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In situ spatial pharmaco-proteomics reveals that andrographolide sulfonate resolves cytokine storm by restoring immune restraint and blocking death execution

Zhen Ma
China Academy of Chinese Medical Sciences
Jinli Hou
Shanxi University
Yanmiao Ma
Shanxi University of Chinese Medicine
Xingyue Xu
First Affiliated Hospital of Guangzhou Medical University
Lei Song
China Academy of Chinese Medical Sciences
Mei Liu
China Academy of Chinese Medical Sciences
Chunhong Jiang
Jiangxi Qingfeng Pharmaceutical Co.,Ltd
Ning Xie
Jiangxi Qingfeng Pharmaceutical Co.,Ltd
Hongjun Yang
China Academy of Chinese Medical Sciences
Xianyu Li
phd_xianyuli@foxmail.com

China Academy of Chinese Medical Sciences

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Abstract

Background: Cytokine storm (CS) is a life-threatening hyperinflammatory syndrome. Although andrographolide sulfonate (Andro-S) is clinically used for respiratory inflammation, its tissue distribution and molecular mechanisms in CS remain insufficiently defined. This study aims to investigate the protective mechanism of Andro-S against LPS-induced CS.

Methods: In a murine model, the efficacy of Andro-S was evaluated. A self-developed 384 In situ Tissue Acquisition Device (384 IsTad) was coupled with MALDI mass spectrometry imaging to visualize Andro-S distribution within pulmonary lesions. An integrated spatial pharmacoproteomic strategy, molecular docking, and cellular thermal shift assays (CETSA) were applied to identify targets and inflammatory pathways, supported by statistical analysis.

Results: Andro-S significantly improved survival, attenuated lung and spleen injuries, and reduced systemic overproduction of TNF-α, IL-1β, and IL-6. Proteomic and target validation identified IFIT2 as a key candidate target, through which Andro-S suppressed TLR4/Caspase/GSDMD-associated pyroptotic signaling. Notably, chemically distinct components of Andro-S exhibited differential target preferences, demonstrating molecular complementarity.

Conclusion: Andro-S alleviates CS by restoring IFIT2-associated immune regulation and limiting Caspase-1/GSDMD-dependent pyroptosis. This study establishes a spatially resolved framework for investigating complex botanical derivatives.

Highlights

1. Developed an *in situ* strategy integrating a novel 384 IsTad device with MALDI-MSI to precisely map the intrapulmonary distribution and molecular signatures of Andro-S during cytokine storms.
2. Revealed distinct target preferences among Andro-S components, establishing a spatially resolved, synergistic model for evaluating complex multi-component botanical formulations.
3. A dual-channel regulatory network that concurrently restores IFIT2-associated immunity and blunts Caspase/GSDMD-driven pyroptosis were unveiled.

1. Introduction

Cytokine storm (CS) is an uncontrolled and self-amplifying inflammatory reaction characterized by excessive production of cytokines and chemokines, dysregulated innate immune activation, and progressive tissue injury [1, 2]. Since its introduction as a term describing hyperinflammatory pathology in severe immune disorders, CS has become a unifying pathogenic concept across life-threatening conditions, including sepsis, severe viral infection, hemophagocytic syndromes, autoinflammatory disease, acute respiratory distress syndrome, and coronavirus disease 2019 [3, 4]. In these settings, the pathological process involves not only quantitative cytokine elevation but also disruption of immune

homeostasis, in which inflammatory amplification, aberrant danger sensing, and maladaptive cell death reinforce one another [5, 6]. Once established, this state can rapidly lead to diffuse tissue damage, endothelial leakage, coagulation abnormalities, organ dysfunction, and death [6, 7].

The lung is among the organs most vulnerable to CS-associated injury. Pulmonary tissue is exposed to circulating inflammatory mediators, pathogen-associated molecular patterns, infiltrating myeloid cells, and tissue-derived danger signals [8–10]. Accordingly, CS can culminate in severe alveolar injury, interstitial edema, hemorrhage, inflammatory infiltration, and respiratory failure. Elevated levels of IL-1 family cytokines, IL-6, TNF-α, IFN-γ, IL-10, and chemokines such as CCL-2 and CCL-3 are recognized features of this syndrome and are often associated with disease severity [4, 11–13]. Additionally, IL-12 P70 plays a role in stimulating natural killer (NK) cells and T cells to produce IFN-γ [11, 14]. Despite these advances, clinical intervention remains challenging. Therapeutic strategies that selectively neutralize a single cytokine can be effective in some contexts, but may be insufficient in severe systemic inflammation, where multiple interconnected inflammatory modules rather than a single dominant linear pathway sustain disease progression.

The complexity of CS suggests that interventions acting on multiple disease-relevant nodes may have therapeutic value. However, the pharmacological understanding of multi-component or multi-target agents often remains limited. In many studies, efficacy is evaluated mainly through serum cytokines or histopathology, whereas tissue delivery, spatial localization in diseased organs, and direct target engagement remain insufficiently resolved [15–20]. This gap is especially relevant in the case of clinically used botanical derivatives and complex formulations. Without evidence of organ localization and tissue-resolved target engagement, mechanistic interpretation of such agents may be reduced to descriptive claims of multiple targets and multiple pathways, which are difficult to evaluate rigorously.

Andrographolide, the principal diterpene lactone derived from Andrographis paniculata, has attracted attention because of its anti-inflammatory, antiviral, antibacterial, anti-fibrotic, immunomodulatory, and metabolic activities [21–24]. Nevertheless, poor water solubility limits its pharmaceutical applicability. Andrographolide sulfonate (Andro-S), generated by sulfonation of andrographolide, improves water solubility and formulation compatibility. In China, injectable Andro-S has been used clinically for more than 30 years in the treatment of upper respiratory tract infection, viral pneumonia, bronchitis, tonsillitis, influenza, and bacillary dysentery. Pharmacological studies have reported that Andro-S reduces excessive cytokine release, ameliorates inflammatory lung injury, modulates host defense functions, and reduces mortality in severe infectious settings [23–25].

Despite these applications, the mechanism by which Andro-S attenuates cytokine storm remains incompletely defined. Most prior work has emphasized downstream inflammatory readouts, whereas less is known about whether Andro-S reaches inflamed target organs in a spatially definable manner, how it alters tissue-level proteomic networks, and which proteins are engaged in vivo or in near-physiological contexts [26–28]. These questions are important for mechanistic interpretation. For a complex formulation, therapeutic efficacy may depend not only on overall exposure but also on whether

active constituents penetrate diseased tissue microenvironments and engage molecular nodes involved in inflammatory escalation and inflammatory cell death.

In the present study, an LPS-triggered murine CS model was combined with a self-developed 384 IsTad, MALDI-MSI, TMT-based quantitative proteomics, molecular docking, CETSA, qRT-PCR, and western blotting to investigate the mode of action of Andro-S. Rather than treating the formulation as a generic multi-target anti-inflammatory drug, the analysis was organized around a defined mechanistic hypothesis. Specifically, the analysis examined whether Andro-S alleviates cytokine storm through two coordinated processes: restoration of IFIT2-associated immune regulation and suppression of TLR4/Caspase/GSDMD-associated pyroptotic signaling. Within this framework, chemically distinct components of Andro-S were not interpreted as evidence of phenotypic synergy. Instead, they were evaluated as potential contributors to broader target coverage through molecular complementarity and differential target preference. By integrating spatial drug localization with tissue-resolved proteomics and target validation, this work sought to develop a mechanistically coherent account of Andro-S in cytokine storm and provide a methodological framework for investigating complex therapeutic formulations in inflammatory disease.

2. Materials and Methods

2.1. Experimental workflow

Figure 1A outlines the experimental workflow used to investigate the molecular mechanisms by which Andro-S alleviates LPS-triggered CS. This workflow was used to identify and validate differentially expressed proteins and mRNA changes in lung and spleen tissues following LPS-triggered CS. To ensure model reliability, LPS-triggered CS was established in mice according to previously reported protocols [29–31]. As shown in Fig. 1A(a), after 24 h of Andro-S treatment in LPS-triggered CS mice, blood, lung, liver, kidney, and spleen samples were collected. Serum cytokine levels were subsequently measured using the Bio-Plex Pro Mouse Chemokine Panel 21. The distribution of Andro-S-derived signals within lung tissue was assessed using matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI), as depicted in Fig. 1A(b). A 384 IsTad was further used to capture core lung tissue regions, and total protein was extracted for proteomic analysis to investigate potential mechanisms of Andro-S treatment in LPS-triggered CS (Fig. 1A(c)). Pathway enrichment analysis, heatmap visualization, and protein–protein association network analysis were then performed. Molecular docking was used to evaluate predicted interactions between Andro-S and candidate target proteins. Cellular thermal shift assay (CETSA), western blotting, cell viability assay, ELISA, and qRT-PCR were used for further validation of candidate targets and related inflammatory responses (Fig. 1A(d)). This integrated workflow enabled systematic assessment of molecular changes associated with Andro-S-mediated alleviation of LPS-induced CS.

2.2. Animal experimental protocol

Andro-S injection was purchased from Qingfeng Pharmaceutical Group Co., Ltd. (Ganzhou, Jiangxi, China). Male BALB/c mice weighing 20–22 g were provided by Beijing HFK Bioscience Co., Ltd. (Beijing, China; Certificate of Quality No. SCXK (Jing) 2019-0008). All mice were housed under controlled environmental conditions, including a temperature of 23–27 ℃, humidity of 50%-70%, and a 12 h light/12 h dark cycle. Mice were fed a standard chow diet ad libitum.

All animal experiments were approved by the Ethics Committee of the Experimental Research Center (Ethics approval number: ERCCACMS20-16-2002). Animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (Council, 2010) and approved by the Animal Care and Use Committee of the China Academy of Chinese Medical Sciences.

Before the main experiment, a dose-optimization study was performed to identify an appropriate LPS dose for inducing CS. LPS doses ranging from 5 to 30 mg/kg were tested in 5 mg/kg increments. At 48 h after LPS administration, a dose corresponding to the half-lethal level was selected for subsequent experiments. Following a 7-day acclimatization period, mice were randomly allocated into four experimental groups (n = 10 per group): Sham group, LPS-injected group (5 mg/kg), LPS plus low-dose Andro-S group (125 mg/kg), and LPS plus high-dose Andro-S group (500 mg/kg).

Before treatment, all animals were fasted for 6 h with free access to water. All groups except the Sham group received a single intraperitoneal injection of LPS (5 mg/kg, dissolved in 0.9% saline) to induce CS. At 1 h after LPS administration, treatment groups received a single tail-vein injection of Andro-S at 125 or 500 mg/kg, and the Sham group received 0.9% normal saline only. Samples were collected 24 h after treatment.

At the end of treatment, mice were euthanized by CO_2 inhalation at a flow rate of 6 L/min for 3 min. Blood was subsequently collected from the retro-orbital plexus and centrifuged at 3000 rpm at 4 ℃ for 15 min to separate serum, which was stored at − 80 ℃ for cytokine assays. Fresh lung, spleen, liver, and kidney tissues were harvested and immediately fixed in 10% buffered formalin for histopathological analysis or stored appropriately for molecular assays.

2.3. Measurement of cytokines in the serum

After euthanasia, serum samples were collected. To meet the serum volume requirements, samples from two mice within each group were pooled into one sample for analysis. Serum inflammatory mediators were measured using the Bio-Plex Pro Mouse Chemokine Panel 21 (Bio-Rad, USA) to evaluate the inflammatory response in CS mice. This panel included CCL2, CCL4, CCL11, G-CSF, IFN-γ, IL-1β, IL-3, IL-5, IL-10, IL-13, TNF-α, CCL3, CCL5, CXCL1, GM-CSF, IL-1α, IL-2, IL-4, IL-6, IL-12 p70, and IL-17. Cytokine levels were calculated from mean fluorescence intensity (MFI) using Bio-Plex Data Pro™ software. Standard curves were generated using reference cytokine concentration gradients, and cytokine levels in mouse serum were calculated from the standard curves.

2.4. Hematoxylin and eosin, and immunohistochemistry staining

Lung, spleen, liver, and kidney samples were embedded in paraffin. Tissue sections were cut at a thickness of 5 μm, deparaffinized in xylene, and rehydrated through graded alcohol solutions. Sections were stained with hematoxylin and eosin (H&E), and histopathological changes were observed under a light microscope (Nikon Eclipse CI, Nikon, Tokyo, Japan).

For immunohistochemistry (IHC), after antigen retrieval in citrate buffer solution (0.01 M, pH 6.0) for 20 min, sections were blocked with 1% bovine serum albumin (BSA) for 10 min. Sections were incubated overnight at 4 ℃ with primary antibodies against TNF-α (Bioss, bs-10802R), IL-1β (Proteintech, 26048-1-AP), and IL-6 (Bioss, bs-6309R), followed by incubation with goat anti-rabbit secondary antibody (DAKO, K5007) for 30 min. Nuclei were counterstained with DAPI for 10 min and examined under a light microscope. Positive staining was identified by brownish-yellow signals in tissues and quantified using ImageJ software.

2.5. Matrix-assisted laser desorption/ionization mass spectrometry imaging

The MALDI-MS imaging was performed using an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The instrument was equipped with a smartbeam Nd:YAG 355 nm laser operating at 2,000 kHz. The laser spot size was set to medium focus, corresponding to an approximate spot diameter of 50 μm, and laser power was kept constant after optimization before each run. The pulsed ion extraction period was 130 ns, with acceleration voltage of 20.00 kV, extraction voltage of 17.95 kV, lens voltage of 7.5 kV, and reflector voltage of 21.10 kV. Mass spectra were acquired over an m/z range of 50-1000 Da with 200 laser shots per pixel and a spatial resolution of 200 μm across lung tissue sections. External mass calibration was performed using standards before data acquisition. MALDI imaging data were processed using SCiLS Lab software; mass spectra were segmented and normalized to the total ion current. Signal intensities were standardized across imaging datasets. The LIFT™ mode of the MALDI-TOF/TOF instrument was used for MS/MS fragmentation to further confirm structural information of detected small molecules [32]. Quantitative results for compound-associated m/z signals were generated and exported using SCiLS Lab software.

2.6. 384 IsTad-assisted tissue capture and proteomic sample preparation

To improve correspondence between pathological microregions and downstream proteomic analysis, a self-developed 384 IsTad was used to capture core microtissue regions from whole lung sections. In each experimental group, lung samples from six mice were pooled, and total protein was extracted in 384-well plates for 1 h using RIPA buffer (MF072-01, Mei5bio, China) containing a complete protease inhibitor cocktail (11697498001, Roche, USA).

Captured microtissues were then rehydrated in digestion solution containing 0.01 μg/μL modified sequencing-grade trypsin and 50 mM ammonium bicarbonate (Promega, USA). After overnight incubation at 37 ℃, digested peptides were extracted using 1% trifluoroacetic acid in 60% acetonitrile

and dried in a vacuum centrifuge. Peptides were purified using ZipTip according to an in-house protocol. The resulting peptides were collected and dried by vacuum freeze-drying. A TMT™ Isobaric Mass Tagging Reagent kit (Thermo Fisher Scientific, USA) was used to label peptide mixtures according to the manufacturer's instructions.

Peptide mixtures were loaded onto a C18 column (25 cm × 75 μm, 1.6 μm; IonOpticks, Melbourne, Australia). The mobile phases were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The nano-flow LC gradient increased mobile phase B from 2% to 17% over 20 min, then to 25% over 20 min, 37% over 10 min, and 95% over 10 min. Ions were accumulated for 100 ms in the TIMS device of a timsTOF Pro 2 mass spectrometer (Bruker Daltonics, Germany). MS data were acquired over an m/z range of 700–3500 Da, and MS/MS data were acquired over an m/z range of 100–1700 Da. During MS/MS acquisition, the TIMS cycle time was set to 1.1 s and included one MS scan plus 10 PASEF-MS/MS scans [33]. Each raw file contained an average acquisition rate of 12 MS/MS spectra per second (> 100 Hz) [34]. Raw LC-MS/MS data were processed using MSFragger (https://msfragger.nesvilab.org/) with Score > 20 and FDR < 0.01 [35]. Differentially expressed proteins (DEPs) were subjected to Gene Ontology functional analysis using RStudio. Pathway enrichment analysis of DEPs was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and DAVID. Protein–protein associations among DEPs were analyzed and visualized using the STRING platform (https://cn.string-db.org/), and heatmaps were generated using RStudio.

2.7. Molecular docking

Three-dimensional structures of selected proteins were obtained from the RCSB Protein Data Bank (PDB; https://www.rcsb.org/) or the AlphaFold Protein Structure Database accessed via UniProt (https://www.uniprot.org/). The chemical structure of Andro-S was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

Protein structures were prepared using UCSF Chimera by removing water molecules, metal ions, and cofactors, followed by the addition of polar hydrogen atoms, merging of nonpolar hydrogens, and assignment of Gasteiger charges. Putative binding regions were identified using BIOVIA Discovery Studio Visualizer (Dassault Systèmes, France).

Docking simulations were performed using AutoDock Vina implemented in the PyRx virtual screening tool. Receptor grids were generated based on the identified binding regions. Docking calculations were conducted using default parameters. Predicted protein–ligand binding poses and corresponding binding energies (kcal/mol) were obtained from the docking output. Protein-ligand interaction patterns and docking conformations were visualized using PyMOL software.

2.8. Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from lung tissues using the FastPure Cell/Tissue Total RNA Isolation Kit V2 (RC112-01, Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. RNA

concentration and purity were determined using a NanoDrop Lite spectrophotometer. Complementary DNA (cDNA) was synthesized using SuperScript™ III reverse transcriptase (ABI-Invitrogen, 11752050) following the manufacturer's protocol.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using SYBR qPCR Mix (Q121-02, Vazyme Biotech, Nanjing, China) on an Applied Biosystems real-time PCR system. The amplification conditions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 20 s, and extension at 72°C for 20 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene for normalization. Primer sequences used for qRT-PCR analysis are provided in Table S1.

2.9 Cellular thermal shift assay (CETSA)

A549 cells were incubated with Andro-S at the indicated concentration (control: DMSO) for 1 h at 37°C. After treatment, samples were aliquoted into PCR tubes and heated for 3 min at a series of temperatures (e.g., 37, 42, 47, 52, 57, 62, and 67 ℃) using a thermal cycler, followed by immediate cooling on ice for 3 min. Cells were lysed by three freeze–thaw cycles (liquid nitrogen/37°C water bath) in ice-cold lysis buffer (e.g., 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5-1% NP-40, supplemented with protease inhibitor cocktail). Lysates were clarified by centrifugation (20,000 ×g, 20 min, 4°C) to remove precipitated proteins. The soluble fractions were collected and protein concentrations were determined by BCA assay. Equal amounts of soluble protein were mixed with loading buffer, separated by SDS-PAGE, and analyzed by western blotting using antibodies against selected targets (e.g., TLR4, IFIT2, and other candidates) and a loading control (β-actin). Band intensities were quantified by densitometry and normalized to the loading control. Thermal stability curves were generated by plotting the normalized soluble protein abundance against temperature, and ligand-associated stabilization or destabilization was evaluated by comparing Andro-S-treated samples with vehicle controls. All CETSA experiments were performed with at least three independent biological replicates.

2.10. Western blotting analysis

In each experimental group, lung samples from six mice were pooled together, and their total protein were lysed using PierceTM RIPA Buffer (Thermo Fisher Scientific, Rockford, IL, USA) supplemented with a protease inhibitor cocktail (GenDEPOT, TX, USA). Subsequently, cell lysates were collected by centrifugation at 12,000 rpm for 20 min. The Protein Gel Electrophoresis Chamber System was used to load the protein onto a 10% SDS-PAGE gel and transfer it to the PVDF membrane (Thermo Fisher Scientific, Rockford, IL, USA). To block nonspecific binding, membranes were incubated in a PBST solution containing 5% skim milk for 1 h at room temperature (25 ℃). The membranes were then incubated overnight at 4 ℃ with various primary antibodies, including interferon-induced protein with tetratricopeptide repeats 2 (IFIT2, sc-398610), IFIT1 (123247-1-AP), IFIT3 (15201-1-AP), Caspase-1 (22915-1-AP), Toll-like receptor 4 (TLR4, 66350-1-Ig), polymeric immunoglobulin receptor (PIGR, A01624), and Gasdermin-D (GSDMD, #97558). Following primary incubation, membranes were treated

with appropriate secondary antibodies for 1 h Protein bands were visualized using West-Q Pico ECL solution (GenDEPOT, TX, USA) and quantified using ImageJ software.

2.11. Statistical analysis

Data values are presented as the mean ± standard deviation. Statistical analyses were conducted using GraphPad Prism 8.0 software (GraphPad, La Jolla, CA), employing one-way ANOVA for multiple comparisons. Significance between two groups was determined at various levels: $p < 0.05$ was considered significant, $p < 0.01$ as highly significant, and $p < 0.001$ as extremely significant.

3. Results

3.1. Andro-S attenuates LPS-induced cytokine storm, tissue injury, and lethality

The LPS-induced cytokine storm model was first verified, followed by evaluation of the protective effect of Andro-S (Fig. 1A). During model optimization, escalating doses of LPS were assessed using survival analysis and measurement of representative cytokines. LPS exposure triggered a systemic inflammatory response, with increased serum levels of IL-6, IL-1β, TNF-α, and CCL-2 detected at 12 h (Fig. 1B). Survival analysis showed that 10 mg/kg and 20 mg/kg LPS caused complete mortality within 2 days, whereas 5 mg/kg induced an acute but partially survivable condition (Fig. 1C). This half-lethal condition was selected for the intervention study because it enabled disease induction while allowing pharmacodynamic assessment.

Under the selected model conditions, Andro-S reduced serum inflammatory responses. LPS challenge increased IL-6, TNF-α, IL-10, IL-17, CCL-2, CCL-3, and CCL-5, while reducing IL-12 p70. Andro-S treatment reversed these changes in a dose-dependent manner. The reduction in IL-6 was notable, consistent with its role as a major mediator of acute inflammatory escalation in cytokine storm. Decreased CCL-2, CCL-3, and CCL-5 levels further indicated reduced chemokine responses associated with immune-cell recruitment and inflammatory amplification. Partial restoration of IL-12 p70 suggested that Andro-S may modulate, rather than uniformly suppress, the inflammatory response (Fig. 1D).

3.2. 384 IsTad-assisted MALDI-MSI maps Andro-S-derived signals in inflamed lungs

Histopathological assessment showed that LPS caused tissue injury, particularly in the lung and spleen. In lung tissue, thickened alveolar walls, inflammatory-cell infiltration, partial alveolar collapse, and vascular congestion were evident. In the spleen, lymphocyte necrosis, nuclear fragmentation, and increased macrophage-rich regions in the red pulp were observed. Liver and kidney injury was present but less pronounced, with partial hepatocyte necrosis, inflammatory infiltration, mild glomerular cystic changes, and erythrocyte accumulation in the renal interstitium. Andro-S reduced these pathological

changes in a dose-dependent manner. Improvement was most evident in the lung and spleen, the two organs showing the strongest response to LPS in this model (Fig. 2A).

The tissue findings were supported by immunohistochemistry. LPS increased TNF-α, IL-1β, and IL-6 expression in the lung and spleen, with more selective changes observed in the liver and kidney. After Andro-S treatment, these inflammatory markers were reduced across affected tissues. These results showed that the effect of Andro-S was reflected not only in serum mediators but also in tissue inflammatory burden and organ pathology (Fig. 2B and Fig. S1). Together, these findings showed that Andro-S reduced systemic and tissue-level inflammatory injury in LPS-triggered cytokine storm. Because the lung exhibited prominent inflammatory injury and is a clinically relevant target organ in cytokine storm, subsequent analyses focused on pulmonary tissue.

An unresolved issue in Andro-S pharmacology is whether the formulation or its derived molecular species reach inflamed lesions in target organs. To examine this issue, in situ MALDI-MSI was performed on lung tissue sections. The imaging data showed an Andro-S-related signal at m/z 429.1, corresponding to the principal Andro-S-associated ion [M-Na], in the lungs of treated animals. This signal was not observed in Sham or untreated LPS-model lungs (Fig. 2C). The presence of this molecular signature in pulmonary tissue indicated that Andro-S-derived material reached the injured target organ. By capturing specific tissue regions after imaging-guided evaluation, the device linked localized drug-derived signals, lesion-associated microanatomy, and downstream proteomic analysis within the same disease context. This integration is less accessible using routine bulk tissue preparation. After spatial localization of Andro-S-derived signals in inflamed lungs, proteomic analysis was performed to examine associated inflammatory signaling changes.

3.3. Spatial pharmacoproteomics identifies Andro-S-associated changes in immune and cell-death-related proteins

To investigate mechanisms associated with Andro-S-mediated protection, 384 IsTad was used to capture lung tissue regions from all groups for proteomic analysis (Fig. 3A). The proteomic workflow generated a dataset suitable for downstream analysis. Peptide distributions fell predominantly within the expected range of 7–20 amino acids, consistent with proper tryptic digestion and appropriate MS fragmentation behavior. In total, 301,880 spectra, 19,291 unique peptides, 3,931 identified proteins, and 3,929 quantifiable proteins were obtained, supporting downstream analysis (Fig. 3B). Quantitative distributions in each group approximated normality, indicating suitable data quality for between-group comparison (Fig. 3C).

These metrics supported the reliability of the qualitative and quantitative proteomic data. A heatmap of the proteomic profiles showed clustering among the Sham, Model, Drug-L, and Drug-H groups, indicating that LPS altered protein expression patterns in lung tissues. High-dose Andro-S partially shifted the proteomic profile toward that of the Sham group (Fig. 3D). As shown in Fig. 3E, 35 upregulated and 141

downregulated DEPs were identified in the Model group compared with the Sham group. In the high-dose Andro-S-treated group, 64 upregulated and 57 downregulated DEPs were identified compared with the Model group.

Cellular component and functional enrichment scatterplots of DEPs showed that Andro-S-associated changes were enriched in categories including extracellular region, response to stimulus, extracellular space, response to stress, defense response, and extracellular organelle (Fig. 3F). To further characterize functional changes associated with Andro-S-mediated protection against CS, functional annotation clustering was performed for DEPs across treatment comparisons. As shown in Fig. 3G, the highest enrichment score was observed in an immunity-related cluster, with a value of 5.97. This cluster included innate immunity, immunity, innate immune response, and immune system process. The next enriched cluster was response to virus, with an enrichment score of 3.21. Pyroptosis is an inflammatory cell-death process involved in host defense against infection.

A network-based approach was then used to organize the DEPs into functionally related groups. The proteomic changes were grouped into immune-related and cell-death-related functional modules. One module involved IFIT-family proteins and related innate immune nodes. Another module involved TLR4, Caspase-1, Caspase-3, and GSDMD, which are associated with inflammatory cell death. STRING analysis identified IFIT1, IFIT2, IFIT3, TLR4, Caspase-1, Caspase-3, GSDMD, CFH, TTR, PIGR, and FBP1 within the protein association network (Fig. 3H).

Among these nodes, IFIT2 showed a distinct expression pattern compared with other IFIT-family members. IFIT1 and IFIT3 were increased after LPS exposure, whereas IFIT2 was reduced. This differential pattern suggested that IFIT2 may have a distinct role within the IFIT-family response. In parallel, the involvement of Caspase-1, GSDMD, and TLR4 suggested activation of pyroptosis-related signaling in this model. These proteomic data provided a basis for further target-level analysis. Specifically, Andro-S-associated changes involved both immune-related proteins and pyroptosis-related proteins. Because cytokine storm involves both inflammatory escalation and inflammatory cell death, these protein changes were further examined in subsequent analyses. To further evaluate potential target engagement, candidate proteins from this network were examined by molecular docking and CETSA.

3.4. Molecular docking and CETSA evaluate potential interactions between Andro-S and candidate targets

Candidate proteins from the proteomic dataset were subjected to molecular docking to evaluate potential compound–protein interactions. This analysis assessed whether selected proteins showed predicted structural compatibility with Andro-S (Fig. 4A). Docking results suggested that Andro-S may interact with multiple targets related to innate immune regulation and pyroptotic signaling.

Among the examined proteins, IFIT2 showed the strongest predicted binding affinity with Andro-S, with a binding energy of − 9.3 kcal/mol. Four hydrogen bonds were predicted at Asp-248, Arg-184, Leu-147, and Lys-148. Strong binding was also predicted for TTR and Caspase-1, with binding energies of − 8.2 kcal/mol. IFIT1 and Caspase-3 each displayed binding energies of − 8.1 kcal/mol. Interactions were also observed with PIGR, CFH, and GSDMD, with binding energies around − 7.3 kcal/mol, while FBP1 showed a somewhat lower but still favorable interaction. Taken together, these results suggested that Andro-S has predicted structural compatibility with multiple proteins within the proteomic network, with IFIT2 showing the strongest docking score (Fig. 4B). These docking results were consistent with the proteomic findings. LPS challenge reduced IFIT2 while increasing IFIT1 and IFIT3, implying that the IFIT family did not behave as a uniformly regulated cluster. The predicted interaction with IFIT2 supported further evaluation of this protein in the context of Andro-S treatment. Predicted interactions with Caspase-1, Caspase-3, TLR4-related nodes, and GSDMD were consistent with possible modulation of pyroptosis-related signaling.

As shown in Fig. 5A, CETSA was used to evaluate the effect of Andro on the thermal stability of related proteins. Across the 45–63 ℃ temperature gradient, protein abundance gradually decreased in the DMSO control group, whereas several proteins showed slower thermal degradation after Andro treatment. Western blotting showed that TLR4, IFIT3, Caspase-3, PIGR, and Caspase-1 retained stronger band signals at higher temperatures after Andro treatment, while β-actin remained comparable between groups. Quantitative analysis was consistent with the western blotting results, showing higher relative abundance of TLR4, IFIT3, Caspase-3, PIGR, and Caspase-1 in the Andro-treated group than in the DMSO group at higher temperatures, especially 57–63 ℃. In contrast, IFIT2 did not show significant thermal stability changes. These results suggest that Andro enhanced the thermal stability of several inflammation-related proteins, consistent with direct or indirect interactions with these targets (Fig. 5A).

Functional and expression-level assays were then performed for further validation. Within the tested concentration range, Andro affected cell viability with an IC_{50} of 70.12 μM, while no excessive toxicity was observed at 30–90 μM, supporting the use of these concentrations in subsequent experiments (Fig. 5B). LPS induced IL-6 secretion, while Andro reduced IL-6 levels at both 12 and 24 h in a dose-dependent manner (Fig. 5C). Furthermore, LPS increased the expression of TLR4, IFIT3, PIGR, and Caspase-3, whereas Andro reduced their expression to varying degrees. Western blotting results were consistent with the quantitative analysis, with more pronounced reductions observed for TLR4 and IFIT3 (Fig. 5D).

3.5. Distinct Andro-S components show differential target-stabilization profiles

The role of different constituent molecules was further examined for this complex botanical derivative. Because phenotypic synergy requires dedicated combination studies, the component analysis was interpreted at the level of target stabilization. Representative components of Andro-S were therefore

examined using CETSA-based target-stabilization readouts. The results were interpreted as evidence of differential molecular interaction rather than broad pharmacological synergy.

To assess component-specific effects, three Andro-S components, including 17-hydroxy-9-dehydroandrographolide, its 3,19-disulfated derivatives, and 14-deoxy-11-oxoandrographolide, were compared for their effects on the thermal stability of multiple target proteins (Fig. 6). The results showed component-dependent target-stabilization profiles. For TLR4, 17-hydroxy-9-dehydroandrographolide showed a stronger thermal-stabilizing effect than 14-deoxy-11-oxoandrographolide. For PIGR and Caspase-3, 14-deoxy-11-oxoandrographolide maintained higher protein stability. For IFIT3 and IFIT2, differences among compounds were relatively limited, and protein abundance generally decreased with increasing temperature. Quantitative curves further showed that, at higher temperatures (57–63 ℃), different components contributed differently to residual target protein abundance rather than producing uniform enhancement. These results suggest that different components preferentially stabilize different targets, consistent with molecular complementarity at the target-interaction level rather than uniform synergistic enhancement of the same target.

3.6. Andro-S modulates IFIT-family and Caspase-1/GSDMD-associated signaling in lung tissue

Key transcripts and proteins were examined in lung tissue to validate the proteomic, docking, and CETSA findings. qRT-PCR analysis showed that LPS increased the expression of IFIT1, IFIT3, GSDMD, IFN-γ, Caspase-1, TLR4, TTR, and PIGR, while reducing IFIT2 expression (Fig. 7A). Andro-S treatment reversed these changes to varying degrees. The effect on IFIT2 was notable because Andro-S increased IFIT2 expression that had been reduced by LPS, while decreasing several pro-inflammatory and pyroptosis-associated markers. IFIT1, TLR4, Caspase-1, GSDMD, IFN-γ, and PIGR were elevated in lungs from LPS-treated mice, whereas IFIT2 was decreased. After Andro-S administration, these changes were partially reversed. Both low and high doses showed regulatory effects, with the high dose generally producing a stronger effect (Fig. 7B). Andro-S increased IFIT2 expression and modulated IFIT-family-associated signaling. IFIT2 has been implicated in regulation of innate immune output and may limit TLR4-driven pro-inflammatory cytokine production under certain conditions. Increased IFIT2 expression therefore suggests modulation of inflammatory responsiveness rather than uniform suppression of innate immune signaling. In parallel, Andro-S reduced TLR4, Caspase-1, and GSDMD, consistent with inhibition of pyroptosis-associated signaling.

Based on the systemic inflammatory readouts, tissue pathology, MALDI-MSI localization, proteomic profiling, molecular docking, CETSA, qRT-PCR, and western blotting results, a working mechanism was proposed (Fig. 8). LPS activates TLR4-associated innate immune signaling and promotes cytokine/chemokine production, IFIT-family dysregulation, and Caspase-1/GSDMD-associated pyroptotic signaling. Andro-S treatment was associated with restoration of IFIT2 expression and reduction of TLR4, Caspase-1, and GSDMD, suggesting coordinated modulation of immune-response regulation and pyroptosis-associated inflammatory injury.

4. Discussion

Cytokine storm (CS)-related diseases continue to pose a major threat to human health. The COVID-19 pandemic, caused by SARS-CoV-2, highlighted the clinical impact of uncontrolled inflammatory amplification and the need for effective therapeutic strategies [1]. Accordingly, considerable efforts have been made to identify agents capable of mitigating CS-related inflammatory injury [36]. Recent studies have suggested that Andro-S may improve outcomes in patients with mild to severe CS-related COVID-19 [24, 37]. However, the mechanisms by which Andro-S counteracts CS and the molecular pathways involved remain incompletely defined. The present findings support that Andro-S alleviates LPS-induced CS by reducing cytokine and chemokine overproduction, restoring IFIT2 expression, and inhibiting Caspase-1/GSDMD-associated pyroptotic signaling.

IL-6 is a critical cytokine in CS-related diseases and is often accompanied by changes in other inflammatory mediators, including TNF-α, IL-1β, IL-10, IL-17, and IL-12 p70. Chemokines such as CCL-2, CCL-3, and CCL-5 also contribute to inflammatory-cell recruitment and amplification of tissue injury [1, 36, 38, 39]. In the present study, Bio-Plex Pro Mouse Chemokine Panel 21 analysis showed that Andro-S reduced LPS-induced production of multiple cytokines and chemokines while partially restoring serum IL-12 p70 levels. IL-12 p70 is known to promote the proliferation and activation of T cells and NK cells, including CD4 + and CD8 + TCR-αβ + T cells as well as TCR-γδ + lymphoblasts [40]. Therefore, the partial restoration of IL-12 p70 suggests that Andro-S may not act through broad immunosuppression alone, but may contribute to partial normalization of the inflammatory response.

The observed decreases in CCL-2 and CCL-3 are also relevant to CS-associated tissue injury. CCL2 is a CC chemokine with chemotactic activity toward monocytes and basophils and is involved in monocyte/macrophage migration and infiltration [41, 42]. CCL3, also known as macrophage inflammatory protein 1-alpha, contributes to immune modulation and inflammatory-cell recruitment [42]. The reduction of these chemokines after Andro-S treatment suggests that Andro-S may attenuate not only cytokine release but also chemokine-mediated inflammatory recruitment. Immunohistochemical staining further supported this interpretation, showing reduced TNF-α, IL-1β, and IL-6 expression in lung and spleen tissues after Andro-S treatment. These observations are consistent with previous studies showing that Andro-S suppresses excessive cytokine production triggered by LPS [43–45]. Further studies are needed to identify the specific cell types that respond to Andro-S treatment, including macrophages, T cells, B cells, and other immune-cell populations involved in CS.

A key feature of the present study is the integration of spatial drug localization with tissue-resolved proteomics. Mass spectrometry imaging provides in situ molecular information from biological samples and enables molecular signatures to be interpreted in relation to histological and morphological features [46, 47]. In this study, MALDI-MSI detected an Andro-S-associated signal in inflamed lung tissue, supporting the presence of Andro-S-derived material within the injured target organ. This spatial information is important because inflamed lung tissue is heterogeneous, with regional differences in edema, inflammatory infiltration, hemorrhage, alveolar collapse, and tissue integrity. Conventional bulk

tissue homogenization can obscure this heterogeneity and may weaken the association between drug-localized regions and downstream molecular alterations.

To address this limitation, a self-developed 384 IsTad was used to capture microregions of lung tissue for proteomic analysis after imaging-guided localization. This workflow enabled spatially informed sampling of pathologically relevant regions and helped link drug-derived signals, lesion-associated tissue architecture, and proteomic changes. In this context, the 384 IsTad was not simply a sample collection tool but a methodological bridge between MALDI-MSI and quantitative proteomics. This integrated strategy may be useful for studying complex formulations whose activity depends on tissue delivery, spatial localization, and multi-target molecular effects.

Differential protein expression analysis is a powerful approach for identifying proteins and pathways altered under specific biological conditions [48, 49]. In this study, TMT-based proteomics indicated that Andro-S treatment was associated with changes in immune-related and pyroptosis-related protein networks. Functional enrichment analysis highlighted innate immunity, immune system processes, response to stimulus, response to stress, and defense response. Network analysis further identified IFIT1, IFIT2, IFIT3, TLR4, Caspase-1, Caspase-3, GSDMD, CFH, TTR, PIGR, and FBP1 as proteins positioned within the Andro-S-associated response network.

These findings support a mechanistic model in which Andro-S affects two interconnected processes in LPS-triggered CS: regulation of innate immune responsiveness and suppression of pyroptosis-associated inflammatory injury. This model is biologically plausible because CS is not maintained solely by elevated soluble cytokines. Instead, inflammatory amplification, innate immune activation, and inflammatory cell death can reinforce one another and promote progressive tissue injury. Therefore, a therapeutic effect that involves both immune signaling and pyroptosis-related pathways may be particularly relevant in severe systemic inflammation.

The IFIT family emerged as an important component of the immune-related response. IFIT proteins are conserved interferon-induced antiviral proteins that participate in host defense and regulation of innate immune signaling [53, 54]. IFIT1, IFIT2, and IFIT3 can form functional complexes, with IFIT3 acting as a scaffold that modulates the activity of other IFIT proteins. In the present study, IFIT1 and IFIT3 were increased after LPS stimulation, whereas IFIT2 was reduced. This divergent expression pattern suggests that IFIT2 may have a distinct regulatory role rather than reflecting a uniform IFIT-family response.

IFIT2 has been reported to regulate inflammatory output and limit TLR4-driven pro-inflammatory cytokine production under certain conditions [55]. Because LPS derived from Gram-negative bacteria is recognized by TLR4 and initiates downstream inflammatory signaling through pathways including MyD88, the decrease in IFIT2 after LPS exposure may be relevant to dysregulated inflammatory amplification. Andro-S restored IFIT2 expression while reducing TLR4, IFIT1, IFIT3, and several inflammatory markers. These results suggest that restoration of IFIT2-associated immune regulation may contribute to resetting inflammatory responsiveness in LPS-triggered CS. However, this interpretation should be viewed as a model supported by expression and network data rather than direct

proof of IFIT2-dependent causality. Future studies using IFIT2 overexpression, knockdown, or knockout systems will be necessary to determine whether IFIT2 is required for the protective effect of Andro-S.

Pyroptosis also appeared to be involved in the Andro-S-associated response. Pyroptosis is an inflammatory form of programmed cell death that contributes to host defense but can also amplify inflammatory injury when excessively activated [56]. Caspase-1 plays a central role in this process by promoting maturation of inflammatory cytokines such as IL-1β and IL-18 and by cleaving Gasdermin D (GSDMD), which mediates membrane pore formation and pyroptotic cell death [56, 57]. In the present study, LPS increased Caspase-1 and GSDMD expression, whereas Andro-S reduced these pyroptosis-associated markers. These findings are consistent with inhibition of Caspase-1/GSDMD-associated pyroptotic signaling and suggest that Andro-S may limit the transition from inflammatory signaling to inflammatory cell death.

Molecular docking and CETSA provided further insight into potential target engagement. Docking analysis predicted structural compatibility between Andro-S and several proteins within the proteomic network, including IFIT2, TTR, Caspase-1, IFIT1, Caspase-3, IFIT3, GSDMD, PIGR, CFH, and FBP1. IFIT2 showed the strongest predicted docking score, suggesting that it is a protein of interest in the Andro-S response. However, CETSA did not show significant thermal stabilization of IFIT2, whereas TLR4, IFIT3, Caspase-3, PIGR, and Caspase-1 showed increased thermal stability after Andro treatment. Therefore, IFIT2 should be described as a candidate functional node or docking-prioritized protein rather than as a conclusively validated direct target. This distinction is important because docking predicts potential binding, while CETSA provides experimental evidence of altered protein thermal stability. The combined data suggest that Andro-S may influence IFIT2-associated signaling through direct or indirect mechanisms, while more direct engagement evidence was observed for several other inflammation-related proteins.

The component-level CETSA analysis further suggested that chemically distinct Andro-S components have different target-stabilization profiles. For example, 17-hydroxy-9-dehydroandrographolide showed stronger stabilization of TLR4, whereas 14-deoxy-11-oxoandrographolide showed stronger effects on PIGR and Caspase-3. Differences among compounds were more limited for IFIT3 and IFIT2. These results do not establish phenotypic synergy, which would require dedicated combination experiments at cellular or animal levels. Instead, the data are more appropriately interpreted as molecular complementarity at the target-interaction level. Distinct components may broaden target coverage by preferentially interacting with different proteins, thereby contributing to the multi-node regulatory profile of Andro-S.

PIGR and TTR were also identified among the Andro-S-associated proteins. PIGR mediates transcytosis of polymeric IgA and IgM across epithelial cells and is regulated by pro-inflammatory cytokines such as TNF-α and IFN-γ [51, 52]. The reduction of PIGR expression after Andro-S treatment may reflect decreased inflammatory stimulation in epithelial or mucosal immune contexts. TTR was also modulated by Andro-S, although its precise role in this CS model remains to be clarified. These proteins may

represent additional components of the tissue-level inflammatory response rather than isolated primary targets.

Taken together, the present findings support a model in which Andro-S alleviates LPS-induced CS through coordinated effects on systemic cytokine production, tissue inflammatory injury, spatially localized drug exposure, immune-related protein networks, and pyroptosis-associated signaling. The proposed mechanism is not a single linear pathway but a multi-level regulatory process. On one side, Andro-S restores IFIT2-associated immune regulation and modulates IFIT-family signaling, which may reduce excessive inflammatory responsiveness. On the other side, Andro-S suppresses TLR4/Caspase-1/GSDMD-associated signaling, which may limit pyroptosis-related inflammatory injury. These two processes are consistent with the biological structure of CS, in which inflammatory amplification and inflammatory cell death can reinforce each other.

Several limitations should be considered. First, although the spatial pharmacoproteomic workflow links drug-derived signals with proteomic changes in injured lung tissue, it does not by itself prove that each observed protein change is caused by direct compound binding. Second, docking results require experimental validation, and CETSA provides evidence of thermal stabilization but does not define binding sites or functional consequences. Third, the causal role of IFIT2 remains to be established through gain- and loss-of-function experiments. Fourth, the contribution of individual immune-cell populations was not resolved in this study. Future studies integrating single-cell analysis, spatial transcriptomics, genetic manipulation of IFIT2, and targeted validation of pyroptosis markers would help clarify the cell-type-specific and causal mechanisms underlying Andro-S-mediated protection.

Overall, these findings extend the pharmacological understanding of Andro-S in cytokine storm. Rather than supporting a generic “multi-target anti-inflammatory” description, the data suggest a spatially and molecularly organized mechanism involving tissue delivery, immune-response modulation, and suppression of pyroptosis-associated inflammatory injury. This framework may also provide a useful strategy for investigating complex botanical derivatives and other multi-component therapeutic formulations in inflammatory diseases.

5. Conclusion

This study supports the therapeutic potential of Andro-S in an LPS-induced CS mouse model. Andro-S reduced pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-10, IL-17, CCL-2, CCL-3, and CCL-5, while partially restoring IL-12 p70 levels. MALDI-MSI enabled spatial detection of Andro-S-derived signals in lung tissue, and 384 IsTad-assisted proteomic analysis helped link spatial drug localization with tissue-level molecular changes. Proteomic, docking, CETSA, qRT-PCR, and western blotting analyses collectively suggested that Andro-S modulates immune-related and pyroptosis-related pathways in LPS-triggered CS. IFIT2 showed the strongest predicted docking affinity with Andro-S and was restored after Andro-S treatment, although CETSA did not show significant IFIT2 thermal stabilization. Therefore, IFIT2 is best interpreted as a candidate functional node within the Andro-S-

associated immune regulatory network. In parallel, Andro-S reduced TLR4, Caspase-1, and GSDMD expression, consistent with inhibition of pyroptosis-associated signaling. Together, these findings support a model in which Andro-S alleviates LPS-induced cytokine storm by modulating IFIT2-associated immune responsiveness and limiting Caspase-1/GSDMD-associated pyroptotic injury. The integrated spatial pharmacoproteomic strategy used here may provide a useful framework for investigating the tissue distribution, target engagement, and molecular mechanisms of complex therapeutic formulations in inflammatory disease.

Declarations

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Author contributions

Zhen Ma: Data curation, Writing - original draft. Xingyue Xu: Data curation, Validation, Writing - original draft. Jinli Hou: Data curation, Investigation. Yanmiao Ma: Data curation, Investigation. Lei Song: Methodology, Data curation. Chunhong Jiang: Resources, Formal analysis. Ning Xie: Resources, Formal analysis. Hongjun Yang: Conceptualization, Funding acquisition. Xianyu Li: Conceptualization, Writing - review & editing Methodology; Mei Liu: Software.

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The datasets generated during the current study are available and the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository [58,59] with the dataset identifier PXD041074.

Ethics approval and consent to participate

The experimental procedures were approved by the China Academy of Chinese Medical Science's Administrative Panel on Laboratory Animal Care (No. ERCCACMS20-16-2002). All animal experiments were performed in accordance with institutional guidelines and ethics. All animal experiments have taken place in the experiment research center of CACMS.

Consent for publication

The authors declare that they all consent to publication.

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