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Integration of Network Pharmacology and Experimental Validation to Investigate the Anti-atherosclerotic Mechanism of Xiong-Gui-San

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Date: 2022-11-02T00:00:00+00:00

Abstract

Qiong Gui San (QP) is a classic prescription in traditional Chinese medicine that has demonstrated potential in treating atherosclerosis over the past few decades. However, the mechanisms mediating these cardiovascular benefits remain to be fully elucidated. Here, we investigated the effects and mechanisms of QP against atherosclerosis using network pharmacology approaches and in vitro models. Active components of QP and related targets were collected from public databases. Through extensive application of bioinformatics methods, including protein-protein interaction (PPI) networks, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG), the central targets and signaling pathways of QP against AS were identified. The predicted key targets were validated in LPS-stimulated mouse macrophage RAW264.7 cells. The anti-inflammatory properties of QP were also evaluated in this model. Through in silico investigation of QP, 18 active components and 49 chemical targets intersecting with AS-related genes were identified. KEGG pathway analysis revealed significant enrichment of lipid and atherosclerosis pathways among these chemical targets. Biochemical analysis showed that QP had significant effects on the expression of predicted chemical targets (PPARγ, CAT, PTGS2) and LPS-induced inflammatory genes (IL-1, IL-6, and TNF-α). These inhibitory effects were associated with suppression of the NF- B signaling pathway activated by LPS stimulation. Our findings reveal the therapeutic potential of QP in the prevention and treatment of atherosclerosis.

Full Text

Preamble

Integrating Network Pharmacology and Experimental Verification to Explore the Mechanism of Qionggui Power Against Atherosclerosis

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Abstract

Qionggui Power (QP), a classic prescription in Traditional Chinese Medicine (TCM), has demonstrated therapeutic potential in atherosclerosis treatment over recent decades. However, the mechanisms underlying these cardiovascular benefits remain incompletely understood. Here, we investigated the effects and mechanisms of QP against atherosclerosis using network pharmacology approaches and an in vitro model. Active ingredients and related targets of QP were collected from public databases, and hub targets and signaling pathways were identified through comprehensive bioinformatics analyses, including protein-protein interaction (PPI) networks, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment. The predicted major targets were validated in LPS-stimulated murine macrophages (RAW264.7), and the anti-inflammatory properties of QP were evaluated in this model. Our in silico investigation identified 18 active ingredients and 49 chemical targets that intersect with atherosclerosis-related genes. KEGG pathway analysis revealed significant enrichment of these targets in lipid and atherosclerosis pathways. Biochemical analysis demonstrated that QP markedly affected the expression of predicted chemical targets (PPARγ, CAT, PTGS2) and LPS-induced inflammatory genes (IL1, IL6, and TNFα). These inhibitory effects were linked to suppression of the NF- B signaling pathway, which was activated by LPS stimulation. Our findings reveal the therapeutic potential of QP in preventing and treating atherosclerosis.

Keywords: Network Pharmacology, Ligusticum chuanxiong hort, Angelica sinensis, atherosclerosis, inflammation

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1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, causing an estimated 17 million deaths annually—approximately one-third of all deaths [1-3]. Risk factors for CVD, including aging, family history, hypertension, hypercholesterolemia, obesity, diabetes, and smoking [4], are closely associated with altered arterial intima structure [5] and modified plasma-derived lipoproteins [6]. These alterations lead to plaque accumulation and thrombus formation in arterial walls, driving the complex pathological process known as atherosclerosis (AS) [7]. Among the various molecular mechanisms underlying AS, lipid-driven inflammation plays a central role in disease pathogenesis [8]. Intensified inflammatory activation can trigger local proteolysis, plaque rupture, and thrombus formation, with pro-inflammatory mediators secreted by recruited macrophages—such as IL1, IL6, and TNFα—contributing to local inflammation and plaque progression [8]. Consequently, inflammatory markers are valuable for identifying active chemical components and therapeutic potential for AS treatment.

Qionggui Power (QP) is composed of two herbs: Chuanqiong (Ligusticum chuanxiong hort) and Danggui (Angelica sinensis). The dried rhizomes of these herbs have long demonstrated clinical benefits in gynecological diseases. In recent years, several experimental studies have evaluated the cardioprotective effects of QP using modern scientific methods [9], raising the possibility of its application in AS treatment. Although modern pharmacological experiments have suggested various anti-inflammatory, anti-oxidative, and anti-aging activities associated with QP [10, 11], the bioactive components and underlying pharmacological mechanisms remain obscure.

Network pharmacology establishes a new paradigm in drug discovery with significant implications for addressing the two major sources of attrition in drug development—efficacy and toxicity [12]. This approach provides an effective means to validate target combinations and optimize multiple structure-activity relationships while maintaining drug-like properties [13]. Using this paradigm, the holistic philosophy of Traditional Chinese Medicine is contextualized through pharmacological mechanisms, efficiently defining interacting networks among multiple components, drug targets, and pathways [14].

In this study, we obtained QP ingredients and drug targets from the public Traditional Chinese Medicine Systems Pharmacology (TCMSP) database. Active ingredients were screened using criteria of oral bioavailability (OB) $ $30% and drug-likeness (DL) $ $0.12. Through PPI network analysis, we identified 49 molecular targets of these chemicals against AS. Functional enrichment via GO and KEGG analyses revealed strong involvement in lipid and atherosclerosis pathways. Furthermore, 15 hub targets were identified using CytoNCA, a Cytoscape plugin for calculating, evaluating, and visualizing multiple centrality measures, with screening conditions including BC, CC, DC, EC, LAC, and NC. The major proteins in the predicted pathways were validated using an LPS-

induced inflammatory model in the macrophage cell line RAW264.7 in vitro.

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2. Materials and Methods

2.1. Screening for Active Ingredients and Related Targets from QP

Active ingredients and related targets were screened from the Traditional Chinese Medicine Systems Pharmacology database (TCMSP; https://tcmsp-e.com/tcmsp.php) using the keywords "Chuanqiong" and "Danggui." Screening criteria for active ingredients were set as oral bioavailability (OB) $ $30% and drug-likeness (DL) $ $0.12. All related target names were standardized according to the UniProt database.

2.2. Establishing a Collection of AS-Related Genes

A collection of genes linked to AS pathogenesis and therapeutics was established using data from five public genetic and clinical databases: GeneCards (https://www.genecards.org/), OMIM (https://omim.org/), PharmGKB (https://www.pharmgkb.org/), TTD (http://db.idrblab.net/ttd/), and DrugBank (https://go.drugbank.com/). Using the keyword "AS," we obtained 1,714 targets (1,350 from GeneCards, 197 from OMIM, 13 from PharmGKB, 34 from TTD, and 120 from DrugBank). After removing duplicates, 1,492 target genes remained for further analysis.

2.3. Mapping Targets of Active Ingredients and AS-Related Genes

Targets of active ingredients from QP and AS-related genes were input into Cytoscape, an open-source software platform, to visualize complex networks. A mapping of these targets and active ingredients was constructed with integrated topological parameters, which reflect the importance of genes in AS pathogenesis and therapeutics.

2.4. Constructing Protein-Protein Interaction (PPI) Network and Hub Proteins Network

Identified genes from target mapping were imported into the STRING database (https://string-db.org/) to build the PPI network. The minimum required interaction score for medium confidence was set at 0.4. After removing disconnected nodes, short tabular text containing edge lists was derived and input into Cytoscape. Hub target proteins were then identified using the Cytoscape plugin CytoNCA through combined screening of six topological factors: Betweenness centrality (BC), Closeness centrality (CC), Degree centrality (DC), Eigenvector centrality (EC), local average connectivity-based method (LAC), and network centrality (NC). Screening criteria were set as values greater than or equal to the median for each factor.

2.5. Functional Enrichment Analyses

Potential target gene names were converted to entrezID format. KEGG and GO enrichment analyses were performed using R software for statistical computing and graphics. The top 30 KEGG entities were mapped as bar plots, while the top 10 GO entities were mapped as bubble plots.

2.6. Preparation of Qionggui Power (QP)

Raw herbs were purchased from Henan Zhang Zhongjing Pharmacy Co. (Henan, China). Ligusticum chuanxiong hort roots were grown in Gansu province, and Angelica sinensis roots were grown in Xichuan province. Both herbs were dried and cut at their origins after harvest. Voucher specimens of Chuanqiong (2021-1117b) and Danggui (2021-1118b) were deposited in the museum of the Scientific Research Center at Nanyang Medical College.

The aqueous extract of QP was prepared as follows: Ligusticum chuanxiong hort and Angelica sinensis were mixed in a 2.33:1 weight ratio and macerated in 10-fold volume of water for 1.5 hours. The mixture was extracted at 100°C for 1 hour, and residues were subjected to two additional extractions for 1 hour and 30 minutes, respectively. The water extract was concentrated by rotary evaporation and dried by vacuum freeze-drying. The extraction yield of the herbal formula QP was approximately 36% [Figure 4: see original paper].

2.7. Cell Culture

The mouse macrophage cell line RAW264.7 (ATCC number: TIB-71) was obtained from the cell bank of the Chinese Academy of Sciences (Beijing, China). Cells were cultured in growth medium consisting of Dulbecco' s modified Eagle' s medium (DMEM) with 17.5 mM D-glucose, 15.1 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 2.5 mM glutamine, and 0.5 mM sodium pyruvate (Dalian Meilun Biotechnology Co., Dalian, China), supplemented with 10% v/v fetal bovine serum (FBS) (Tianhang Bio, Hangzhou, China) and 1% (v/v) penicillin/streptomycin sulfate cocktail at 37°C in a humidified atmosphere containing 5% CO_2. Upon reaching full confluence, cells were seeded in plates at a density of 4$\times 10^{\{5\}/cm}${2}.TheincubationmediumconsistedofDMEMsupplementedwith1^{2}. Cells were allowed to reattach and settle for 12 hours at 37°C before incubation under the following experimental conditions: (a) incubation medium alone; (b) 100 ng/ml LPS (Beyotime Institute of Biotechnology, Haimen, China); (c) 100 ng/ml LPS + 0.25 mg/ml QP; (d) 100 ng/ml LPS + 0.5 mg/ml QP; (e) 100 ng/ml LPS + 1 mg/ml QP.

2.8. Formazan Formation Assay

Cell viability was assessed using the Cell Counting Kit-8 (Dalian Meilun Biotechnology Co., Dalian, China), a water-soluble tetrazolium salt (WST)-based assay.

After incubation, cells in 96-well plates were treated with incubation medium containing 10 l/well WST-8 (10% Cell Counting Kit-8 solution). Following 1 hour incubation at 37°C, absorbance was measured at 450 nm using a microplate spectrophotometer (Bio Tek, US).

2.9. RNA Extraction and cDNA Synthesis

Total RNA was isolated from cultured cells in six-well plates using an Ultrapure RNA Kit (Jiangsu Kangwei Century Biotechnology Co., Ltd, China) according to the manufacturer' s instructions. Briefly, culture medium was completely removed and 1 ml TRIzol was added to each well. After shaking on a circumferential shaker for 20 minutes, the lysis solution was collected and mixed with 200 l chloroform. Following vigorous vortexing, the mixture was centrifuged at 12,000g for 10 minutes at 4°C. A total of 420 l aqueous-phase supernatant was collected, mixed with an equal volume of 70% ethanol, and loaded into Spin Columns with silica cartridges for purification. Purified RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) at 260 nm ($A_{260}/_{280}$ = 1.8-2.0). Reverse transcription was performed using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biomedical Technology Co., Ltd., Japan) after DNase treatment to remove potential DNA contamination. cDNA synthesis conditions were: 37°C for 15 minutes, 85°C for 5 seconds, and 4°C hold.

2.10. Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed using 2× Real-Time PCR master mix containing SYBR Green I and LOW ROX (Code No. RR820, Takara, Japan). The standard PCR mixture contained 2.0 l template cDNA, 10 l TB Green Premix Ex Taq II, 0.8 l forward primer, 0.8 l reverse primer, 6.0 l RNase-free water, and 0.4 l ROX Reference Dye II. The cycling protocol included initial denaturation at 95°C (20 seconds), followed by 40 cycles of 95°C (3 seconds) and 60°C (30 seconds annealing). A melt curve was generated to verify amplification specificity. Primer sequences were: IL1β (5' -CCACCTCAATGGACAGAATATCA-3', 5'-CCCAAGGCCACAGGTATTT-3'), IL6 (5'-CCAGAGTCCTTCAGAGAGATACA-3', 5'-CCTTCTGTGACTCCAGCTTATC-3'), and TNFα (5'-TTGCTCTGTGAAGGGAATGG-3', 5'-GGCTCTGAGGAGTAGACAATAAAG-3'). Reactions were performed in quadruplicate. mRNA levels were normalized to endogenous GAPDH and quantified using the comparative CT method (2^(-ΔΔCT)).

2.11. Protein Isolation and Immunoblotting Analysis

Total cellular protein and nuclear extracts were prepared using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology CO., Shanghai, China). Protein concentration was determined using a Bicinchoninic Acid (BCA) Protein Assay Kit (Dalian Meilun Biotechnology Co., Dalian, China) by measuring absorption at 562 nm.

Cellular lysates were mixed with 6× loading buffer (Beyotime Institute of Biotechnology, Haimen, China) and heated at 95°C for 5 minutes. Twenty micrograms of protein were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immun-Blot, Bio-RAD, USA). After blocking with 5% nonfat dry milk in phosphate-buffered saline plus 0.1% Tween 20 (PBST), membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Signal detection was performed using enhanced chemiluminescence with the G:BOX Chemi XRQ system (Syngene, USA). Band intensity was analyzed using ImageJ software. Antibodies are listed in .

2.12. Statistical Analysis

Statistical differences between control and treatment groups were determined using independent two-tailed Student' s t-tests. Data are presented as mean ± SD. Differences were considered significant at $P < 0.05$. All experiments were repeated independently three times.

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3. Results

3.1. Active Ingredients in QP and Related Targets

To reduce the complexity of chemical components and mechanisms, active ingredients of QP were screened using combined pharmaceutical parameters: oral bioavailability (OB) >30% and drug-likeness (DL) >0.12. A total of 18 active ingredients were selected, including 15 from Chuanqiong and 3 from Danggui . From these, 95 related targets were retrieved from the TCMSP database for further analysis [Figure 1: see original paper].

3.2. Prediction of Potential Targets for AS

AS-related targets were compiled from five public genetic and clinical databases using the keyword "AS," yielding 1,714 targets (1,350 from GeneCards, 197 from OMIM, 13 from PharmGKB, 34 from TTD, and 120 from DrugBank). After removing duplicates, 1,492 target genes remained for analysis [Figure 1: see original paper]. The intersection between QP targets and AS targets is shown in [Figure 1: see original paper].

3.3. Ingredient-Target-Disease Network

Venn diagram analysis identified 49 intersecting target genes between QP and AS, which are associated with 15 active ingredients of QP. A network between active ingredients and intersected target genes was constructed using Cytoscape 3.8 software, illustrating the pharmaceutical potential of QP against AS [Figure 1: see original paper].

3.4. PPI Network Construction and Key Targets

To further understand QP' s pharmaceutical potential, functional connectivity among target proteins was analyzed topologically. These proteins were queried in the STRING database, constructing a PPI network with 956 nodes and 1,753 edges after removing disconnected nodes [Figure 2: see original paper]. Hub proteins were identified through two consecutive screenings with CytoNCA based on six topological factors: BC, CC, DC, EC, LAC, and NC. Values for each factor are listed in . Fifteen hub proteins were identified: CAT, NR3C1, PPARG, BDNF, SOD1, CASP3, PPARA, PTGS2, JUN, ESR1, NCOA2, RXRA, SERPINE1, NOS2, and LPL [Figure 2: see original paper]. These proteins were selected for further validation in the LPS-induced inflammatory model.

3.5. Functional Enrichment Analysis

To investigate QP' s functional activities in signaling pathways and biological processes, KEGG and GO enrichment analyses were performed based on potential target proteins. KEGG analysis revealed involvement in 90 signaling pathways ($p < 0.05$), with the top three being neurodegeneration, chemical carcinogenesis, and lipid and atherosclerosis [Figure 3: see original paper]. GO enrichment analysis showed tight association with response to nutrient levels and extracellular stimulus, membrane raft and microdomain, and DNA-binding transcription factor binding [Figure 3: see original paper]. These data demonstrate QP' s potential anti-atherosclerotic activities.

3.6. Effects of QP Treatment on Macrophage Cell Viability

To determine QP' s effects on macrophage viability, RAW264.7 cells were incubated with various QP concentrations and assessed for formazan formation via WST-8 reduction assay (CCK-8). Cells exposed to 0-4 mg/ml QP showed significantly increased WST-8 reduction after 6 hours ($p < 0.05$), though this increase normalized after 12 hours at 1 mg/ml [Figure 4: see original paper]. QP treatment also altered RAW264.7 morphology in a concentration-dependent manner at 12 hours, characterized by increased cytoplasmic vacuoles [Figure 4: see original paper]. Vacuole formation may reflect altered biological processes. Since concentrations >1 mg/ml decreased formazan formation and increased vacuole visibility, 0-1 mg/ml QP was selected for further biochemical analysis.

3.7. Effects of QP on PPARγ, CAT, and PTGS2 Expression

To verify QP' s predicted pharmacological mechanism against AS, we measured effects on the top three hub targets using the LPS-stimulated macrophage model, which displays markers typical of AS-associated macrophages. QP treatment increased CAT expression in LPS-treated RAW264.7 cells in a concentration-dependent manner. QP (1 mg/ml) attenuated LPS-induced PTGS2 expression and significantly upregulated PPARγ expression, though this effect was not

observed at lower concentrations. These data generally align with in silico predictions [Figure 5: see original paper].

3.8. Effects of QP on Macrophage-Derived Inflammatory Mediators

Inflammation plays a central role in AS pathogenesis, and LPS-stimulated RAW264.7 cells represent a well-established in vitro inflammation model. QP exhibited anti-inflammatory activity by reducing expression of inflammatory mediators including IL1, IL6, and TNFα—known markers of LPS-induced inflammation in RAW264.7 cells [Figure 6: see original paper]. This effect was accompanied by suppression of NF- B signaling, as QP treatment alleviated abnormal phosphorylated-p65 distribution in cytosol and nucleus while increasing I B levels [Figure 6: see original paper].

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4. Discussion

We have described network pharmacology analysis and functional verification of the Chinese herbal complex Qionggui Power (QP), composed of Chuanqiong (Ligusticum chuanxiong hort) and Danggui (Angelica sinensis). In silico analysis revealed active ingredients and hub molecular targets highly enriched in signaling pathways and biological processes related to AS pathogenesis. These findings are functionally significant, as QP treatment modulated expression of core molecular targets that are abnormally expressed in an inflammatory macrophage model. Moreover, in vitro experiments demonstrated anti-inflammatory activity through reduced expression of inflammatory mediators. Our data indicate QP' s potential anti-atherosclerotic properties, which may contribute to further pharmaceutical development.

Screening active ingredients from herbal medicines is crucial in network pharmacology analysis. Oral bioavailability [15] and drug-likeness [16] are essential pharmacokinetic parameters for this screening. Oral bioavailability indicates the efficiency of ingredients reaching systemic circulation after oral administration, while drug-likeness reflects molecular similarity to existing drugs. By quantitatively assessing physicochemical properties' impact on molecular behavior, drug-likeness has been widely used to filter undesirable compounds in early drug discovery phases [17, 18]. The suggested TCMSP screening criteria are OB $ $20% and DL $ $0.1. In this study, we used more stringent criteria (OB $ $30% and DL $ $0.12), which may improve selection of therapeutic bioactive molecules with optimized pharmacokinetic properties.

Atherosclerosis involves pathophysiological alterations in medium to large arteries driven primarily by lipid abnormalities and dysregulated inflammation [19]. Therapies targeting lipid or inflammatory homeostasis effectively reduce morbidity and mortality from atherosclerotic cardiovascular disease [20]. Our in silico data revealed 15 core pharmaceutical targets of QP enriched in multiple pathways, including lipid and atherosclerosis. The top three ranked hub

proteins were PPARγ, CAT, and PTGS2. PPARγ, together with PPARα and PPARβ, forms a nuclear hormone receptor subfamily [21]. Previous studies suggest PPARα and PPARγ activation reduces AS progression by correcting metabolic disorders and exerting direct vascular wall effects [22], involving lipid metabolism and inflammatory response modulation. PTGS2 (COX-2), induced by cytokines and mitogens, contributes to inflammation, pain, angiogenesis, and cancer [23]. PTGS2 inhibition reduces vascular prostacyclin, which blocks vascular inflammation and atherosclerosis [24]. CAT catalyzes hydrogen peroxide decomposition into water and oxygen; enhanced CAT activity was observed in foam cells from rabbit atherosclerotic lesions, and combined CAT/SOD1 overexpression alleviates atherosclerosis [25]. In this study, QP treatment demonstrated atheroprotective effects by increasing PPARγ and CAT protein levels while reducing PTGS2 expression, consistent with network pharmacology predictions.

Macrophages are key contributors to arterial inflammation. As the most abundant leukocytes in atherosclerotic lesions, macrophages decisively determine the plaque inflammatory environment by secreting cytokines such as IL1, IL6, and TNFα [26, 27]. Clinical agents antagonizing these cytokines have proven effective in reducing cardiovascular events [28, 29]. Interestingly, QP-exposed macrophages showed suppressed expression of LPS-induced IL1, IL6, and TNFα. This suppression was accompanied by altered NF- B signaling activity, a critical regulator of inflammatory processes [30]. This anti-inflammatory effect was not captured in our network pharmacology analysis, possibly due to the stringent active ingredient screening criteria (OB $ $30% and DL $ $0.12), which may have excluded components with anti-inflammatory potential. Thus, an in vivo atherosclerosis model will be required for comprehensive assessment of QP' s cardioprotective effects. Notably, this may complicate validation of network pharmacology analysis due to the various cell types in atherosclerotic lesions and differential functions of target genes across these cells.

Collectively, our data provide insights into QP' s potential for pharmaceutical development against atherosclerosis-related diseases. In silico analysis identified active ingredients with favorable pharmacokinetic parameters (OB and DL) and hub targets intimately involved in atherosclerosis pathogenesis and clinical intervention. Key predicted cardioprotective targets were validated in an inflammatory macrophage model, which comprises a major contributor to atherosclerotic lesion formation. Further in vivo studies are required to fully determine QP' s value in pharmaceutical development.

Author Contributions

Conceptualization: Y.W.; **Methodology:** X.J., G.Z., Q.Z., D.S.; **Software:** Y.M.; **Validation:** S.L.; **Formal Analysis:** X.J., Y.M.; **Writing–Original Draft Preparation:** Y.M.; **Writing–Review and Editing:** Y.W.; **Visu-**

alization: X.J.; **Supervision:** Y.W.; **Project Administration:** Y.W., S.L.; **Funding Acquisition:** Y.W. All authors have read and agreed to the published version of the manuscript.

Funding

This work was funded by Key Research Projects of Henan Higher Education Institutions in 2020 (20A310010, to Y.M.) and the Technology Development Plan of Nanyang in 2019 (KJGG192, to Y.M.).

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

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