Proteomics Analysis of Hepatoprotective Effects for Scoparone Using MALDI-TOF/TOF Mass Spectrometry with Bioinformatics

Aihua Zhang, Hui Sun, Gelin Wu, Wenjun Sun, Ye Yuan, and Xijun Wang

Abstract

Scoparone is an active ingredient of Yinchenhao (*Artemisia annua* L.), a well-known Chinese medicinal plant, and has been utilized in prevention and therapy of liver damage. However, the molecular drug targets associated with the pharmacological effects of scoparone are largely unknown. In the present article, we extend the previous research on Yinchenhao through a study of its active ingredient and thus the putative targets of scoparone. We employed two-dimensional gel electrophoresis, and all proteins expressed were identified by MALDI-TOF/TOF MS and database research. Protein-interacting networks and pathways were also mapped and evaluated. The possible protein network associated with scoparone was constructed, and contribution of these proteins to the protective effect of scoparone against the carbon tetrachloride-induced acute liver injury in rats are discussed herein. Hepatoprotective effects of scoparone on liver injury in rats were associated with regulated expression of six proteins which were closely related in our protein-protein interaction network, and appear to be involved in antioxidation and signal transduction, energy production, immunity, metabolism, and chaperoning. These observations collectively provide new insights on the molecular mechanisms of scoparone action against hepatic damage in rats.

Introduction

NATURAL PRODUCTS HAVE BEEN REGARDED as excellent sources for drug discovery and development, given their structural diversity and variety of biological activities. Natural products play key roles as biological probes due to their ability to interact with high affinity of biological targets. Knowledge about the protein targets and protein-drug interactions of natural products is critical for understanding drug mode of action (Cheng et al., 2010). Recently, proteomic technology features high-throughput parallel analysis of proteins expressed, and thus provides the possibility of details on the molecular mechanisms of natural products (Wang et al., 2012a; Yao et al., 2008). The identification of their targets is required for a comprehensive understanding of their pharmacological effects and mechanism of action. Proteomic approaches combined with bioinformatics method can reconstruct regulatory networks, signaling cascades, and metabolic pathways upon drug treatment (Wong et al., 2008). It has played an important role in significantly accelerating

stages of drug discovery and development such as target discovery or mechanism of drug action (Tang et al., 2006; Wang et al., 2012b).

Identification of the putative targets of natural products is an important aspect of current drug discovery and will aid drug development (Gersch et al., 2012). Yinchenhao (*Artemisia annua* L.) is one of the most popular traditional Chinese medicinal plants for treatment of liver disorders (Sun et al., 2013; Wang et al., 2013; Zhang et al., 2011). It can be used clinically to treat liver fibrosis, cholestasis, hepatitis C, and cholestatic diseases (Yin et al., 2012). Interestingly, scoparone (Fig. 1), one of the main constituents of Yinchenhao, shows hepatoprotectivity and has been proven effective for liver injury (Wang et al., 2011; Zhang et al., 2012). Furthermore, scoparone was reported to be an effective drug to enhancing secretion of bile acids for normalizing liver function (Yang et al., 2011). It suggested that scoparone may be a good lead compound for further studies.

Among the reported pharmacological properties of scoparone, its hepatoprotective effects are of particular interest.

FIG. 1. Chemical structure of scoparone.

However, the molecular drug targets associated with the pharmacological effects of scoparone are largely unknown. Proteomic approaches could provide a recent breakthrough for the clarification of target proteins. Here, we will use proteomics approaches to explore the mechanisms and implications and molecular drug targets of scoparone. Moreover, a computational program, STRING (Jensen et al., 2009), was applied to verify the drug targets of scoparone and to mine the functional association of the defined proteins.

Materials and Methods

Reagents

Ultra-pure reagents for polyacrylamide gel preparation were obtained from Bio-Rad. Dithiothreitol (DTT), TPCK-Trypsin, iodoacetamide, thiourea, and α -cyano-4-hydroxycinnamic acid were provided by Sigma Chemical (St. Louis, MO, USA). IPG buffer and 18-cm immobilized pH gradient (IPG) strips (pH 3–10) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were purchased from Bio-Rad.

Animal sampling and handling

Animals were randomly assigned to scoparone, control, and model groups of 8 rats per group. The rats in the animal model group were orally administrated 20% CCl_4 (1 ml/kg body weight) and scoparone for 8 consecutive days (at 8:00 PM), and on day 9, the rats were anesthetized via an intraperitoneal injection of 1% pentobarbital sodium (0.15 mL/100 g body weight). The control group was given distilled water for 8 consecutive days and anesthetized on day 9 as in the model group. Blood (5 mL) was collected from the hepatic portal vein, and the serum was separated through centrifugation at 4500 rpm for 5 min at 4°C, and stored at -80°C until the proteomics analyses were performed.

Proteomics analysis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed. Protein spots with scores that were

higher than 61 were considered to be differentially expressed, and were selected and subjected for further identification by MALDI-TOF/TOF MS analysis. Two-dimensional gel electrophoresis, image analysis, protein identification, and database searching were performed. Detailed procedures are described in our previously published work that used the identical methodology (Sun et al., 2013).

Bioinformatics analysis of proteomic data

Identified proteins were further analyzed using STRING for protein–protein interactions, to statistically determine the functions and pathways most strongly associated with the protein list. Prior to upload and analysis using STRING, the mean ratio of each quantified protein in a group was calculated and the fold change between the groups calculated. The KEGG (www.genome.jp/kegg/) pathway database were used to further examine the significantly expressed molecules.

Results

Two-dimensional gel electrophoresis

Proteins from the control, model, and scoparone groups were analyzed by 2DE. Approximately 1000 protein spots were detected on the 2DE gels. A representative protein profile is shown in Figure 2. In total, fifteen spots which showed remarkable change ($p < 0.05$) between the model and control groups had been observed, of which 7 spots were upregulated and 8 spots downregulated.

Identification of significantly modified proteins

The proteins extracted from the 2-DE gel were applied to MALDI-TOF/TOF-MS and analyzed by the Mascot search engine to differentially expressed proteins. Finally, we identified 15 proteins which are shown in Supplementary Table S1 (Supplementary data are available online at www.liebertpub.com/omi). A representative peptide mass fingerprint (PMF) map and database query result of protein prothrombin and transthyretin are shown in Figure 3. Specifically, relative expression of the significantly modified proteins is shown in Figure 4. They appear to be involved in energy production, immunity, metabolism, signal transduction, and chaperoning. Hepatoprotective effects of scoparone action on liver injury was associated with regulated expression of six proteins including Ig kappa chain C, haptoglobin, alpha-1-antitrypsin, zinc finger protein 407, transthyretin, and prothrombin.

Visualization and analysis of protein–protein interaction network

To facilitate access to the protein–protein interaction data, STRING integrates information about interactions from metabolic pathways and drug–target relationships. As seen in Figure 5, association network of six differentially expressed proteins using STRING was constructed, where the nodes are the entities and the edges their interactions. Modes of action are shown in different colors. Differentially expressed

FIG. 2. Two-dimensional electrophoresis (2-DE) representative proteomic maps of control (A), model (B), and scoparone (C) groups.

proteins are represented as pill-shaped nodes, while proteins are shown as spheres. A possible network associated with scoparone target-related proteins was constructed, suggesting that these proteins were closely related in the protein-protein interaction network. From all the GO categories covered, the identified proteins could be divided into several main groups according to their functions. These closely connected proteins in networks are regarded as the signatures of the underlying targets.

Discussion

The tide of drug discovery programs has been turned back to Nature in the search for new drug candidates. Natural products, on the other hand, have evolved to interact with biomolecules, which have been dominating the pharmaceutical industry for a long time, increasingly turning out to

be successful. They have contributed to the development of important therapeutics to combat diseases over the past decades (Yang et al., 2012). The value of natural products to drug discovery is still considerable, however, the protein target(s) of action of these fascinating compounds are largely unknown. Recently, MS-based proteomics has the potential to identify novel proteins that are related to the action mechanisms of natural products (Barnett et al., 2012; Schrattenholz et al., 2010; Zhang et al., 2013). Various types of biological networks are being constructed, including protein-protein interaction networks, and protein regulatory networks, to help drug discovery (Abu-Asab et al., 2011; Ge et al., 2012).

Scoparone is an important ingredient for the therapeutic effect of Yinchenhao. Evidence shows that scoparone could yield potential benefits in the treatment and prevention for liver diseases, such as a potent inhibitor of hepatic fibrosis induced by carbon tetrachloride (Wang et al., 2011; Yin et al., 2012; Zhang et al., 2011 and 2012). All these results suggest that it may be a potent drug candidate for further study and up to now, its therapeutic mechanism has still been completely unclear. Therefore, in this study, we have conducted a comprehensive analysis of the possible target proteins following 2-DE and MALDI-TOF/MS. Protein interaction networks were also mapped using STRING analysis program. As a result, hepatoprotective effects of scoparone action against acute hepatic injury rats was associated with regulated expression of six proteins, including Ig kappa chain C, zinc finger protein 407, prothrombin, haptoglobin, alpha-1-antitrypsin, and transthyretin. Furthermore, the change in the levels of these proteins caused by scoparone treatment appear to be involved in antioxidation and signal transduction, energy production, immunity, metabolism, and chaperoning. As seen in Figure 5, association networks of differentially expressed proteins in the scoparone-treated group using STRING was constructed, which may help to increase the understanding of molecular networks for scoparone treatment.

Modern drug discovery is time-consuming and expensive (Savino et al., 2012). The rapid advancement of proteomics approaches has provided a vast of significant information to drug discovery that are recruited for application to pharmaceutical research (Shen et al., 2007). Furthermore, the networks may also play an important role in discovering biomarkers. It has been shown that identified markers are more reproducible if their identification is based on networks rather than single proteins (Heo et al., 2011). Novel protein targets and the protein-associated subsystems provide new insights into the mechanisms underlying pathogenesis. Here, we developed a practical solution particularly to address the bottleneck in the identification of authentic target proteins among a number of candidates. These identifications, undoubtedly, have significantly contributed to the elucidation of the underlying biological mechanism of drug action.

Conclusion

Target-based drug discovery has become a major pharmacological approach since the emergence of -omics technologies. Proteomics holds the key to revolutionize the target identification for natural products. Scoparone is a

FIG. 3. Peptide mass fingerprint spectrum of transthyretin (A) and prothrombin (B). The spot was in-gel digested with trypsin. After desalted, the peptide mixture was analyzed by MALDI-TOF/TOF MS. Spectral masses (in mass per charge unit, M/z) obtained by MALDI-TOF/TOF MS were analyzed by using bioinformatics through the Mascot and Profound search engines. The *x-axis* represents the mass-to-charge ratio (m/z), and the *y-axis* represents the relative abundance. All protein identifications are provided in Supplementary Table S1. Down: the probability-based Mowse scores obtained using the Mascot search engine. Among predicted proteins with differential Mowse scores shown as multiple bars on the *x-axis*, only proteins with Mowse scores greater than 61 were considered significant, which were 397 and 156 ($p < 0.05$) for transthyretin and prothrombin, respectively.

natural compound with recognized effects against hepatic damage properties. Our study implemented proteomics to identify potential protein targets for liver injury rats affected by scoparone. Six proteins with statistically significant altered expression levels changed under scoparone treatment were identified and might be considered as possible targets of scoparone. These results provide new details on the therapeutic mechanism of scoparone from a molecular perspective. On the other hand, it is unlikely that

a compound has only one target protein, and therefore the present method may be useful for the identification of multi-target proteins that are at the basis of therapeutic effects to the compound. Network-based drug discovery protocols that emphasize the value of small compounds will likely be re-evaluated. The methodology reported herein is anticipated to make an important contribution for elucidating the underlying biological mechanism of action at a molecular level toward discovery research in this area.

FIG. 5. Protein interaction network generated and visualized with STRING 9.0 for proteins identified. Modes of action are shown in different colors. Differentially expressed proteins are represented as *pill-shaped nodes*, while other proteins are shown as *spheres*. Nodes that are associated to each other are *linked by an edge*. Thicker lines represent stronger associations. Lines and for directed edges, *arrows* of different colors stand for different edge types in the actions view: binding (*blue*), activation (*green*), inhibition (*red*), catalysis (*magenta*), same activity (*cyan*), and reaction (*black*). (A) Ig kappa chain C; (B) zinc finger protein 407; (C) haptoglobin; (D) alpha-1-antitrypsin; (E) transthyretin; (F) prothrombin.

FIG. 4. Relative expression level of the differentially expressed proteins obtained by MALDI-TOF/TOF MS.

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Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Address correspondence to:

Prof. Xijun Wang

National TCM Key Lab of Serum Pharmacochemistry
Key Pharmacometabolomics Platform of Chinese Medicines
Heilongjiang University of Chinese Medicine
Heping Road 24
Harbin 150040
China

E-mail: xijunwangls@126.com