Quantitative MALDI-MSI combined with LC-MS/MS metabolomics analysis to study the inhibition of solasonine in lung cancer

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Abstract

Background and purpose: The Chinese medicine monomer solasonine has been shown to be an effective inhibitor of Lung adenocarcinoma in vitro and in vivo. The research on the application of solasonine in lung cancer mostly involves the cell level, the lack of information on the spatial distribution of drugs and related metabolic pathways are common problems faced by many Chinese medicine monomers. Experimental Approach: LC-MS/MS metabolomics analysis was performed to reveal the underlying regulatory mechanism, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) and 3D computational reconstruction were applied to illustrated the spatial-temporal distribution of solasonine. Solamargine was chosen as the internal standard to correct the calibration curve due to the similarity in structure. Key Results: Metabolomics analysis illustrated that solasonine promotes A549 cells ferroptosis via GPX4-induced destruction of the glutathione redox system. Detailed distribution features of solasonine in different organs were revealed by MALDI-MSI after intravenous administration in the mice. The heterogeneity of solasonine distribution and penetration in tumor demonstrated that significant drug deposits around the necrotic area. Conclusion and Implication: The anti-tumor mechanism of solasonine associated with ferroptosis is identified for the first time. It provides an additional basis for the previous conclusion that solasonine promotes tumor necrosis. Quantitative spatial-temporal information obtained here can improve our understanding of pharmacokinetics (PK), pharmacodynamics (PD), potential transient toxicities of solasonine in organs, and possibly direct further optimization of drug properties to reduce drug-induced organ toxicity and broaden the scope of application.

Abbreviations

MALDI-MSI: matrix-assisted laser desorption/ionization mass spectrometry imaging;

LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry;

PK: pharmacokinetics; PD: pharmacodynamics; DHB: 2,5-dihydroxybenzoic acid;

i.v: intravenous injection; i.p: intraperitoneal injection;

IS: internal standard; ITO: indium tin oxide; ROI: region of interest;

LLOQ: lower limit of quantification; TCC: time-concentration curve;

Tmax: time of maximum concentration; 3D: three-dimension;

HCC: Hepatocellular carcinoma;

GSS: Glutathione Synthetase; GPX4: Glutathione Peroxidase 4;

Key words : solasonine, lung cancer, MALDI-MSI, metabolomics, ferroptosis

Introduction

According to the American cancer society's "global cancer statistics 2018" report, there were nearly 2.1 million new cases of lung cancer, with 1.76 million deaths. The new incidence and mortality rates were 11.6% and 18.4%, respectively (Bray et al., 2018). Both the incidence and mortality of lung cancer ranked the first among all kinds of cancers in the world.

At present, the combined application of multiple methods and the development of gene-based precision medicine have greatly improved the efficacy and prognosis of tumors. However, the efficacy and prognosis of lung cancer are not ideal, due to the shortage of early diagnosis, high metastasis and recurrence, and insensitivity to treatment.

In recent years, the development and application of a variety of Chinese medicine monomers show the merits. Solasonine is a potential candidate for the treatment of malignant tumors (Ding et al., 2013). Studies have shown that solasonine can inhibit the growth of a variety of tumor cells, including human lung cancer cells (Shih et al., 2007; Zhang et al., 2016), human glioma cells (Wang et al., 2017), hepatocellular carcinoma cells, colon cancer cells, cervical adenocarcinoma cells, breast cancer cells (Munari et al., 2014; Lee et al., 2004). Solasonine may target anti-inflammatory signaling pathway, and more specifically p-p38 and p-JNK MAPKs (Wang et al., 2017). Solasonine can change the transcriptional activity of p53 gene and its encoded protein, and increase cell membrane permeability to promote apoptosis (Yang et al., 2006). The majority of previous studies focusing on inhibitory effect of solasonine on tumors was limited to the in vitro experimental stage, while the in vivo research studies are insufficient.

Metabolomics is the study of the totality of endogenous metabolites in living organisms and their changing rules. It can reflect the pathophysiological state of the organism more directly and accurately. Combined with the biological information analysis method, it provides a large amount of potential information and is widely applied in many research fields. To reveal the underlying regulatory mechanism, metabolomics analysis was performed in this study.

Accurately obtaining the specific distribution, permeability and deposition characteristics of drug in target area is very important for understanding the PK and PD of the drug (Tang et al., 2019).The limited knowledge on the PK profile has focused mainly on the measurements of compounds from the blood, urine and occasionally from tissue homogenates by LC-MS/MS (Sharma et al., 2015), the spatial distribution of the drug within a sample is lost (Jove et al., 2019).

MALDI-MSI has emerged as a key technology for unlabeled bioanalysis of the spatial and quantitative distribution of biomolecules, pharmaceuticals and other xenobiotics in tissue sections with high spatial resolution (Schulz et al., 2019; Ryu et al., 2018). MSI is ideally suited to provide insights into the spatial distribution of a small molecule drug in target tissues (Pirman et al., 2013; Rzagalinski et al., 2017; Giordano et al., 2016; Torok et al., 2017).

Here, we established a method to quantity the concentration of solasonine in multiple tissues. The results visualize the spatial-temporal information of the drug in vivo, explore the correlation between efficacy and drug permeability and distribution, evaluate the clinical characteristics and potential toxic and side effects of the drug. Combined with 3D reconstruction technology, the drug distribution and osmotic differences in tumors in the long-term treatment group provides the detail information for drug related studies.

Method

In vitro experiment

Cell culture and treatment

The human LAC (lung adenocarcinoma) cell lines A549 and PC9 were purchased from the Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). All cells were cultivated in DMEM medium

with 10% FBS (both from Gibco-BRL, Gaithersburg, MD, USA) in a humidified incubator at 37 containing 5% CO_2.

Cell proliferation assay

Human LAC cell lines (cell density, $5*10^3$ cells per well for all) were seeded into 96-well plates followed by treatment with various concentrations of solasonine (0, 5, 10, 15,20 and 40 μg*mL^{-1}) for 24, 48, or 72 h. Then 10 μL CCK-8 solution was added to incubate the cells at 37°C for 1 h. The absorbance was detected at an OD of 450 nm using a microplate reader (Bio-Tek, Winooski, VT, USA). Cell growth inhibitive rates were calculated using the following formula:100%* (OD control-OD experiment)/(OD control-OD blank).

Cell cycle and apoptosis assay

The cells (5 $*10^4$ cells per well for all) were seeded in the plates with 6 wells containing 2 mL of complete culture media overnight and then synchronized for different concentrations of solasonine (0, 15 and 20 μg*mL^{-1}) for 24 h. We labeled cells with propidium iodide (PI)/RNase Staining Buffer for cell cycle analysis in accordance with a standard procedure. We determined DNA content using the Cytomics FC 500 Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA) and analyzed data through Modfit software (Verity Software House, Inc., Topsham, ME, USA).

Annexin V/PI staining was performed to assess cell apoptosis rate. Cells were seeded in 6-well plates for exposure to solasonine (0, 15, or 20 μg*mL^{-1}) for 24 h, then collected after trypsinization and washed twice with cold PBS. Cells were resuspended in 500 μL binding buffer and finally stained with 5 μL annexin V-FITC and 5 μL PI at room temperature for 15 min in the dark. The apoptotic rate was determined by above-mentioned flow cytometry.

Transwell assay

Cell invasion ability was examined by Transwell membrane filter inserts (8-μm pore size; Costar, Corning, NY, USA) in 24-well dishes. Cells ($1*10^4$) suspended in 200 μL serum-free medium with solasonine were seeded into the upper chambers; 500 μL complete medium was added to the lower chamber for exposure to solasonine (0, 15, or 20 μg*mL^{-1}) for 24 h. Invaded cells were fixed in 4% paraformaldehyde and stained with 0.05% crystal violet for observation under an inverted microscope (Bio-Tek). The stained cells in five randomLy selected fields were counted and photographed.

Scratch wound assay

All cells were seeded into 6-well plates as confluent monolayers and then scratched by a 200 μL pipette tip. The cells were then washed twice with PBS to remove detached cells and underwent incubation with various doses of solasonine for 24 h. Wound images were acquired through an inverted microscope.

Animal experiments

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; Mc Grath and Lilley, 2015).All animal studies complied with the NIH guide for the care and use of laboratory animals and approved by the Animal Ethics and Research Committee of Shanghai Traditional Chinese Medicine (Shanghai, China).

BALB/c nude mice (4-6 weeks old, male, 14–16 g body weight) were from Shanghai JiKai Laboratory Animal Technology (Shanghai, China). A549 cells ($8*10^5$) were suspended in 100 μL PBS and injected s.c. into the right flank of all mice. Mice were randomly assigned to three groups (PBS, 20 or 30 mg*kg^{-1}solasonine) with eight animals in each group. When the tumors reached a volume of approximately 180 mm^3, each group received i.p of PBS or solasonine once a day for 2 weeks. The mean tumor volumes were measured using the formula: volume = (length * $width^2$)*2^{-1}. All mice were killed and tumors were excised and weighed on the last day. Tumors were stored at -80 °C for MALDI-MSI 3D reconstruction and further research.

After 6 h of fasting, tumor-bearing mice were administered i.v at a single dose of 20 mg*kg^{-1} solasonine. Lung, liver, kidney, spleen, and tumor samples were collected at 0, 5, 10, 20, 30, 45 and 60 min, each time

point with six animals. The organs were surgically dissected, washed with ice-cold saline, snap frozen in liquid nitrogen and stored at -80 ℃ until use.

Preparation of samples for metabolomics analysis

A549 cells treated with or without solasonine were used for metabolomics analysis. We seeded cells into the plates with 6 wells at an initial density of $1*10^6$ each well. After an appropriate culture period, the supernatant was discarded and each well was washed three times with PBS. Then, 2 mL of cold (4 ℃) methanol were added to each well and the adhered cells were scraped free and lysed with a cell pulverizer to fully extract the metabolites. Finally, we centrifuged the supernatant at 14,000 × g for 10 min before LC-MS/MS analysis. We processed all resulting data via Compound Discoverer 2.1 software (Thermo Fisher Scientific). We conducted orthogonal partial least squares discriminant analysis (OPLS-DA) and principal component analysis using SMICA-P 14.0 software (MKS Umetrics AB, Umeå, Sweden). Metabolite identification was based on product ion spectra and accurate mass. We performed pathway analysis through MetaboAnalyst 4.0 metabolomics software.

Extraction and derivation of cell samples

The logarithmic growth phase A549 cells were inoculated into 6-well plates, $5*10^5$ per well, and treated with or without solasonine. After 24 h culture, discard the supernatant and place the plate on ice; Wash each well with 4 mL PBS, then add 2 mL 4 ℃ methanol to stop the metabolism and scrape the cells; Lyse the fully cells with a cell pulverizer for metabolite extraction, and place the extraction in an ice bath for 20 min; Transfer the supernatant to an EP tube after centrifugation at 14000 g for 10 min, then 10 μL Fenclonine (2.9 $g*L^{-1}$, internal standard) was added. Frozen dry the supernatant with nitrogen and store it in -80 ℃; Reconstitute it with 100 μL of methanol before analysis.

Preparation method of samples

400 μL methanol and 5 μL Fenclonine (2.9 $g*L^{-1}$, internal standard) were added into 100 μL sample and then mixed thoroughly. Sample were centrifuged at 12000 $r*min^{-1}$at 4 for 15 min. 200 μL supernatant was transferred into vials for analysis.

UPLC-Q-Orbitrap-MS analysis

Chromatography Condition

Column: ACQUITY UPLC (HSS T3, 100 mm×2.1 mm, 1.8 μm); The mobile phase consisted of mixture of water with 0.1% formic acid (A) and acetonitrile (B). The gradient elution conditions at a flow rate of 0.3 $mL*min^{-1}$ for 5 min were as follows: 0-2 min, 95%A; 2-12 min, 5%A; 12-15 min, 5%A; 15-17 min, 95%A.

Mass Condition

Positive mode: (1) heater temperature: 300 ℃; (2) sheath gas flow: 45 psi; (3) auxiliary gas flow: 5 $L*min^{-1}$; (4) tail gas flow: 0.3 $L*min^{-1}$; (5) electrospray voltage: 3.0kV; (6) capillary temperature: 350 ; (7) S-LensF Level, 30%. Negative mode: (1) heater temperature 300 ; (2) sheath gas flow: 45 psi; (3) auxiliary gas flow: 5 $L*min^{-1}$; (4) tail gas flow: 0.3 $L*min^{-1}$; (5) electrospray voltage: 3.2 kV; (6) capillary temperature: 350 ; (7) S-LensF Level, 60%.

Metabolic data analysis

The LC/MS data were extracted and pre-processed by SIEVE software (Thermo Company). Then data were normalized and edited in Excel 2013. Finally, the data were arranged into two-dimensional data matrix. The principal component analysis (PCA) was performed on both serum and cell samples of mice in each group by SMICA-P software. Then the samples were analyzed by orthogonal partial least squares discriminant analysis (OPLS-DA).

Metabolite identification and pathway analysis

Discriminant metabolic features were identified based on their accurate masses and/or product ion spectra in negative and positive mode. HMDB, KEGG, mzCloud were searched to assist metabolite identification. The metabolomics pathway analysis was performed by using MetaboAnalyst 4.0.

Total RNA extraction and RT-QPCR

Total RNA was extracted from A549 cells with TRIzol reagent (Invitrogen Corporation) following standard procedures. We measured concentration and purity of the RNA samples at an absorbance of 230, 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). For this analysis, OD260/280 and OD260/230 ratios of 1.8-2.1 and > 1.8 were considered acceptable.

We reverse transcribed RNA (1μg) into complementary DNA through SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). We performed RT-qPCR via an AB 7300 Real-Time System (Applied Biosystems, Foster City, CA, USA) with primer pairs specific for glutathione peroxidase 4 (GPX4), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GenePharma Co., Ltd., Shanghai, China), and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). We utilized GAPDH as a reference gene. We quantified gene expression using the 2-ΔΔCt method. The primers used to assay GSS expression were (forward) 5'-GTA CTC ACT GGA TGT GGG TGA AGA-3' and (reverse) 5'-CGG CTC GAT CTT GTC CAT CAG-3', GPX4 were (forward) 5'-AGT GCC ATC AAG TGG AAC TTC AC-3' and (reverse) 5'-TTC AAG GCA GGC CGT CAT-3', and those for GAPDH were (forward) 5'-GCA CCG TCA AGG CTG AGA AC-3' and (reverse) 5'-GGA TCT CGC TCC TGG AAG ATG-3'.

ROS content measurements

Frozen sections were rewarmed, circles were drawn with pap pen. The sections

were incubated with diluted DHE stain (Sigma-Aldrich) for 30 minutes at 37 in the dark. The slides were placed in PBS and washed on the decolorization shaker for 3 times, 5 min each. After slicing and drying, DAPI dye solution (Shanghai Biyuntian Bio-Technology Co., Ltd.) was added to dye the core at room temperature for 10 min. Repeat the above decolorization steps, shake the section dry and seal the slice with anti-fluorescence sealing tablet (Shanghai Biyuntian Bio-Technology Co., Ltd.).

To detect the extent of oxidative stress to A549 cells, the production of reactive oxygen species (ROS) was assessed via an ROS Assay Kit (Shanghai Biyuntian Bio-Technology Co., Ltd., Shanghai, China). After treatment with 15 μg*mL^{-1} of solasonine for 24 h, A549 cells were incubated with a fluorescent probe (dichlorofluorescein diacetate; Shanghai Biyuntian Bio-Technology Co., Ltd.) for one hour under the room temperature. Fluorescence was assessed under a FluoView FV1000 Laser Scanning Confocal Microscope (Olympus Corporation, Tokyo, Japan) equipped with a digital camera.

Sample preparation for MALDI MS imaging

Multiple 10 μm thick serial sections were obtained at -20 °C using a cryomicrotome (Leica, Germany). Each sample was serially slice , three slices at a time. One for H&E staining, another for MALDI-MSI, and the last for future use. Sections thaw-mounted onto indium tin oxide (ITO)-coated glass slides (Bruker-Daltonics). Prior to matrix coating, the tissue sections were placed in a vacuum desiccator to dehydrate for 15 min at room temperature. HTX Imaging Sprayer assisted matrix coating setup was used to homogenously deposit matrix onto tissue sections. Briefly, 40 mg*mL^{-1} DHB matrix solution containing 100 ng *mL^{-1} IS (internal standard, solamargine) was deposited onto tissue sections at a flow rate of 0.075 mL*min^{-1}. The automatic sprayer parameters for matrix coating were as follows: 50% Methanol as solvent; sheath gas flow, 10 Psi; heated temperature, 85 ^{o}C; drying time between each cycle, 10 s; total number of cycles, 8.

MALDI MS imaging

MALDI-MSI was performed using an Autoflex Speed MALDI-TOF (Bruker Daltonics, USA) with a frequency tripled Nd:YAG solid-state laser ($\lambda = 355$nm). The laser was set to the 'Ultra' footprint setting at an ~200 μm diameter. Mass spectrometer calibration was performed using DHB matrix ions and a Peptide Calibration Standard Kit II (Bruker Daltonics). Tissue sections were analyzed in positive reflection ion mode with 200

laser shots fired at 1000 Hz and imaged with a 200 μm laser step size. MSI data was analyzed using flex Analysis 3.4 and flex Imaging 4.1 (Bruker Daltonics). Data were analyzed using Data Analysis 4.0 (Bruker Daltonics). For 3D MALDI reconstruction of tumor, 16 series sections, in 200 μm steps throughout the half tissue volume, were subjected to MALDI-MSI analysis. The data for all 16 sections were imported into the software SCi LS Lab version 2017b (SCi LS, Bremen, Germany) to reconstruct the original relations between the sections.

Quantitative of MALDI-MSI data

The data for standard curve generation and quantitation were imported into the software SCi LS Lab version 2017b. The m/z intensity data for each region of interest (ROI) was labelled of different concentration of solasonine. The data for quantitative imaging was normalized to the signal from the IS via pixel by pixel.

Hematoxylin and eosin (H&E) staining

After Multiple tissues (tumor, lung, liver, kidney, spleen)were rinsed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and subsequently, H&E staining was performed at room temperature (hematoxylin, 7 min; eosin, 5 min) to observe the pathological changes of diverse tissues and the images were observed using a digital pathological scanner (Precice 500; UNIC Technologies, Inc.) at x200 magnification.

Statistical analyses

We assessed significance of differences between two groups through the unpaired or paired two-tailed t-test. We utilized Pearson's correlation coefficient to identify correlations between two groups. The data are denoted by the mean ± standard deviation (SD). A probability (p) value of < 0.05 was regarded as statistically significant. We performed statistical analyses through GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Material

Solasonine (purity[?]98%) and Solamargine (purity[?]98%) were obtained from Yuanye Biotech (Shanghai, China), dissolved in 100% DMSO, and stored at -20degC. HPLC-grade methanol was purchased from Merck (LiChrosolv(r) Reag. Ph, Europe). Deionized water was prepared by a Milli-Q water purification system (Millipore, Billerica, MA, USA). The MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was obtained from Bruker. H&E staining was purchased from Shanghai Biyuntian Bio-Technology Co., Ltd.

The cell counting kit-8 (CCK-8) and the apoptosis detection kit were purchased from Dojindo and BD Biosciences (San Diego, CA, USA), respectively. The cell cycle detection kit was from Absin Bioscience Co., Ltd. TRIzol reagent and Power SYBR Green PCR Master Mix were from Life Technologies (Grand Island, NY, USA). The tissue RNA kit was from Biomiga. The Prime Script RT reagent kit with gDNA Eraser was from TaKaRa (Dalian, China).

Results

Solasonine treatment inhibited the LACs growth both in vitro and in vivo

Solasonine prohibits proliferation of LAC cells.

The CCK-8 assay was carried out with the purpose of investigating the effects of solasonine on LAC cells (A549 and PC9) growth in vitro. Solasonine dramatically inhibited the proliferative capability of two types of LAC cells dose- and time-dependently (Fig. 1a-b).

According to the results, we chose 24 h as the optimal treatment time for the following studies as the IC50 values of A549 and PC9 were 14.468 $\mu g*mL^{-1}$ and 16.496 $\mu g*mL^{-1}$respectively.

Solasonine inhibits LAC cells invasion and migration

Transwell assay was used to verify whether solasonine inhibits LAC cells invasive ability. As shown in (Fig. 2a-d), the number of cells that invaded the lower chamber was clearly reduced in response to solasonine for 24

h. The scratch wound assay also showed that solasonine-treated cells migrated into the wound region slower than cells in the control group (Fig. 2e-h). Suppression of LAC cells invasion and migration by solasonine both showed a dose-dependent trend.

Solasonine induces apoptosis &G2/M-phase cell cycle arrest in LAC cells

To evaluate the effects of solasonine treatment on LAC cells apoptosis, flow cytometric experiments were carried out. Compared with the control group, the apoptosis rate of LAC cells in solasonine-treated groups (15 or 20 $\mu g*mL^{-1}$ for 24 h) was significantly improved (Fig. 3a-c).

FACS analyses with PI staining was applied to further assess the influence of different concentrations of solasonine on the cell cycle. The results showed that the percentage of two types cells in G2/M phase increased in response to solasonine treatment for 24h, suggesting the ability of solasonine to induce G2/M-phase cell cycle arrest in LAC cells dose-dependently (Fig. 3d-e).

Solasonine suppresses xenograft tumor growth

We established a xenograft model with A549 cells in order to further research the influence of solasonine on tumor development in vivo. Control group and administration group were set according to the LD50 value of solasonine in previous studies (Zhong et al., 2018). Tumors in both treated groups developed slower and their final volumes were conspicuously lower compared to the control group (Fig. 4). There was no statistical difference in tumor volume between the 20 $mg*(kg*d)^{-1}$ and 30 $mg*(kg*d)^{-1}$ groups. The P value was greater than 0.05 (P value: 0.1637).

Metabolomics study in vitro

Quality control (QC) samples were determined for instrument precision. The QCs was clustered together on the PCA score plots and separated from the other group.

The PCA was performed with L-02 induced by APAP in both positive ionization (ESI+) and negative ionization acquisition (ESI-). In both modes, samples were separated completely in PCA. The cumulative values of R2X and Q2Y were over 0.876, which indicating a high predictive degree.

Differential metabolites were found by OPLS-DA and variable importance in projection (VIP>1) and T-test (P<0.05) was used for screening.

MetaboAnalyst software was used to elucidate the pathways underlying alterations of metabolic products (Fig. 5a). The levels of hippuric acid, threonine, pyroglutamic acid, alanine, and glutamic acid in the intervention group were significantly increased compared with the control group, while the contents of glutathione and cysteine glycine were significantly reduced compared to the control group. At the metabolomics level, many pathways in the solasonine-treated group have changed significantly, including glutathione metabolism, malate-aspartate shuttle, urea cycle, and methyl histidine metabolism, etc. (Fig. 5b).

Compound reaction networks regarding genes and metabolites were visualized using MetScape software (http://metscape.ncibi.org/). The results showed that glutathione metabolism was significantly dysregulated after solasonine treatment.

Solasonine treatment contributed to ferroptosis of A549 cells

Studies have shown that intracellular cysteine reduction, glutathione (GSH) depletion, and related lipid peroxidation are all closely related to glutathione-dependent enzymes (Glutathione Peroxidase 4, GPX4). The GPX4-GSH-cysteine axis mediated by GPX4 is an important node of the ferroptosis cascade (Mou et al., 2019; Lu et al., 2018; Shen et al.,2018).

The study results verified that GSS and GPX4 expression levels of A549 cells decreased after exposure to solasonine for 24 h (Fig. 5c). Immunofluorescence detection revealed that lipid ROS levels increased of tumor tissues and A549 cells were both increased after exposing to solasonine (Fig. 5d-e), which function importantly in triggering ferroptosis.

We draw an oxidative stress network which mainly related to glutathione metabolism (Fig. 5f). Inside the box is the intracellular environment, outside the box is the extracellular environment. Intracellular: Two-way transformation of GSSG and GSH. The key enzyme GPX4 promotes GSH synthesis. Decreased levels of GPX4 cause intracellular ROS accumulation and GSH consumption. Cysteine and L-glutamate are raw materials for GSH synthesis. Extracellular: GSH precursor pyroglutamate, citric acid cycle related products L-alanine and L-aspartate participate in the regulation of intracellular oxidative stress. The results showed that glutathione metabolism was significantly dysregulated after solasonine treatment.

Optimization of parameters for quantitative MALDI-MSI

To obtain high quality MALDI-MSI data. DHB was chosen as the matrix for MALDI-MSI experiment. Based on previous studies (Tang et al., 2019), the internal standard (IS) was incorporated in the matrix solution to compensate signal variation resulting from the complex surface properties and varied matrix-analyte interaction (Pirman et al., 2011; Chumbley et al., 2016). Solamargine was considered as an IS candidate due to their similarity in structures. As shown in Fig. S1, protonated ions of solasonine and solamargine are the major adducted ions detected with DHB. The formula of solamargine is $C_{45}H_{73}NO_{15}$, the protonated ion of solamargine with m/z 868.5±0.06 and the formula of solasonine is $C_{45}H_{73}NO_{16}$, the protonated ion of solasonine with m/z 884.5±0.06 were observed in mass spectrometry. Additionally, considering that the possible interference peaks arose from different tissues may affect the detection signal, solasonine and IS were spotted on five tissues and no interference peaks were observed by comparing with the blank tissues (Fig. S2).

Validation of quantitative MALDI MSI

To further investigate the correction ability of the IS for the accurate quantitative MALDI-MSI, linear regression analysis was performed on standard curves generated with IS-based normalization method. 0.5 μL of standard solutions of solasonine with concentrations ranging from 2.5 to 20 μg *mL^{-1} were spotted on blank mouse tissue sections, and the mixture of DHB and IS was homogeneously sprayed on the tissue sections. The standard curve for solasonine was generated by plotting average ion intensities of mass spectra in defined ROI as a function of the quantities of standard spotted on different tissue sections. As shown in Fig. 6 and Table S1, non-normalized data yielded the lower linear correlation coefficients (R^2=0.972912˜0.981795), whereas the higher correlation coefficients were obtained using the IS-based normalization (R^2=0.985178˜0.995074). Meanwhile, the LLOQ was acquired by analyzing standards spotted on different tissues. The LLOQ ranges from 2.25 μg*$mL^{-1}$to 3.29 μg*mL^{-1}.

Spatial-temporal distribution of solasonine in different tissues

The detailed spatial-temporal information on solasonine distribution in multiple tissues have not been reported previously. In this work, we applied the quantitative method to investigate the spatial-temporal distribution of solasonine in tumor, lung, liver, kidney and spleen. The identity of the drug was verified by in situ mass spectrometry. We conducted two different schedules of solasonine, either 20 mg*kg^{-1} iv. as a single dose, or 20 mg*$(kg*d)^{-1}$ or 30 mg*$(kg*d)^{-1}$ ip. for 14 days. After drug delivery by injection of solasonine, sections were collected for quantitative MALDI-MSI.

Tumor

As mentioned above, solasonine shows prominent inhibitory effects on LAC cells and promotes tumor necrosis by triggering ferroptosis mechanism. Therefore, monitoring its distribution and dynamic changes in tumor tissues were first conducted.

As shown in Fig. 7a-b, the single dose group shows no difference of drug concentration and distribution intra-tumor (iv. 20 mg*kg^{-1}, single-dose). The quantitative results of drug signals inside tumor at different time points were all lower than the LLOQ of the standard curve, so the time-concentration quantitative curve could not be drawn. However, after serial section of the tumor with long-term treatment group (i.p 20 mg*$(kg*d)^{-1}$ or 30 mg*$(kg*d)^{-1}$, 2 weeks), the image showed that there was a significant differences of drug concentration and distribution intra-tumor.

After merging the MALDI-MSI images with H&E staining figures and 3D reconstruction, referring to Fig. 7c-e, the portion colored in yellow, representing the highest drug concentration, it was found that the drugs were deposited locally around necrosis boundary of the tumor, and the concentration was above the LLOQ, the average concentration of the necrosis margin reaching to 0.82-5.28 $\mu g*g^{-1}$. While in a large part of the sample the signal intensity was below our limit of detection (blue-green or uncolored), indicating that the diffusion of solasonine inside tumor is seriously hampered by the heterogeneity of tumor structure. In Fig. 7f, showed that the distribution of solasonine in tumor was associated with proliferating non-necrotic areas.

Lung

In view of the inhibitory effect of solasonine on lung cancer, this study monitored the distribution of solasonine in lung tissue (Fig. 8a). As shown in the figure, after the tail vein injection of drugs (single-dose samples), the time-concentration curve (TCC) of the solasonine in the lung is similar to the plasma drug metabolism feature in previous study (Chen et al., 2015). After intravenous injection, the drug quickly enters the lung tissue from the blood and reaches a peak at the time of about 5 min (Tmax). Subsequently, the concentration of the drug in the tissues and blood decreased rapidly. As shown by the MADLI-MSI heat map, the uptake and clearance of solasonine by lung tissue were not uniform. Compared with H&E staining, the lung tissue divides into three parts, the apex, the central and the base of the lung. The base and the periphery of the lung took more drugs and the clearance rate was slightly slower, in comparison to the other two parts.

Liver

The liver is an important drug metabolic organ (Asha et al., 2010). As shown in the Fig. 8b, the MALDI-MSI heat map and the drug TCC show that the summit of solanine in the liver is about 30 min. Solasonine exhibited no differential distribution in the liver. The retention time and amount of the drug in the liver are significantly higher than those of other monitored organs.

Kidney

The kidney is also an important organ for drug metabolism. MALDI-MSI heat map (Fig. 8c) and drug TCC show that the peak of solasonine in the kidney is about 10 min. After tail vein injection, drug accumulation can be seen in the kidney tissue upon 5 min, and then quickly excreted through the kidney. The renal parenchyma is mainly divided into the outer cortex region and the inner medulla region. As shown in the figure, the drug signal intensity in the medullary region is significantly lower than that in the cortical region.

Spleen

Spleen, as the secondary largest lymphoid organ in the body, is of great importance in red blood cell production and in the immunity system. The spatial-temporal distribution of solasonine in the spleen and H&E staining results are shown in Fig. 8d. The spleen is mainly divided into three main functional regions, the serosa, blood-derived red pulp, and lymphoid white pulp (Pislyagin et al., 2013). MALDI-MSI images suggested that solasonine can penetrate the serosa and accumulate in the red and white pulp regions, and maintain a high concentration until 30 min before showing a rapid removal trend.

Discussion & Conclusion

In this study, experiments prove that solasonine can inhibit the proliferation, invasion, and migration of LACs in vitro, which is consistent with previous researches. Combined with metabolomics analysis and molecular biology results, solasonine can inhibit tumor growth by promoting ferroptosis in lung cancer.

Studies have shown that iron disorders are common in patients with lung cancer and closely associated with the initiation and development of lung cancer (Buck et al., 2009). TFR1 and ferritin expression was significantly increased in NSCLC patients and in 88% of patients and in 62% of patients (Alemán et al., 2002; Yildirim et al., 2007). EGFR mutation frequently occurs in NSCLC, which can regulate iron balance in the body by redistributing TFR1 in vivo. It mainly increases the accumulation of intracellular iron and restrict the transport of iron elements to promote tumor development (Paez et al., 2004; Lynch et al., 2004; Pardinas et al., 2006). EGFR activation is positively correlated with TFR1 expression and iron levels (Wang

et al., 2016). The process of ferroptosis is iron-dependent. The accumulated iron intracellular of lung cancer patients can promote lipid oxidation to produce reactive oxygen species and promote the occurrence of ferroptosis. At the same time, anti-tumor drugs that related ferroptosis may be a viable treatment strategy to reverse drug resistance in cancer treatments (Mou et al., 2019; Lu et al., 2018; Shen et al., 2018).

According to the results of MALDI-MSI, aimed at the different results of two tumor groups, three aspects may contribute to the outcomes: firstly, the poor targeting of solasonine results in the persistent low intra-tumoral concentration in the single injection group. Secondly, due to the influence of solasonine characteristics and local blood perfusion, the water solubility of solasonine is extremely poor, which means that the solubility of drug in vivo after intravenous administration is limited by local blood perfusion. Different from inherent organs, the tumor microenvironment shows an abnormal vasculature that cannot support a homogeneous drug distribution. In addition to the altered extracellular matrix and the lower lymphatic return, the abnormal vasculature leads to the accumulation of extravasated macromolecules, increasing the interstitial fluid pressure that is a further obstacle for drug penetration and elimination (Wang et al., 2016; Carmeliet et al., 2000; Heldin et al., 2004). Finally, the limitations of mouse strain. It has been reported that the sensitivity order of solasonine was divergent with mouse strains (among 4 strains of mouse, BALB/c mice performed the worst) (Zhong et al., 2018). Better results may be obtained by changing the animal models and adjusting the dose within a reasonable range.

The results of MALDI-MSI showed that the intra-tumoral drug in the single administration group had a persistently low concentration and there was no significant difference in distribution; On the contrary, the intra-tumoral drug in the long-term administration group was significant unevenly distributed. The different distribution of drugs inside tumor may lead to the failure to reach and maintain the effective drug concentration and weaken the drug effect in the target area. The heterogeneity of the drug distribution in vivo can explain the animal experimental results, which increase of drug dose from 20 $mg*(kg*d)^{-1}$ to 30 $mg*(kg*d)^{-1}$ failed to give statistically significant difference between two groups.

The difference in drug distribution and clearance rate of lung may be related to the perfusion characteristics, that is, the lung base perfusion is slightly higher. Lung adenocarcinoma occurs initially in periphery of the lung then diffuses and invades the lung lobe. If solasonine is used in the treatment of orthotopic lung adenocarcinoma, the immediate residence time of the original drug in the lung is short, which enhances the role of the drug in the target area. Time and permeability may become the important factors in monitoring the efficacy of the drug.

Primary liver cancer is the sixth most common cancer overall and the second most common cause of cancer mortality worldwide. Hepatocellular carcinoma (HCC) accounts for up to 90% of all primary hepatic malignancies and represents a major international health problem. Most patients are diagnosed with advanced or intermediate stages, and effective treatment options for advanced HCC are limit, so their 5-year survival rate is very low (Park et al., 2015). Pharmacological studies have confirmed (Pham et al., 2019) that solasonine can effectively inhibit the interaction of Hepg2 cells that simultaneously express p53 and mortalin (hsp70 chaperone protein, lethal protein) and activate the apoptotic process. MSI results suggest that the efficacy of solasonine in orthotopic liver lesions may be highly correlated with the high permeability and sustained maintenance of the drug. However, the continuous high concentration of the drug in the organ will increase the risk of liver injury. Studies have shown that solasonine has hepatotoxicity, which can significantly reduce the expression of cyp450 gene during and after transcription (Tang et al.,2019), slow down the metabolism of the drug in liver, enhance the pharmacological activity of the drug and increase the toxic side effects.

According to the metabolomics results, solasonine can be used to inhibit lung tumor growth by triggering ferroptotic, then the inactivation of GPX4 will induce acute renal failure with a complex lipid oxidation signature (Friedmann et al., 2014). MALDI-MSI results suggest that solasonine mostly accumulates in the renal cortex. Therefore, how to reduce or avoid potential kidney damage should be taken into account.

Studies have shown that solasonine, solamargine and its hydrolysate, soladine, have a risk of hemolysis when used alone (Roddick et al., 2001), the MALDI-MSI distribution results show when solasonine is used

alone, attention should be paid to the hemolytic response, and the effect of the drug can also be improved by referring to its spatial-tempol distribution characters. Utilizing the liposome-encapsulated drug release system can reduce the dosage of a drug, improve the targetability and thereby decreasing the risk of hemolytic reactions. This might be a solution for clinical application of solasonine.

Previous studies (Chen et al., 2015), traditional LC-MS / MS methods were used to measure the concentration of solasonine in rat blood by i.v. The results showed that the solasonine distribution showed a rapid trend in absorption and elimination. In this study, only the pulmonary distribution trend and peak time close to result which mentioned above. In this study, we successfully established an internal standard calibration method for the quantitative study of solasonine and acquiring the MALDI-MSI information of solasonine in multiple tissues as a supplement to PK and PD. In addition, the relationship between tumor structural heterogeneity and different drug distribution results providing a basis for the clinical application of solasonine. Finally, it was clarified that the mechanism of solasonine inhibiting lung cancer is closely related to ferroptosis through metabolomics analysis and molecular biological method verification. However, there are some further work can be carried out. For example, the use of an animal model of lung cancer in situ can better show the relationship between drug distribution and tumors.

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Author contributions

C.-Z.S. performed tissue sectioning, LC-MS/MS and MALDI-MSI data acquisition, data analysis and wrote the manuscript. M.-M.J. performed the analysis and interpretation of data and wrote and revised the manuscript. S.-J.L. and X.-Y.L performed the RT-QPCR. J.-R.W performed tissue H&E staining .G.H and X.-Y.H. supervised the study and revised the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Animal Experimentation, and as recommended by funding agencies, and publishers and other organisations engaged with supporting research.

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