear regression analysis was also performed to estimate dose-dependency: *p < 0.05, **p < 0.01. (a) Betaine; (b) Phosphatidylcholine; (c) N,N-Diacetylchitobiose.
Fig 6. Merged networks of the identified metabolites in different BFP treatment groups. Metabolites are represented as nodes, and the biological relationship between two nodes is represented as a line. Note that the colored symbols represent metabolites that occur in our data, while the transparent entries are known molecules from the Ingenuity Knowledge Database. Red symbols represent up-regulated metabolites; green symbols represent down-regulated metabolites; yellow symbols represent canonical pathways that were related to the identified specific metabolites. Solid lines between molecules indicate direct physical relationship between molecules, while dotted lines indicate indirect functional relationships. (a-d) The merged network of CB1, CB2, CB3 and CB4, respectively.
Fig 7. Perturbed metabolic regulatory network in response to BFP toxicity. Two metabolites within red box, up-regulated betaine and down-regulated phosphatidylcholine, were found to be closely related to the toxicity of the treatment. "f" represents the fold change value of CB vs. C. (BHMT, betaine homocysteine methyltransferase; MS, methionine synthase.)