Trametenolic Acid B Triggers HSP90AA4P and Autophagy in HepG2/2.2.15 Cells by Proteomic Analysis

Feifan Shi, II Yihe Fu, II Junzhi Wang, * Lie Li, Ailing Wang, Yuan Yuan, Huajun Luo, Haibo He, and Gaigai Deng

**ABSTRACT:** Our previous studies have demonstrated that trametenolic acid B (TAB) extracted from the *Laetiporus sulphureus* (Fr.) Murrill owned effective anti-proliferation of HepG2/2.215 cells and induced autophagy activity. The present aim was to further investigate its mechanisms involved by proteomic analysis. The iTRAQ of TAB on HepG2/2.215 was carried out and the western blot was used to verify the results of the proteomics analysis. According to the peptide segment quantitative standard (FDR ≤ 1%), a total of 5324 proteins were identified in HepG2/2.215 by proteomic analysis. The results identified that the major up-regulated proteins were HSP90AA4P, MYB, SERPINE1, and down-regulated proteins were Rho C, SERPINE1, and PIK3R4, which were related to PI3K/Akt signaling pathway, cell metastasis, and autophagy. HSP90AA4P and Rho C's proteomics analysis were further confirmed by the western blot. The proteomic results demonstrated that the anti-hematoma effect of TAB was closely related to the increase of HSP90AA4P protein expressions and autophagy, which may be a critical target of TAB, which was expected to be a candidate drug for the treatment liver cancer.

**INTRODUCTION**

Hepatocellular carcinoma (HCC) is one of the malignant tumors that seriously threaten human health worldwide. The incidence of HCC is related to many factors, such as hepatitis virus infection, alcoholism, smoking, environmental pollution, aflatoxin, and so on. Most patients with HCC are already in the middle and advanced stages when they are discovered. Although the five year survival rate can reach 80% or even more than 90% after surgery for very early stage liver cancer patients, whose tumor mass was less than 2 cm, the five year recurrence rate is as high as 70%. Therefore, drug treatment is crucial for HCC. However, for both traditional chemotherapy and targeted therapy, the efficacy is severely decreased because of drug resistance. Even for Sorafenib, the first-line molecule-targeting drug, its efficacy has been negatively affected due to the emergence of drug resistance. Therefore, it is of great significance to find more efficient therapeutic drugs for hepatocellular carcinoma.

Proteome refers to all proteins translated and transcribed by a cell or tissue or even a biological genetic information in a specific period, which does not only include the proteins directly translated and transcribed by the genome, but also the modified proteins after transcription and translation. Traditional research methods mainly focus on a single protein, but it cannot get all the protein information of an organism, tissue, or cell. In recent years, the relative and absolute quantitative technique of isotope labeling (iTRAQ) is a new quantitative technique of proteomics, which can accurately quantify and identify all proteins expressed in a genome or in a complex system. ITRAQ technology can not only realize the separation and identification of proteins, but could also qualitatively and quantitatively analyze the dynamic changes of proteins in cells, tissues, or body fluids under different physiological and pathological conditions, truly reflecting the comprehensive information of cell function, process mechanism, etc.

Traditional Chinese medicine has the advantages of small side effects and good curative effects in the treatment of tumors. *Laetiporus sulphures* (Fr.) Murrill is a Traditional Chinese medicine with a long history and is safe and reliable. Trametenolic acid B (TAB) is a triterpenoid compound extracted from it, which has the effects of anti-cancer, anti-gastric ulcer, hypoglycemic, and neuroprotection functionalities. Previous studies had shown that TAB reversed paclitaxel resistance. However, its effect was not through apoptosis but through autophagy. Our previous studies have demonstrated that TAB owned effective anti-proliferation of HepG2/2.215 cells and induced autophagy activity, and the current study was to further investigate the mechanism of autophagy by proteomic analysis.

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RESULTS AND DISCUSSION

TAB-Suppressed HepG2/2.2.15 Cell Proliferation. To assess the influence of TAB on the cytotoxicity and proliferation on HepG2/2.2.15 cells, they were treated with TAB (10–80 μM) on HepG2/2.2.15 cells for 12 and 24 h, respectively. Following 12 and 24 h of treatment with TAB in HepG2/2.2.15 cells, its proliferations of HepG2/2.2.15 cells were significantly depressed by 20, 40, 60, and 80 μM TAB. The IC50 values were 46.40 and 27.31 μM for HepG2/2.2.15 cells at 12 and 24 h, respectively. These aforementioned results indicated that TAB had a good growth inhibitory effect on HepG2/2.2.15 cells in a dose-dependent manner and relatively high selectivity (Figure 1A). Compared with SGC7901 cells, TAB had a higher inhibition rate on HepG2/2.2.15 cells (Figure 1B). For all subsequent experiments, TAB (40 μM) was used.

Proteomic Differential Protein Was Identified and Differential Expression Protein Used in Clustering Analysis. A total of 5324 human proteins were identified by iTRAQ, quantitative proteomics analysis, and the peptide segment quantitative standard was FDR ≤ 1%. In the 3 h group, there were 7 differentially expressed proteins up-regulated and 11 differentially expressed proteins down-regulated; in the 6 h group, there were 93 differentially expressed proteins up-regulated and 123 differentially expressed proteins down-regulated; in the 12 h group, there were 91 differentially expressed proteins up-regulated and 107 differentially expressed proteins down-regulated. Statistics of quantitative results of protein changes are displayed in the form of volcano charts (Figure 2).

In the proteomics determination of the three time periods (3, 6, and 12 h), the proteins of 6 h changed the most and was not much different from 12 h, so we mainly displayed 6 h figures. Hierarchical clustering was used to cluster the differentially expressed proteins among groups, and the data were displayed in the form of a heatmap. As indicated in Figure 3, the differentially expressed proteins were screened by the criteria of multiple change points greater than 1.2 times, which could effectively separate the comparison groups (P < 0.05). It showed that the screening of differentially expressed proteins was reasonable. Through analysis, we found that the remarkable elevated proteins were HSP90AA4P, MYB, and SERPINE1, and the continuous up-regulation proteins were HSP90AA4P and SERPINA1; the significantly reduced proteins were Rho C, SERPINA1, and PIK3R4, and the continuous down-regulation proteins were Rho C and SERPINA1 (Figure 3 and Tables 1 and 2). Since HSP90AA4P and Rho C had obvious changes and had direct influence on the development of liver cancer, we studied the mechanism of TAB from these proteins, which were marked in the volcanic map (Figure 2).

Differential Expression Proteins Were Used in Gene Ontology (GO) Enriched Analysis. GO is a functional classification system that provides a set of dynamically updated standardized vocabulary to describe the properties of genes and gene products based on three different perspectives, which involve biological process, molecular function, and cellular component. GO analysis was performed at level 2. As shown in Figure 4A, in the analysis of the biological process (BP), the regulation of wound healing, negative regulation of blood coagulation, regulation of fibrinolysis and cell matrix adhesion, and other biological processes are involved. In molecular function (MF) analysis, differential proteins show molecular functions such as structural constituent of ribosome and fibronectin binding. In cellular component (CC) analysis, most of the identified proteins belong to cellular components such as ribosomes and ribosome subunits. The process of enriching more differential proteins mainly includes the metabolic process and cellular process. The main functions involved are the combination function, signal conduction function, etc. The cell components mainly involved include cells, organelles, and cell parts (Figure 4B). These processes were related to proliferation, invasion, and metastasis of liver cancer cells and also indicated that TAB played an important role through these pathways.

Differential Expression Proteins Were Used in Enrichment Analysis Based on the KEGG Pathway. As is well known, proteins do not perform their functions independently in organisms, but coordinate with each other to complete a series of biochemical reactions to perform their biological functions. Therefore, pathway analysis was the most direct and necessary way to systematically and comprehensively understand the biological process of cells and the mechanism of drug action. As shown in Figure 5, the results of the KEGG pathway enrichment analysis (taking the first 20 change pathways) on the differential expression of the 6 h group indicated that important pathways such as the PI3K/AKT signaling pathway, ribosome, HTLV-1 infection, chronic myeloid leukemia, complement, and coagulation cascades had significantly changed.

According to the analysis results, PI3K/Akt pathway was one of the most significant biological processes after treatment with...
The PI3K/Akt signaling pathway had been demonstrated to play an important role in regulating autophagy in cancer cells. It had reported that the up-regulation of HSP90α might activate Akt and promote metastasis. However, TAB up-regulated the expression of HSP90AA4P, but inhibited the expression of p-Akt and affected the activity of the PI3K/Akt signaling pathway.

**Figure 2.** (A) Three hour volcanic maps. (B) Six hour volcanic maps. (C) Twelve hour volcanic maps. The number of changed proteins in the three groups of HepG2/2.2.15 cells treated with TAB was determined in the iTRAQ experiment. The red dot was the significant difference expression protein (multiple change >1.2 and \( P < 0.05 \)), and the black dot had no difference.
The mechanism of TAB might be the result of the multitarget function, which needed further study. The realization of this regulatory or mediating action requires the binding or interaction between proteins. It is of great significance to study the interaction between proteins in the network formed by the interaction. In this project, we compared the 6 h differential expression proteins of the group to construct the protein interaction network. As shown in Figure 6, in the protein interaction network, the nodes indicated the protein, and the lines represented the interaction between proteins.

### Table 1. Optimized Differentially Expressed Proteins of the 6 h Group (B/K: Up-Regulated > 1.5, Down-Regulated < 0.83)

| protein IDs | gene name | B/K | p value |
|-------------|-----------|-----|---------|
| F8WDK3     | PPP4R2    | 1.50089006 | 0.048677167 |
| A0A0F7G8J1 | PLG       | 1.503073935 | 0.000208879 |
| Q99988     | GDF15     | 1.514167727 | 0.001903171 |
| H3RRL9     | NTHL1     | 1.519907503 | 0.027455871 |
| P02751     | FN1       | 1.58872742 | 0.013061431 |
| L0R828     | C1orf162  | 1.818890495 | 0.034425598 |
| D6REL8     | FGB       | 2.046280943 | 0.006190145 |
| Q58FG1     | HSP90AA4P | 2.892780808 | 0.005527898 |
| H7C5K1     | C1orf61   | 2.901809599 | 0.038950658 |
| H0YC6N     | MYB       | 5.716990532 | 0.009060596 |
| E9PQH6     | RHOC      | 0.505766888 | 0.02464391 |
| Q8NGA1     | OR1M1     | 0.508985457 | 0.10749728 |
| A0A042R6J7 | SERPINA1  | 0.510066593 | 0.000264749 |
| D6RA3C     | PIK3R4    | 0.549099553 | 0.00212212 |
| Q16626     | MEA1      | 0.574748462 | 0.001073967 |
| X6R9N0     | PTAR1     | 0.57805257 | 0.00927024 |
| D6RBY8     | CDC23     | 0.585079745 | 0.03403597 |
| F8W150     | ANKRD13A  | 0.587166697 | 0.02756821 |
| P04350     | TUBB4A    | 0.588530545 | 0.008034129 |

### Table 2. Optimized Differentially Expressed Proteins of the 12 h Group (C/K: Up-Regulated > 1.5, Down-Regulated < 0.83)

| protein IDs | gene name | C/K | p value |
|-------------|-----------|-----|---------|
| Q15439     | ABCC4     | 1.531327818 | 0.020074382 |
| Q9ULX9     | MAFF      | 1.54381155 | 0.01007169 |
| P60468     | SEC61B    | 1.580072763 | 0.004671853 |
| Q6NT15     | STON2     | 1.58181982 | 0.000831852 |
| H0YM2A     | TMOD2     | 1.650370931 | 0.03471262 |
| A4F4K3     | CYP1A1    | 1.711202889 | 0.006433227 |
| R7Z553     | ABCC4     | 1.716976352 | 0.018910851 |
| Q99888     | GDF15     | 1.772384797 | 0.00133458 |
| P1T301     | ITGA2     | 1.784474594 | 0.003542275 |
| Q8WUJ3     | CEMIP     | 1.852765943 | 0.002410563 |
| F6M4D4     | HRA1      | 2.048330488 | 0.00007 |
| Q15742     | NAB2      | 2.09426093 | 0.03820169 |
| C9JX9F     | IGFBP1    | 2.192014574 | 0.00883387 |
| P05121     | SERPINE1  | 2.451743869 | 0.000184744 |
| Q58FG1     | HSP90AA4P | 9.012655171 | 0.038736037 |
| E9POH6     | RHOC      | 0.535146891 | 0.03874584 |
| Q5EC54     | HNRPK     | 0.555578714 | 0.03443851 |
| P47915     | CEBPA     | 0.597204285 | 0.02469818 |
| J3KQ66     | RELN      | 0.626574895 | 0.00123722 |
| A0A024R4P4 | ZNF444    | 0.630660267 | 0.00620777 |
| Q562M3     | ACT       | 0.64158606 | 0.04133265 |
| P02390     | BTF3      | 0.643345699 | 0.04588412 |
| O00767     | SCD       | 0.648399493 | 0.00845196 |
| E5RGR5     | C8orf59   | 0.650300672 | 0.047407105 |

Protein Interaction Network Was Analyzed. The realizations of this regulatory or mediating action require the binding or interaction between proteins. It is of great significance to study the interaction between proteins and the network formed by the interaction. In this project, we compared the 6 h differential expression proteins of the group to construct the protein interaction network. As shown in Figure 6, in the protein interaction network, the nodes indicated the protein, and the lines represented the interaction between proteins.
The results showed that the up-regulated protein (HSP90AA4P) was not directly related to others, such as MYB, SERPINE1, FGB, etc. After screening, we considered that HSP90AA4P was the most valuable target; therefore, the research focused on HSP90AA4P. HSP90AA4P belongs to the HSP90α protein family. The function of HSP90α was closely related to the growth and differentiation of cancer cells. It was reported that the increase of HSP90AA4P effectively promote autophagy.20−23 Meanwhile, the down-regulated proteins (RhoC, TUBB4A, SEPRINA1, etc.) were also not directly connected with HSP90AA4P except TUBB4A, which inferred that TAB might directly affect the expression of HSP90AA4P and regulate the function of HSP90AA4P’s customer proteins, such as inhibiting the ubiquitination of Beclin1, ULK1, and the degradation of protein enzyme body, thus causing autophagy.24,25 In addition, Rho C was associated with HSP90AA4P through two proteins (MYO5B and ACT), but they were nonsignificant differential proteins, so we did not discuss them carefully. According to Figure 7, TAB significantly decreased Rho C in both SGC7901 and HepG2/2.215 cells, indicating that the down-regulation of Rho C was not completely relevant to HSP90AA4P. In other words, TAB may directly affect the

Figure 4. (A) Top 20 of the GO function enrichment (color represents p value, the label above the bar chart represents the richFactor (richFactor ≤ 1); richFactor indicates the ratio of the number of differentially expressed proteins annotated to a GO functional category to the number of all identified proteins annotated). (B) Number of differentially expressed proteins related to the GO functional classification.
expression of HSP90AA4P and Rho C, which were both the targets of TAB. It is suggested that TAB may play the anti-hepatoma role through multiple targets.

Protein Expression Verified by Western Blot Analysis.
As shown in Figure 7A, TAB significantly elevated the expression of HSP90AA4P protein in HepG2/2.2.15 cells, while HSP90AA4P was moderately elevated in SGC7901 cells, indicating that HSP90AA4P might cause the death of HepG2/2.2.15 cells, which is also partly relevant to autophagy. Recent studies have shown that HSP90α was an important positive regulator of autophagy. It can stabilize the expression of Beclin-1, ATG7, and ULK1 and promoted autophagy death.

TAB significantly reduced the protein expression of Rho C in HepG2/2.2.15 cells and SGC7901 cells. The results were consistent with the result of proteomics analysis. We inferred that HSP90AA4P is an important target on the anti-hepatoma mechanism. Previous studies have shown that HSP90α may promote cell migration, while if the decrease of Rho C would overcome this question needed further study. The result of LC3 also showed that HSP90AA4P was associated with autophagy.

As shown in Figure 7B, TAB significantly elevated the expression of HSP90AA4P and LC3II/I proteins in HepG2/
2.2.15 cells. The results indicated that autophagy was related to the up-regulation of HSP90AA4P in HepG2/2.2.15 cells. Meanwhile, 17AAG, the N-terminal inhibitor of HSP90α could inhibit the up-regulation of HSP90AA4P with TAB. Based on the previous study, TAB could inhibit the activity of H+K+-ATPase, which inferred that TAB may bind to the N-terminal ATP binding region of HSP90α and thus activated heat shock reaction by negative feedback regulation and induced the up-regulation of HSP90AA4P expression. It was also necessary to further determine the effect of HSP90AA4P expression induced by TAB on the function of related proteins through proteomic studies of phosphorylation, so as to determine the specific mechanism of TAB against liver cancer.

The results showed that HSP90α accelerated autophagic death of tumor cells. We also found that a certain dose of TAB could induce autophagic death of hepatoma cells, which suggested that the expression of HSP90AA4P induced by TAB could promote autophagic death of cancer cells, which might be a new method for the treatment of hepatoma.

TAB had a great growth inhibitory effect on HepG2/2.2.15 cells. Our results first demonstrated that TAB caused autophagy and death of cancer cells, which may be related to the up-regulation of HSP90AA4P and other proteins. TAB led to the down-regulation of Rho C and other proteins, which may help inhibit the metastasis of cancer cells. The anti-hepatoma effect may be due to the multiple targets of TAB.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** MEM medium, phosphate buffered saline (PBS), trypsin, penicillin and streptomycin, dimethyl sulfoxide (DMSO), and fetal bovine serum were obtained from Gibco Company (Carlsbad CA, USA); trypsin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Company (St. Louis, MO, USA); ITRAQ Kit was obtained from AB SCIEX Company (Washington, USA); sodium pyruvate (NaP) and nonessential amino acids (NEA) were purchased from McLean Biochemical Technology Co., Ltd. (Shanghai, China); G418 (neomicina) was obtained from Soleboard Technology Co., Ltd. (Beijing, China); and BCA Kit was obtained from Biyuntian Company (Nanjing, China). All other chemicals were of analytical grade.

**Cell Culture.** Human hepatocellular carcinoma cells (HepG2/2.2.15) were obtained from Kebai Biological Co., Ltd. (Nanjing, China), and the HepG2/2.2.15 cells (1 × 10^5 cells/well) were grown in an MEM medium containing 10% FBS and 1% antibiotic (100 μg/mL streptomycin and 100 units/mL penicillin) at 37 °C in a humidified 5% CO2 atmosphere. Cell growth was observed daily, and the experiments were performed when the monolayer cells were attached to an adherent level of about 80%. TAB was dissolved in DMSO, and finally, 10 mM was prepared for cell experiments. When in use, the culture medium is diluted to the required concentration.

**Cell Viability Assay.** HepG2/2.2.15 cells were seeded in a 96-well plate for 12 h and then treated with TAB (10.0, 20.0, 40.0, 60.0, and 80.0 μM) continuously for 12 and 24 h. After the end of culture, the MTT assay was also used to assess the proliferation of TAB on the HepG2/2.2.15 cells. The experiment was repeated four times to calculate the inhibition rate of TAB on cell proliferation. With this approach, drug dosage suitable for proteomic determination was selected.

**Schematic Flow Chart of TAB Acted on Screening Proteins in HepG2/2.2.15 Cells.** The mechanism of hepatocellular carcinoma (HCC) is complex and uncertain. Based on the proteomic analysis of iTRAQ markers, we determined the differentially changed proteins and their pathways under the action mechanism of drugs. The flow chart is shown in Figure 8.

In this study, HepG2/2.2.15 cells were seeded in culture bottles for 12 h and then treated with TAB (40 μM) continuously for 3, 6, and 12 h. After protein extraction and trypsin was digested, iTRAQ labeling experiments were carried out. In these two iTRAQ experiments, the labeled peptides were
polymerized together and fractionated by HPLC and analyzed by LC–MS/MS. Database search and intensive bioinformatics were analyzed to identify potential tumor-specific biomarkers for HCC. These potential biomarkers were further confirmed by western blot, which was to clarify the mechanism of action of TAB in HCC.

**Protein Extraction of and Peptide Segment Enzymolysis.** HepG2/2.215 cells were seeded in culture bottles for 12 h and then treated with TAB (40 μM) continuously for 3, 6, and 24 h. In this experiment, the SDT (4% (w/v) SDS, 100 mM Tris/HCl pH 7.6, 0.1 M DTT) lysis method was used to extract protein, and then the BCA method was used to quantify protein. Then, the sample proteins were enzymatically hydrolyzed by trypsin using the filtered protein preparation (FASP) method and then desalted by the C18 cartridge. The peptide was lyophilized and redissolved by adding 40 μL dissolution buffer, and the peptide was quantified (OD280).

**iTRAQ Markers and SCX Chromatographic Classification.** Peptide (0.1 mg) from each sample was taken, mixed with the peptide labeled according to the iTRAQ labeling kit, and then graded with AKTA Purifier 100. The detection wavelength was 214 nm, and the flow rate of the column was 1 mL/min. Gradient elution was used for separation with buffer A (10 mM KH2PO4, 25% ACN, pH = 3.0) and buffer B (10 mM KH2PO4, 500 mM KCl, 25% ACN, pH = 3.0), and the eluent was collected every minute before freeze-drying and desalination. The liquid phase gradient is shown in Table 3.

**LC–MS/MS Analysis.** Each fractionated sample was separated by HPLC liquid phase system Easy nLC with a nanoliter flow rate. Buffer A is 0.1% formic acid aqueous solution, and B is 0.1% formic acid acetonitrile aqueous solution (acetonitrile is 84%). The chromatographic column is balanced with 95% of liquid A. The sample is loaded into the loading column (Thermo Scientific Acclaim Pepmap 100, 100 μm × 2 cm, Nanoviper C18) by an automatic sampler and separated by an analysis column (Waters nanoACQUITY, 25 cm) at a flow rate of 300 nL/min.

After chromatographic separation, samples were analyzed by mass spectrometry using a Q-Exactive mass spectrometer (Thermo Fisher Scientific). The detection method is positive ions, the scanning range of parent ions is 300–1800 m/z, the primary mass spectrum resolution is 70,000 at 200 m/z, the AGC (automatic gain control) target is 1e6, the maximum IT is 50 ms, and the dynamic exclusion time is 60.0 s. The mass-to-charge ratio of polypeptide and polypeptide fragments is collected according to the following methods: 10 fragment patterns (MS2 scan) are collected after each full scan and the MS2 activation type is HCD, isolation window is 2 m/z, secondary mass spectrometry resolution is 17,500 at 200 m/z, normalized collision energy is 30 eV, and underfill is 0.1%

**Protein Identification and Quantitative Analysis.** The RAW data for mass spectrometry analysis are RAW files, and the software Mascot2.2 and Proteome Discoverer1.4 are used for library checking, identification, and quantitative analysis. Relevant parameters and descriptions are as follows. Enzyme: trypsin. Max Missed Cleavages: 2. Fixed modifications: carbamidomethyl (C), iTRAQ 4/8plex (N-term), iTRAQ 4/8plex (K). Variable modifications: Oxidation (M), iTRAQ 4/8plex (Y). Peptide mass tolerance: ± 20 ppm. Fragment mass tolerance: 0.1 Da. Protein Quantification: The protein ratios are calculated as the median of only unique peptides of the protein.

**Bioinformatics Analysis.** The original data of mass spectrometry analysis were used to quantify and identify analysis with Proteome Discoverer 1.4 and Mascot 2.2 systems and retrieved by analysis software. All quantitative and qualitative protein analysis results were merged according to the filter parameter FDR < 0.01. GO function annotation/enrichment and KEGG pathway annotation/enrichment, cluster analysis, and PPI protein interaction network analysis were performed for differentially expressed proteins with a ratio of >1.2 and a p value of <0.05, which was considered as significant.

**Western Blot Analysis.** The total proteins of in the HepG2/2.215 cells were extracted by using appropriate separation kits. The protein content was detected by a nucleic acid analyzer (Thermo scientific, USA). After separation by using SDS-PAGE gels, the protein was transferred onto the nitrocellulose membranes. The nitrocellulose membranes transferred protein were incubated with the proteins to be verified and β-actin primary antibodies at 4 °C overnight and then secondary antibody labeled HRP were added. The target protein bands were visualized on an X-ray film by using ECL coloration. Quantitative analysis was performed by using the Image J morphology analysis system (National Institute of Health, USA), and molecular expressions were normalized to β-actin.

**Protein Database Searches.** Proteins related to proteomics were searched in NR and UNIPORT databases. KEGG

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**Table 3. Gradient Elution Condition of Buffer A and Buffer B in SCX Chromatographic Classification**

| t (min) | 0   | 25  | 32  | 42  | 47  | 60  | >60 |
|--------|-----|-----|-----|-----|-----|-----|-----|
| buffer A | 100%| 90% | 80% | 55% | 0%  | 0%  | 100%|
| buffer B | 0%  |10%  |20%  |45% |100%|100%|0%  |
analysis was searched in Kyoto Encyclopedia of Genes and Genomes, GENE ONTOLOGY database, and string database (http://string-db.org/), which have outstanding research on the information of protein interaction. Therefore, in order to obtain accurate interaction information and network with experimental evidence and make it more meaningful to study the precise regulatory relationship between proteins, we usually choose intact (http://www.ebi.ac.uk/intact/main.xhtml) as the main method. Furthermore, In order to obtain more information, the data of string database is usually used for the species with insufficient interaction research.

**Statistical Analysis.** All results were confirmed in at least three independent experiments, and all data were presented as mean ± SD. Database was set up with SPSS 21.0 software package (SPSS Inc., Chicago, USA), multiple variables were performed by one-way ANOVA, Dunn’s multiple comparison test and the Kruskal–Wallis test were used for comparison of variable pairs. \( P < 0.05 \) or \( P < 0.01 \) was considered statistically significant.

## AUTHOR INFORMATION

**Corresponding Author**

Junzhi Wang — Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, Yichang, Hubei 443002, China; orcid.org/0000-0002-8357-993X; Phone: +86-717-6397478; Email: horsedog@163.com; Fax: +86-717-6397478

**AUTHORS**

Feifan Shi — Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, Yichang, Hubei 443002, China

Yihe Fu — Three Gorges food and drug inspection and Testing Center, Yichang, Hubei 443000, China

Lie Li — Yichang Humanwell Pharmaceutical Co., Ltd, Yichang, Hubei 443000, China

Ailing Wang — Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, Yichang, Hubei 443002, China

Yuan Yuan — Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, Yichang, Hubei 443002, China

Huajun Luo — Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, Yichang, Hubei 443002, China

Haibo He — Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, Yichang, Hubei 443002, China

Gaigai Deng — Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, Yichang, Hubei 443002, China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c00962

**Author Contributions**

F.S. and Y.F. contributed equally to this work.

**Notes**

The authors declare no competing financial interest. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD018963.

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**ABBREVIATIONS**

TAB: trametelonic acid B; HSP90AA4P: heat shock protein 90 alpha family class AA4P member; 17AAG: tansopirycin

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