Quantitative Proteomics Reveals the Antitumor Effects of Sodium New Houttuyfonate on Non-small Cell Lung Cancer

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Abstract

Background and objectives: Houttuynia cordata Thunb, which is a traditional Chinese herbal medicine, is commonly used as an anti-inflammatory, antiviral, and antibacterial agent in China. Emerging evidence shows that extracts of H. cordata Thunb have anticancer activity in human colorectal, leukemic, lung, and liver cancer cells; however, the specific active ingredients or compounds that responsible for these anticancer activities and their mechanism of action remain unknown. Sodium new houttuyfonate (SNH) is an additional product of the active ingredient houttuynin from H. cordata Thunb, which possesses anticancer activity; however, the molecular mechanisms that underlie its action have not been clarified. This study aims to explore the antitumor effect and related molecular mechanism of SNH on human non-small cell lung cancer (NSCLC).

Methods: The cytotoxicity of SNH against human lung cancer cells H1299 was investigated using WST-1 and apoptotic assays, and its antitumor molecular mechanism was explored using quantitative proteomics combined with various cellular and biochemical assays.

Results: The results showed that SNH reduced the viability and enhanced the apoptosis of H1299 cells in a dose-dependent manner. Quantitative proteomics and Ingenuity pathway analysis revealed that SNH downregulated the expression of cell cycle-related proteins, which included cyclin-dependent kinase 1 (CDK1), protein tyrosine phosphatase type IVA 2 (PTP4A2), and cyclin-dependent kinase 6 (CDK6), and upregulated the expression of Nrf2 (nuclear factor erythroid 2-related factor 2)-mediated oxidative stress response-related proteins in H1299 cells.

Conclusions: SNH-induced G0/G1 arrest and apoptosis in H1299 cells by the inhibition of cell cycle-related proteins that included CDK1, PTP4A2, CDK6, and activated the expression of Nrf2-mediated oxidative stress response-related proteins. These findings might provide new molecular mechanisms that underlie the antitumor activity of SNH against NSCLC and could implicate SNH as a novel therapeutic drug for NSCLC in the future.

Keywords: Sodium new houttuyfonate; Non-small cell lung cancer; Proteomics; G0/G1 cell cycle; Apoptosis; Nrf2.

Abbreviations: SNH, sodium new houttuyfonate; NSCLC, non-small cell lung cancer; Nrf2, nuclear factor erythroid 2-related factor 2; iTRAQ, isobaric tags for relative and absolute quantitation; IPA, ingenuity pathway analysis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CDK1, cyclin-dependent kinase 1; PTP4A2, protein tyrosine phosphatase type IVA 2; CDK6, cyclin-dependent kinase 6; DMEM, Dulbecco’s modified eagle medium.

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Introduction

Non-small cell lung cancer (NSCLC) accounts for >80% of lung cancers and is one of the most common malignant tumors with the highest mortality rate worldwide. Despite a significant amount of research, surgery, chemotherapy, radiotherapy, and targeted therapies remain the major methods for the treatment of NSCLC; however, their therapeutic efficacy is not satisfactory for NSCLC patients. Recently, the induction of cell cycle arrest has been considered to be a promising treatment strategy for NSCLC; therefore, an increasing number of novel chemical agents that target cell cycle-associated proteins have been reported recently.

Cyclin-dependent kinases (CDKs) are serine or threonine kinases that are important in governing the transition between different phases during cell cycle progression. Thirteen loci in the human genome encode CDKs, but only five CDKs (CDK1-4, and CDK6) are involved in driving the cell cycle. The inhibition of the expression and activity of these five CDKs leads to cell cycle arrest. Therefore, selective CDK inhibition could represent new avenues for cancer therapy. Overall, >10 specific CDK inhibitors have entered clinical trials; in addition, palbociclib, which is a specific CDK4 and 6 inhibitor, has been accelerated and approved by the FDA for the treatment of HR’Her-2’ metastatic breast cancer. Therefore, more cell cycle inhibitors are required for clinical practice.

H. cordata Thunb is a food and medicinal herb that has been reported to possess nutraceutical and anti-inflammatory, anti-viral, and antibacterial activities and has been used for many years in China. Recently, many studies have shown that extracts of H. cordata Thunb have anticancer activity against human colorectal cancer, leukemia, lung cancer, and liver cancer cells. However, the specific molecules that are responsible for these anticancer activities and their mechanistic action remain unknown. Sodium new houttuyniate (SNH) is a derivative of H. cordata Thunb and has been used for the treatment of respiratory tract and skin infections in clinical environments. Recently, SNH has been reported to have antibacterial and anticancer effects. However, further investigations are required to determine the molecular mechanism that underlies its anticancer activity.

Proteomics, which is a powerful tool for the comprehensive characterization of protein alterations in response to nutraceutical and drug treatment, has been widely applied to analyze the molecular mechanisms and to screen targets of anticancer agents. This study aims to investigate the antitumor effect of SNH on NSCLC and to further determine its underlying antitumor mechanisms in NSCLC using quantitative proteomics combined with various cellular and biochemical assays.

Materials and methods

Reagents and antibodies

SNH was purchased from Chembest (Shanghai, China). The chemical structure of SNH is illustrated in Figure 1a. An Annexin V-FITC/PI apoptosis assay kit and antibodies against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were purchased from TransGen Biotech (Beijing, China). WST-1 cell proliferation, cytotoxicity assay, reactive oxygen species assay kits, and 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA) were obtained from Beyotime (Jiangsu, China). Propidium iodide (PI) and isobaric tags for relative and absolute quantitation (iTRAQ) reagent-8plex multiplex kits were obtained from Sigma (St. Louis, MO, USA). Antibodies against protein tyrosine phosphatase type IVA.2 (PTP4A2), CDK1 and 6 were purchased from Abcam (Cambridge, UK), and antibodies against caspase-3 and cleaved caspase-3 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

Human lung adenocarcinoma H1299 and A549 cell lines and human bronchial epithelial (HBE) cell lines (ATCC, Rockville, MD, USA) were cultured in dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a 5% CO₂ incubator.

Cell viability assay

Cell viability was evaluated using a WST-1 Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Jiangsu, China), according to the protocol described in the manufacturer’s manual. H1299 or HBE cells (5 × 10³/well) were cultured into 96-well plates for 12 h and treated in triplicate with various concentrations (0, 25, 50, 75, 100, or 150 µM) of SNH for 24 or 72 h in the dark. The 10 µL of WST-1 solution was added to each well and incubated at 37 °C for 1 h. The absorbance of individual wells was read on a microplate reader at 450 nm. The relative cell viability and 50% inhibitory concentration (IC₅₀) were calculated using GraphPad Prism 6.0 software.

Apoptosis assay

The impact of SNH on the apoptosis of cells was analyzed by flow cytometry using an Annexin V-FITC/PI apoptosis assay kit (TransGen Biotech, Beijing, China), according to the protocol described in the manufacturer’s manual. Briefly, H1299 cells (1 × 10⁵/well) were treated in triplicate with SNH (0, 50, 75, or 100 µM) for 24 h. The cells were harvested and stained with Annexin V-FITC and PI at room temperature for 30 min in the dark. The frequency of apoptotic cells was analyzed by flow cytometry in a BD Accuri C6 flow cytometer (BD Biosciences, San Diego, CA, USA).

Cell cycle assay

The effect of SNH on cell cycling was determined by flow cytometry. Briefly, H1299 cells (5 × 10³/well) were treated in triplicate with vehicle or 75 µM SNH for 24 h. The cells were harvested, washed, and fixed with prechilled 70% ethanol at −20 °C for 1 h. After washing, the fixed cells were stained with PI staining buffer (33 µg/mL PI, 130 µg/mL RNase A, 0.5% Triton X-100, and 10 mM EDTA-Na₃) in the dark for 15 min. Finally, the stained cells were analyzed by flow cytometry in a BD Accuri C6 flow cytometer (BD Biosciences, San Diego, CA, USA), and the percentage of cells in various phases of the cell cycle was analyzed using FlowJo software (Tree Star Inc., Ashland, USA).

Protein sample preparation and iTRAQ-based proteomics analysis

H1299 cells were treated with or without SNH at an IC₅₀ dose for 24 h. The cells were harvested and suspended in SDS lysis buffer, followed by centrifugation. The supernatant fractions were...
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collected to measure the total protein concentration using a BCA protein assay kit (Thermo Fisher Scientific, San Jose, USA).

For the iTRAQ experiments, 200 µg proteins from each group of cell lysates were processed for digestion and iTRAQ labeling according to the manufacturer’s instructions. The resulting peptides were labeled with an iTRAQ Reagent-8Plex kit (AB SCIEX, Framingham, CA, USA) at room temperature for 2 h. The peptides from the control group were labeled with iTRAQ reagents 114, 115, and 116 and the peptides from the SNH treatment group were labeled with iTRAQ reagents 117, 118, and 119. Then, 100 µg of control proteins and 100 µg of SNH-treated proteins were mixed and labeled with iTRAQ reagent 113 as an internal reference. Then, the peptides were fractioned and subjected to LC-ESI-MS/MS analysis, according to the procedures described in a previous report.19

Data processing and Ingenuity Pathway Analysis

All MS raw data files (.wiff) were searched against the Homo sapiens protein database, and proteins were quantified using ProteinPilot Software 4.523 using the search parameters described in a previous paper.19 except for the iTRAQ 4 plex (peptide labeled) and Streptococcus pneumoniae D39_.fasta databases that were replaced with the iTRAQ 8 plex (Peptide Labeled) and Homo sapiens_.fasta databases, respectively. The differentially expressed proteins (DEPs) were defined when fold changes $>1.50$ or $<0.67$ and $p<0.05$, and peptides $>1$ were identified in three biological replicates.

The distribution of individual proteins was analyzed by Volcano plot analysis using MATLAB software. The pathways of DEPs were analyzed using Ingenuity Pathway Analysis (IPA) software to identify the top canonical pathways, as described previously.4

Western blotting analysis

After incubation with or without SNH for 24 h, the cells were collected and lysed in an SDS lysis buffer. The cell lysates were subjected to Western blotting analyses. Briefly, equal amounts of lysate proteins from the control and SNH-treated groups were separated by SDS-PAGE on 12% gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked and incubated with specific primary
antibodies against caspase-3, cleaved caspase-3, CDK1, CDK6, PTP4A2, and GAPDH at 4 °C overnight and reacted with horse-radish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Subsequently, the results were visualized using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and quantified using ImageJ software. The GAPDH protein was an internal control.

Reactive oxygen species assay

The contents of intracellular reactive oxygen species (ROS) in individual groups of cells were determined by flow cytometry using DCFH-DA on a BD Accuri C6 flow cytometer (BD Biosciences, San Diego, CA, USA). Briefly, H1299 cells were cultured into 12 well plates overnight and treated in triplicate with various concentrations of SNH (0, 25, 50, 75, and 100 µM) for 24 h. 500 µL of DCFH-DA was added to each well at a 1:1,000 dilution and incubated for another 20 m at 37 °C in the dark. Finally, the cells were analyzed by flow cytometry at 488 nm. The results were analyzed using FlowJo 10 software (Tree Star Inc., Ashland, USA).

Statistical analysis

Each experiment was performed ≥3 times. All data were analyzed using GraphPad Prism 6.0 and were presented as the means ± standard error of mean (SEM). The difference between groups was analyzed using a two-tailed unpaired Student’s t-test and deemed significant with a p-value of <0.05.

Results and discussion

SNH reduces H1299 cell proliferation

To determine the effect of SNH on the proliferation of lung cancer cells in vitro, H1299 and A549 were treated with various concentrations of SNH (0, 25, 50, 75, 100, and 150 µM) for 24 or 72 h and their proliferation was measured for WST-1 based cell viability. Compared with the control group, the viability of H1299 cells decreased from 75% to 36% after treatment with 50, 75, 100, and 150 µM SNH for 24 h (Fig. 1b), and the IC50 of SNH for H1299 cells was 75 µM. In addition, the treatment of H1299 with SNH for 72 h further reduced their viability (Fig. 1c). A similar pattern of SNH decreasing the viability of A549 cells was observed (Fig. 1d). Of note, SNH was less cytotoxic to HBE cells (Fig. 1e), with an IC50 = 183 µM for 24 h treatment. These results revealed that SNH had selective toxicity against lung cancer cells and their cytotoxic effects were dose-dependent.

SNH induces the apoptosis of H1299 cells

To determine whether SNH could induce the apoptosis of lung cancer cells, H1299 cells were treated with SNH (0, 50, 75, or 100 µM) for 24 h. As shown in Figure 2a, SNH selectively triggered lung cancer cell apoptosis. Treatment with some doses of SNH for 24 h increased the frequency of apoptotic H1299 cells in a dose-dependent manner (14.28 ± 2.20% for 50 µM, 23.09 ± 5.31% for 75 µM, and 42.07 ± 9.92% for 100 µM, Fig. 2b). In addition, SNH
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significantly increased the expression levels of apoptotic marker cleaved caspase-3 (Fig. 2c). In combination, these results indicated that SNH induced the apoptosis of H1299 cells in a dose-dependent manner.

Proteomics revealed proteins that are regulated by SNH

To further investigate the potential antitumor mechanisms of SNH in lung cancer cells, and iTRAQ-based quantitative proteomics strategy was used to analyze the protein profiles between the control and the SNH-treated H1299 cells. The control and SNH-treated samples from three biological replicates were labeled with 114, 115, 116, and 117, 118, 119, respectively. The labeled peptides were mixed and separated into 12 fractions by high pH reverse-phase ultra performance liquid chromatography (UPLC) coupled with an Ultremex SCX column, followed by identified using an AB SCIEX Triple-TOF 5600 mass spectrometer (AB SCIEX, Framingham, CA, USA).

Using stringent criteria, 5,791 proteins were identified. Based on the criteria of fold changes >1.50 or <0.67 and \( p < 0.05 \), and peptides >1, there were 253 DEPs, 99 upregulated, and 154 downregulated DEPs in the SNH-treated cells (Fig. 3a, b). These DEPs are listed in Table S1.

Fig. 3. Proteomic changes in H1299 cells treated with SNH and pathway analysis: (a) Volcano plots of the total protein expression profiles of the SNH-treated group compared to the control group. Each dot represents the mean expression of individual proteins obtained from three independent biological experiments. The red and green dots were considered DEPs according to the following parameters: fold change \( > \mid 1.5 \mid \) and \( p < 0.05 \); (b) numbers of DEPs, which included the upregulated proteins and downregulated proteins, in response to SNH treatment. Red dots/bars indicate the upregulated proteins in response to SNH treatment, green dots/bars indicate the downregulated proteins in response to SNH treatment, and (c) top six canonical pathways involved in the effect of SNH on H1299 cells were determined using IPA.

The IPA revealed that these DEPs were involved in the top six canonical pathways, in particular in the cell cycle control of chromosomal replication, mismatch repair in eukaryotes, pyrimidine deoxyribonucleotides de novo biosynthesis, hereditary breast cancer signaling, mitotic roles of polo-like kinase, and Nrf2-mediated oxidative stress response (Fig. 3c).

Of note, the mitotic roles of the polo-like kinase pathway had a negative z-score and the Nrf2-mediated oxidative stress response pathway had a positive z-score (Fig. 3c). These data suggest that SNH might inhibit the mitotic roles of the polo-like kinase pathway and enhance the Nrf2-mediated oxidative stress response pathway in H1299 cells. A further network analysis exhibited that...
27 DEPs participated in the cell cycle, DNA replication, recombination, and repair functions (Fig. 4a), and 21 DEPs were related to post-translational modification, protein degradation, and molecular transport (Fig. 4b).

SNH reduces the expression of cell cycle-related proteins

As shown in Figure 4a, many downregulated proteins were involved in the regulation of the cell cycle, which included CDK1, DNA replication licensing factors MCM4 and MCM7, DNA polymerase alpha catalytic subunit (POLA1), PTP4A2, and ribonucleoside-diphosphate reductase subunit M2 (RRM2). These suggested that SNH may induce cell cycle arrest, consistent with our findings. Specifically, CDK1 is required for cell cycling and proliferation, regulating G1 progression, and G1-S and G2-M transition; therefore, it could be a novel target for the exploitation of anticancer drugs. SNH treatment significantly decreased CDK1 expression in H1299 cells. The downregulated CDK1 expression by SNH treatment could limit cell proliferation and G1 phase progression in lung cancer cells. Therefore, these findings might provide new pharmacological mechanisms that underlie the antitumor action of SNH.

Human PTP4A2 protein stimulates cell cycle progression from G1 to S phase during mitosis. Many studies have reported that PTP4A2 expression is upregulated in breast and lung cancer. SNH significantly decreased PTP4A2 expression in H1299 cells (Fig. 5a, b, Table S1), which agreed with the observations of SNH-induced G0/G1 arrest in H1299 cells (Fig. 5c, d).

In addition, SNH treatment significantly reduced the expression of CDK6, which participates in the G1-S progression, in H1299 cells (Fig. 5, Table S1). CDK6 expression is upregulated in many types of cancer cells, and the blockade of CDK6 expression by microRNAs inhibits the proliferation of various cancer cells, including lung cancer cells. In addition, a phase I/I clinical drug (PD-0332991) inhibited CDK4 and 6 to arrest cells in the G1 phase, decreased proliferation and caused apoptosis in cancer cells. SNH treatment significantly decreased CDK6 expression, induced cell cycle arrest in G0/G1 phases, which limited the proliferation of lung cancer cells. This suggests that SNH might target several molecules simultaneously to inhibit the growth of lung cancer.

SNH enhances the expression of Nrf2-mediated oxidative stress response-related proteins

Research suggests that ROS function as signaling molecules to activate multiple intracellular signaling pathways that regulate cell proliferation, differentiation, and survival. However, excess ROS are toxic to cancer cells and trigger their apoptosis and cell cycle arrest. Therefore, the induction of excess ROS production in cancer cells is a promising antitumor therapeutic strategy. Nrf2 is a key redox-sensitive transcription factor and its expression is upregulated by oxidative stress that increases ROS production. The Nrf2 signaling is crucial for the growth and survival of cancer cells.

In this study, SNH upregulated the expression of a set of Nrf2-mediated oxidative stress response-related proteins, including heme oxygenase 1 (HMOX1) and sequestosome-1 (SQSTM1/p62) (the downstream targets of Nrf2) (Fig. 4, Table S1). In addition, it has reported that an ethanolic extract of fermented H. cordata elicits the apoptosis of human leukemia cells in vitro via oxidative stress and a mitochondrial pathway. An H. cordata Thunb extract has been reported to increase ROS production and decrease the mitochondrial membrane potential in human primary colorectal cancer cells. In combination, SNH might stimulate intracellular ROS accumulation in lung cancer cells. In addition, SNH treatment significantly increased the levels of intracellular ROS in H1299 cells in a dose-dependent manner, compared with the control group (Fig. 6). These data indicated that the antitumor activity of SNH against H1299 cells was related to the induction of ROS production. Therefore, SNH treatment might induce oxidative stress and upregulate the expression of Nrf2-related proteins, which further inhibited cancer cell proliferation and induced apoptosis.

Fig. 4. Network analysis of the SNH-regulated proteins: (a) SNH-regulated functional network was associated with the cell cycle, DNA replication, recombination and repair; and (b) SNH-regulated functional network was associated with post-translational modification, protein degradation and molecular transport. Red indicates the upregulated proteins; green indicates the downregulated proteins.
expression of Nrf2-mediated oxidative stress response-related proteins to promote ROS accumulation, which induces oxidative DNA damage, impairs DNA replication, and G0 and G1 cell arrest and apoptosis.

**Future directions**

SNH exerted its antitumor effect by inhibiting the expression of cell cycle-related proteins and simultaneously enhanced the expression of Nrf2-mediated oxidative stress response-related proteins in vitro. These data indicated that SNH might act as CDK inhibitors for the treatment of NSCLC. Further research is required to confirm the antitumor effect of SNH on NSCLC in vivo mouse model and clinical trials. *In vitro* and *in vivo* experiments into the anticancer activity of SNH against other cancers, including colorectal, leukemic, liver, and esophageal cancer are required.

**Conclusions**

In this study, the data indicated that SNH, which is a bioactive component derived from nutraceutical *H. cordata* Thunb, had potent antitumor activity against lung cancer. Proteomics combined with other molecular biological analyses revealed that SNH downregulated the expression of cell cycle-related proteins and...
simultaneously enhanced the expression of Nrf2-mediated oxidative stress response-related proteins. Both factors contributed to SNH-induced G0/G1 arrest and apoptosis, which inhibited tumor growth. These findings might provide the potential molecular mechanisms that underlie the antitumor activity of SNH in vitro; however, these effects must be explored further in an in vivo mouse model to evaluate its efficacy and to determine if *H. cordata* Thunb is a nutraceutical drug that could prevent and treat NSCLC.

**Supporting information**

Supplementary material for this article is available at https://doi.org/10.14218/JERP.2021.00007.

**Table S1.** The DEPs identified by iTRAQ-based proteomics.

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**Conflict of interest**

The authors declare no conflicts of interest.

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**Fig. 6.** SNH significantly increases the intracellular ROS level: (a) SNH significantly increased the intracellular ROS level in H1299 cells in a dose-dependent manner. Cells were treated with different concentrations of SNH (0, 50, 75, or 100 µM) for 24 h, and the ROS level was detected by measuring the fluorescence intensity of DCFH-DA using a flow cytometer; and (b) quantity of ROS levels in H1299 cells after SNH treatment. The experiments were performed in triplicate, and the data represent the means ± SEM, *p*<0.05, **p**<0.01, and ***p***<0.001. The red, blue, orange and green bars represent the control group, 50, 75, and 100 µM SNH treatment group, respectively.

**Author contributions**

Study design and performance of experiments (YXY, WGB), analysis and interpretation of data (LYJ, LWT), manuscript writing (YXY), critical revision and critical funding (YXY, HQY), administration, technical or material support, study supervision (HQY).

**Data sharing statement**

No additional data are available.

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