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Luteolin Reverses Methotrexate Resistance in Rheumatoid Arthritis via Targeting Sp1-DHFR Axis and p38 MAPK/STAT3 Signaling

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ABSTRACT: Background: Rheumatoid arthritis (RA) is a disabling autoimmune disease where methotrexate resistance, driven by Specificity protein 1 (Sp1)-mediated upregulation of dihydrofolate reductase, poses a key clinical challenge. Preliminary network pharmacology analysis identified Dihydrofolate reductase (DHFR) as a high-confidence target of luteolin in RA. Given that Sp1 is a known transcriptional activator of DHFR, we further investigated whether luteolin modulates the DHFR-Sp1 axis, thereby prompting this study to explore its potential in overcoming Methotrexate (MTX) resistance. **Methods:** An MTX-resistant human fibroblast-like synoviocyte (FLS) model was established *in vitro* to investigate whether luteolin can reverse MTX resistance and elucidate the underlying mechanisms. Assessments included cell viability, apoptosis, RT-qPCR, Western blotting, DHFR promoter reporter assays, and chromatin immunoprecipitation (ChIP) for Sp1. Translational relevance was evaluated *in vivo* using a collagen-induced arthritis (CIA) mouse model treated with MTX alone or in combination with luteolin. **Results:** Compared to parental cells, MTX-resistant FLS showed ~15-fold higher DHFR mRNA ($p < 0.001$) and increased p38 MAPK/STAT3 phosphorylation. Co-treatment with luteolin (10 μ M) and MTX significantly resensitized resistant cells, reducing viability to 60% of control ($p < 0.001$) and increasing total apoptosis to 16.0% vs. 13.7% with MTX alone ($p < 0.01$). Mechanistically, luteolin suppressed Sp1 binding to the DHFR promoter by ~35% ($p < 0.01$), reducing DHFR promoter activity and downregulating DHFR mRNA/protein. Concurrently, luteolin inhibited p38 MAPK/STAT3 phosphorylation by ~40–50% ($p < 0.01$). In CIA mice, luteolin + MTX outperformed MTX monotherapy: arthritis score decreased from 1.5 to 0.67 ($p < 0.001$), serum IL-6 from 150 pg/mL to 50 pg/mL ($p < 0.001$), and synovial DHFR expression was reduced. These improvements indicate clinically relevant attenuation of disease severity. **Conclusion:** Luteolin reverses MTX resistance in RA synovial fibroblasts through a dual mechanism: Sp1-dependent suppression of DHFR expression and concurrent inhibition of pro-survival signaling pathways.

KEYWORDS: Luteolin; methotrexate resistance; rheumatoid arthritis; dihydrofolate reductase; specificity protein 1

1 Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by persistent synovial inflammation, hyperplasia of synovial fibroblasts, and progressive joint destruction [1]. Fibroblast-like synoviocytes (FLS) in RA proliferate abnormally and produce matrix-degrading enzymes and pro-inflammatory cytokines that mediate cartilage and bone erosion [2,3]. Methotrexate (MTX) is widely utilized as a first-line anchor drug for RA treatment owing to its anti-proliferative and anti-inflammatory properties. At low doses used in RA, MTX acts as a folate antagonist that inhibits Dihydrofolate reductase (DHFR), resulting

in suppression of purine/pyrimidine synthesis, and it also promotes anti-inflammatory adenosine accumulation via inhibition of AICAR transformylase [4]. Clinically, MTX monotherapy induces remission or low disease activity in a substantial proportion of RA patients. However, a significant subset of patients shows an inadequate response or loses responsiveness to MTX over time [5]. Such MTX resistance can manifest as continued joint inflammation and damage despite therapy, necessitating alternative or combination treatments.

Mechanisms underlying acquired MTX resistance are multifactorial. Increased expression or activity of drug efflux transporters, such as P-glycoprotein (ABCB1), can reduce intracellular MTX concentrations; notably, P-glycoprotein is often upregulated in refractory RA and is implicated in MTX resistance [6]. Another key mechanism is alteration of MTX's intracellular targets. In particular, amplification or overexpression of the DHFR gene can blunt MTX's antifolate effect. DHFR overexpression has long been recognized as a cause of MTX resistance in cancer cells [7], and evidence suggests similar adaptations in immune cells of MTX-treated RA patients (e.g., increased DHFR enzyme levels) [8]. Transcriptional regulation plays a major role in DHFR expression: the DHFR promoter is GC-rich and contains tandem Sp1 binding sites that drive its basal transcription [9]. Enhanced Sp1 activity has been linked to elevated DHFR in MTX-resistant cells [10]. For example, forcing Sp1 overexpression increases MTX resistance, whereas inhibiting Sp1-DNA binding with mithramycin selectively downregulates DHFR and resensitizes resistant cells [11]. Therefore, targeting the Sp1-DHFR axis could be a promising strategy to overcome MTX resistance.

Luteolin (3',4',5,7-tetrahydroxyflavone) is a plant-derived flavonoid found in many fruits, vegetables, and medicinal herbs. It is known for diverse bioactivities, including anti-inflammatory, antioxidant, and anti-cancer effects. In the context of RA, luteolin has been reported to inhibit the pathological functions of FLS. Hou et al. showed that luteolin treatment suppresses the proliferation of RA-FLS and reduces their production of IL-6, IL-8, IL-15, TGF- β and matrix metalloproteinases [12]. Luteolin induced cell cycle arrest in FLS and synergized with TNF- α to further inhibit FLS growth [13]. At the signaling level, luteolin can block multiple cascades; for example, it was found to inhibit the MAPK/ERK and PI3K/Akt pathways in RA-FLS [14]. Other studies have demonstrated luteolin's ability to interfere with inflammatory signaling, such as JNK and p38 MAPK, as well as transcription factors NF- κ B and AP-1, in stimulated synovial cells [15]. These multi-targeted actions suggest that luteolin might counteract cellular mechanisms that contribute to MTX resistance (e.g., aberrant activation of survival pathways or transcription factors like Sp1). In addition, as an anti-inflammatory agent, luteolin could potentially enhance the overall therapeutic effect of MTX in RA.

Furthermore, its anti-inflammatory properties suggest that luteolin may have the potential to act synergistically with MTX, thereby enhancing the overall therapeutic efficacy in RA. Therefore, this study aimed to investigate whether luteolin can reverse MTX resistance in RA synovial fibroblasts and to elucidate the underlying mechanisms, focusing on DHFR transcriptional regulation via Sp1 and involvement of the p38 MAPK/STAT3 signaling pathways. To achieve this, we employed an *in vitro* MTX-resistant FLS model and an *in vivo* CIA mouse model to test the hypothesis that luteolin resensitizes resistant cells to MTX through suppressing Sp1-mediated DHFR expression and inhibiting pro-survival MAPK/STAT3 signaling. Herein, we aimed to: (1) identify direct targets of luteolin in RA using network pharmacology and molecular docking, which highlighted DHFR as a core hub; (2) based on the known role of Sp1 as a master transcriptional regulator of DHFR, investigate whether luteolin suppresses Sp1-dependent DHFR expression; (3) elucidate the underlying mechanisms involving Sp1-mediated DHFR transcription and p38 MAPK/STAT3 pathways; (4) verify the *in vivo* efficacy in a CIA mouse model.

2 Materials and Methods

2.1 Target Identification for Luteolin in Rheumatoid Arthritis

The potential protein targets of luteolin were interrogated using five publicly accessible databases [16]: ChEMBL (<https://www.ebi.ac.uk/chembl/>), DrugBank (<https://go.drugbank.com/>), ETCM (<http://www.tcmip.cn/ETCM/index.php/Home/Index/>), the Similarity Ensemble Approach (SEA, <https://sea.bkslab.org/>), and SwissTargetPrediction (<https://www.swisstargetprediction.ch/>). The resulting pool of candidate targets was consolidated and annotated against the UniProt database (<https://www.uniprot.org/>) to standardize gene identifiers and remove non-human entries, yielding a final set of 106 unique putative drug-target genes for luteolin.

Separately, disease-associated targets for rheumatoid arthritis (RA) were acquired. A systematic search was performed in the GeneCards (<https://www.genecards.org/>) and OMIM (<https://www.omim.org/>) databases using “rheumatoid arthritis” as the query term. From GeneCards, only targets meeting a relevance score threshold of ≥ 20 were retained. All retrieved RA-related targets were subsequently processed through UniProt to eliminate duplicates, correct erroneous entries, and ensure standardized gene nomenclature [17].

2.2 Intersection Analysis and Protein-Protein Interaction Network Construction

To elucidate the potential mechanistic targets of luteolin in RA, its putative drug targets were cross-referenced with the compiled RA-related target list to determine their intersection. This set of common genes, representing the most relevant targets, was illustrated using Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>), an interactive online tool for comparing lists using Venn diagram [18].

The functional relationships among these common targets were then investigated by constructing a protein-protein interaction (PPI) network. The gene list was submitted to the STRING database (v11.5), specifying Homo sapiens as the organism and setting a minimum interaction confidence score of 0.4. The resulting network was imported into Cytoscape software (v3.9.1) for visualization and topological analysis. The centrality of each node was assessed using the “Degree” algorithm implemented via the NetworkAnalyzer plugin. Proteins with the top 5 degree centrality values were identified as hub targets within the network, and their established roles in RA pathophysiology were further substantiated through a review of existing scientific literature [19]. While the PPI network identified DHFR as a hub target, the transcription factor Sp1—a known activator of the DHFR promoter—was not directly predicted by this analysis but was investigated based on established biological knowledge of DHFR transcriptional regulation.

2.3 Functional Enrichment Analysis

To interpret the biological significance of the common targets, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted using the DAVID (<https://david.ncifcrf.gov/>) bioinformatics resource. The GO analysis encompassed the three standard domains: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). Statistical significance was defined as a p -value < 0.05 coupled with a False Discovery Rate (FDR) < 0.05 for both GO terms and KEGG pathways. The top 20 significantly enriched terms and pathways were graphically represented as bubble charts, generated using the R programming language (version 4.2.1) with the ‘clusterProfiler’ (version 4.6.0) and ‘ggplot2’ (version 3.4.0) packages [20].

2.4 Molecular Docking Validation

To evaluate the binding potential between luteolin and the identified hub targets at an atomic level, molecular docking studies were performed. The three-dimensional chemical structure of luteolin was

obtained in SDF format from the TCMSP database (<https://tcmsp-e.com/tcmsp.php>). This structure was energy-minimized and converted into PDB format using Open Babel GUI software (version 3.1.1). The high-resolution three-dimensional crystal structures of the hub target proteins were retrieved from the Protein Data Bank (PDB, <https://www.rcsb.org/>). Both ligand and receptor files were prepared for docking using appropriate tools within the AutoDock (version 4.2.6) suite. Docking simulations were then executed using AutoDock Vina, exploring the binding conformation and affinity of luteolin within the active site of each target protein. The most favorable docking poses for each complex were visually inspected and rendered for presentation using PyMOL software (version 2.5.2) [21].

2.5 Synovial Fibroblast Culture and Induction of MTX Resistance

Primary human RA synovial fibroblasts (RA-FLS) were obtained from YaJi Biological (Shanghai, China, Catalog Number: YS1838C). All cells tested negative for mycoplasma contamination using a PCR-based detection kit (Beyotime, Shanghai, China) prior to use in experiments. This cell bank is accredited by the International Organization for Standardization (ISO) and adheres to the ethical guidelines of the World Health Organization (WHO) and the Declaration of Helsinki for human biological materials. Cells were expanded in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Thermo Fisher Scientific, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Grand Island, NY, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Thermo Fisher Scientific, Grand Island, NY, USA), and maintained in a humidified incubator at 37°C with 5% CO₂. The culture medium was replaced every 2–3 days, and cells were passaged every 3–4 days when reaching 80–90% confluency. RA-FLS were used between passages 3 and 8.

To establish MTX-resistant synovial fibroblasts, RA-FLS were continuously exposed to increasing concentrations of methotrexate (MTX) over a 3-month period. The selection started at 0.01 µM MTX, and the dose was gradually doubled every 2 weeks until reaching 1 µM. At each concentration step, cells were maintained until normal proliferation resumed prior to further MTX escalation. Cells that survived and proliferated in 1 µM MTX were designated RA-FLS/MTX (methotrexate-resistant FLS), while parental RA-FLS maintained in parallel without MTX served as the wild-type control. The resistant phenotype of RA-FLS/MTX was validated by comparing cell viability under MTX exposure and by assessing DHFR expression levels relative to parental cells. Cells that survived and proliferated in 1 µM MTX were designated as RA-FLS/MTX (methotrexate-resistant FLS), while the parental RA-FLS maintained in parallel for the same duration without MTX served as passage-matched wild-type controls. Resistant cells underwent approximately 25–30 population doublings during the 3-month induction period. All experiments comparing resistant and parental cells used these passage-matched controls to rule out culture time artifacts. Three independently derived RA-FLS/MTX batches were used to confirm the reproducibility of key findings. The resistant phenotype was validated by calculating the half-maximal inhibitory concentration (IC₅₀) of MTX. The resistance index (RI = IC₅₀ resistant/IC₅₀ parental) was 3.81.

2.6 Reagents and Treatments

Methotrexate (MTX) (Cat# M9929) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 1× PBS (pH 7.4, Thermo Fisher Scientific, Waltham, MA, USA) for *in vitro* experiments. Luteolin (Cat# L9283, purity ≥98% by HPLC, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO to prepare a 20 mM stock solution, aliquoted, and stored at −20°C in the dark to avoid photodegradation. Working solutions were freshly prepared immediately before use by diluting the stock solution in culture medium, and the final DMSO concentration was kept at ≤0.1% (v/v) in all treatment conditions, including vehicle

controls. For cell treatments, luteolin was diluted in culture medium to final concentrations typically ranging from 2.5 to 20 μM , with 0.1% DMSO as vehicle. Unless specified otherwise, luteolin treatment was for 24 h. In some experiments, cells were co-treated with MTX and luteolin simultaneously (MTX at a fixed concentration chosen to partially inhibit parental cells, e.g., 0.1–0.5 μM , and luteolin at 10 μM). The Sp1 inhibitor mithramycin A (Cat# 200184, Calbiochem, San Diego, CA, USA) was used at 100 nM for 24 h to pharmacologically inhibit Sp1-DNA binding, as a comparison to luteolin's effects. For pathway inhibition studies, selective inhibitors were used: SB203580 (Cat# 1202, 10 μM) for p38 MAPK, and stattic (Cat# 2798, 5 μM) for STAT3 (all from Tocris Bioscience, Bristol, UK), treating cells 1 h prior to MTX or luteolin.

2.7 Cell Viability and Proliferation Assays

Cell viability was assessed using the MTT assay. Parental and RA-FLS/MTX cells (5×10^3 cells per well) were seeded into 96-well plates and allowed to adhere overnight. Cells were then treated with MTX at the indicated concentrations for 72 h. After treatment, MTT solution (5 mg/mL in PBS, Cat# M5655, Sigma-Aldrich, Inc., St. Louis, MO, USA) was added to each well to a final concentration of 0.5 mg/mL, and plates were incubated at 37°C for 4 h. Formazan crystals were dissolved in 100 μL DMSO per well, and absorbance was measured at 570 nm using a microplate reader (iMark, Bio-Rad Laboratories, Hercules, CA, USA), with 630 nm as the reference wavelength.

To evaluate the effect of luteolin on MTX responses, RA-FLS/MTX cells were treated with vehicle or luteolin (10 μM) in the presence or absence of MTX (at the concentrations indicated) for 48 h, followed by MTT assays as described above.

In some experiments, cell proliferation was measured by BrdU incorporation using a BrdU ELISA kit (Cat# 6813, Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions to confirm the antiproliferative effect of treatments.

Apoptosis was analyzed by staining cells with Annexin V-FITC/PI (Cat# 556547, BD Biosciences, San Jose, CA, USA). Briefly, after treatment, cells were harvested, washed twice with cold PBS, and resuspended in 1 $\times$ Annexin V Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.4) at a density of 1×10^5 cells per 100 μL . Cells were stained with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI), mixed gently, and incubated for 15 min at room temperature in the dark. An additional 400 μL of 1 $\times$ Binding Buffer was then added to each tube. All samples were analyzed on a flow cytometer within 30 min after PI staining to avoid false-positive signals due to cell death. Flow cytometric acquisition was performed on a BD FACSCalibur (model number 342975, BD Biosciences, San Jose, CA, USA) within 30 min after staining.

2.8 Cell Transfection

Small interfering RNAs (siRNAs) targeting human Sp1 (siSp1) and DHFR (siDHFR), as well as a non-targeting scramble siRNA negative control (siNC), were purchased from GenePharma (Shanghai, China). The siRNA sequences (sense strand, 5'→3') were as follows: siSp1, CCAACAGAUUAUCACAAAU; siDHFR, GUCUAGAUGAUGCCUAAAATT (the DHFR siRNA is a duplex with a dTdT overhang at the 3' end). All siRNAs were used at a final concentration of 50 nM. RA-FLS/MTX cells were seeded in 6-well plates at a density of 2×10^5 cells/well and transfected with siRNAs at a final concentration of 50 nM using Lipofectamine™ RNAiMAX Transfection Reagent (Cat# 13778075, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). For each well of a 6-well plate, 5 μL of Lipofectamine™ RNAiMAX was diluted in 125 μL of Opti-MEM reduced-serum medium, and 50 nM siRNA was diluted in 125 μL of Opti-MEM. The diluted siRNA and Lipofectamine were mixed (total 250 μL), incubated for 15 min at room temperature, and then added to the well containing 1.75 mL of antibiotic-free growth medium, resulting in a total

transfection volume of 2 mL per well. All transfections were performed according to the manufacturer's protocol. After 48 h, knockdown efficiency was confirmed by RT-qPCR and/or Western blotting, and cells were subsequently subjected to viability assays and Annexin V/PI apoptosis analysis as indicated. For Sp1 overexpression experiments, a full-length human Sp1 expression plasmid (pcDNA3.1-Sp1, Cat# SC119175, OriGene Technologies, Rockville, MD, USA) was used. RA-FLS/MTX cells were seeded in 6-well plates at 2×10^5 cells per well and grown to 70–80% confluence. Transfection was performed using Lipofectamine 3000 (Cat# L3000015, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 2.5 μ g of pcDNA3.1-Sp1 or empty vector (pcDNA3.1, as negative control) was mixed with 5 μ L of Lipofectamine 3000 in 250 μ L of Opti-MEM reduced-serum medium, incubated for 15 min at room temperature, and added to each well containing 1.75 mL of antibiotic-free growth medium. After 48 h, transfection efficiency was confirmed by Western blotting and RT-qPCR for Sp1 expression before subsequent assays.

2.9 Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from parental RA-FLS or RA-FLS/MTX (MTX-resistant) cells using TRIzol reagent (Cat# 15596026, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). To eliminate genomic DNA contamination, RNA samples were treated with DNase I (Cat# 2270A, Takara Bio, Kusatsu, Japan) according to the manufacturer's protocol. Subsequently, 1 μ g DNase-treated RNA was reverse-transcribed to cDNA using a High-Capacity cDNA Kit (Cat# 4368814, Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TB Green[®] Premix Ex Taq[™] II (Tli RNaseH Plus, catalog number RR820A, Takara Bio). The thermal cycling conditions were: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. A melting curve analysis was performed after each run to confirm primer specificity. DHFR mRNA levels were quantified with specific primers (forward 5'-ATGGTTGGTTCGCTAAACCT-3'; reverse 5'-CCTTCTTGAACCCCATAGCA-3'). For Sp1 mRNA detection, the following primers were used: forward 5'-AGTTCCAGACCGTTGATGGG-3'; reverse 5'-GTTTGCACCTGGTATGATCTGT-3'. Transcript levels were normalized to GAPDH (forward 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse 5'-GAAGATGGTGTATGGGATTTC-3') as an internal control. Primers for DHFR and GAPDH were designed to span exon–exon junctions to prevent amplification of genomic DNA. Each reaction included a no-template control (NTC) using nuclease-free water instead of cDNA template to monitor potential contamination. All reactions were performed in triplicate wells, and the $2^{-\Delta\Delta C_t}$ method was used to calculate relative expression. Each condition was run in triplicate wells and experiments were repeated at least three times with independent cell preparations.

2.10 Western Blot Analysis

Parental RA-FLS, MTX-resistant RA-FLS/MTX, or treated RA-FLS/MTX cells were lysed in RIPA buffer (prepared in-house: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors cocktail (cOmplete[™], Cat# 4906837001, Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (PhosSTOP[™], catalog number 4906837001, Roche, Basel, Switzerland). Protein concentrations were determined by BCA assay (Cat# 23225, Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein (20–30 μ g) from each sample were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Burlington, MA, USA). Blots were blocked with 5% non-fat milk and incubated with primary antibodies overnight at 4°C. Primary antibodies were diluted in 5% bovine serum albumin (BSA) in TBST (Tris-buffered saline containing 0.1% Tween-20) at the indicated dilutions. The primary antibodies used included: anti-DHFR (Cat# 45710, 1:1000, Cell Signaling Technology, Danvers, MA, USA),

anti-Sp1 (Cat# 07-645, 1:1000, Millipore, Burlington, MA, USA), anti-phospho-p38 MAPK (Thr180/Tyr182, Cat# 4511, 1:1000, Cell Signaling), anti-p38 MAPK (total p38, Cat# 8690, 1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-phospho-ERK1/2 (Thr202/Tyr204, Cat# 4370, 1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-ERK1/2 (Cat# 4695, 1:1000, Cell Signaling), anti-phospho-STAT3 (Tyr705, Cat# 9145, 1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-STAT3 (Cat# 9139, 1:1000, Cell Signaling Technology, Danvers, MA, USA), and anti-GAPDH (Cat# G8795, 1:5000, Sigma-Aldrich, St. Louis, MO, USA) as loading controls. After washing, blots were incubated with HRP-conjugated secondary antibodies (Cat# 111-035-003, 1:5000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Proteins were visualized using ECL substrate (Cat# 34580, Thermo Fisher Scientific, Waltham, MA, USA) and chemiluminescence detection using a ChemiDoc™ MP Imaging System (Model number 12003154, Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were quantified by densitometry using ImageJ software (Version 1.53t, National Institutes of Health, Bethesda, MD, USA) within the linear, non-saturated exposure range. Western blot experiments were performed using three independent biological replicates. (separate cell preparations). In each biological replicate, samples were loaded in duplicate technical replicates on the same membrane. The densitometric values from the two technical replicates were averaged to represent that biological replicate. Statistical analysis was then performed on the three biological replicate means ($n = 3$). Data are presented as mean $\pm$ SD. Phospho-protein signals were normalized to the corresponding total protein, and target protein levels were normalized to GAPDH. Relative expression levels were calculated as fold change compared with the control group.

2.11 Luciferase Reporter Assay

A 1.2-kb fragment of the human DHFR gene promoter (−1158 to +47 relative to the transcription start site), containing the two consensus Sp1 binding sites, was PCR-amplified from genomic DNA and cloned into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). The construct was verified by sequencing. RA-FLS/MTX cells at ~70% confluence were co-transfected with the DHFR promoter luciferase plasmid (0.5 μ g) and a Renilla luciferase internal control plasmid (pRL-TK, 0.05 μ g) using Lipofectamine 3000 (Cat# L3000015, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Transfection efficiency was monitored using the Renilla luciferase internal control and was consistent across experimental groups. After 24 h, transfected cells were treated with luteolin (10 μ M) or vehicle, or with mithramycin (100 nM) as a positive control for Sp1 inhibition. Lysates were collected 16 h later, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Cat# E1910, Promega, Madison, WI, USA). Firefly luciferase readings were normalized to Renilla luciferase. Results are expressed as relative luciferase units (RLU) compared to the control. In some assays, a mutant DHFR promoter reporter was used in which the Sp1 binding sites (at −63 and −28) were mutated (ggggc to ttttc) by site-directed mutagenesis, to assess Sp1 dependence.

2.12 Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed to evaluate Sp1 binding to the DHFR promoter in RA-FLS/MTX, with and without luteolin treatment. Approximately 1×10^6 cells per condition were fixed with 1% formaldehyde for 10 min at 37°C to cross-link DNA-protein complexes. Glycine (125 mM) was added to quench the reaction. Nuclei were isolated and chromatin was sheared by sonication using a Branson Sonifier 250 (Model number 101-063-700, Branson Ultrasonics, Danbury, CT, USA) to an average length of 200–500 bp. Sheared chromatin was incubated overnight at 4°C with 5 μ g of anti-Sp1 antibody (Millipore, Burlington, MA, USA) or with normal IgG as a negative control. Protein-DNA complexes were pulled down with Protein

A/G agarose beads (Thermo Fisher Scientific, Waltham, MA, USA). After extensive washing, cross-links were reversed by heating at 65°C, and DNA was purified using a spin column. The recovered DNA was analyzed by RT-qPCR using primers flanking the Sp1 binding region of the DHFR promoter (forward 5'-GCTGTGCCTCCGAGTTCATA-3'; reverse 5'-GGCTCAGCCTCTTCCCTATA-3', amplifying a region -150 to +10). To validate ChIP efficiency, a positive control primer pair targeting a known Sp1-binding region within the GAPDH promoter was included in each ChIP-qPCR run. ChIP-qPCR data are presented as percent input, calculated as (ChIP signal/input chromatin signal) × 100%. The IgG control was used as a negative control to assess non-specific background but was not used for normalization. Specific enrichment is shown relative to the input sample. ChIP-qPCR experiments were performed using three independent biological replicates (separate cell culture preparations), each with duplicate technical qPCR measurements. Data are presented as mean ± SD (n = 3 biological replicates).

2.13 CIA Mouse Model and Treatment

Male DBA/1 mice (8–10 weeks old, 20–25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China, Certificate No. SCXK 2021-0006). Mice were housed individually under specific pathogen-free conditions at Kangtai Medical Laboratory Services Hebei Co., Ltd. (Baoding, China; 12 h light/dark cycle, 22 ± 2°C, 50 ± 10% humidity, sterile corn cob bedding, ad libitum water and chow). Animals were acclimated for 7 days before experiments. CIA was induced, as described [17]. Mice were immunized on Day 0 with 100 µg of bovine type II collagen (Cat# 20021, Chondrex, Inc., Woodinville, WA, USA) emulsified in complete Freund's adjuvant and injected intradermally at the base of the tail. On Day 21, a booster injection of 100 µg type II collagen in incomplete Freund's adjuvant was administered. Starting from Day 25 (onset of arthritis in initial animals), mice were randomly assigned to four treatment groups (n = 8 per group) using a computer-generated random number table (Excel 2019). Allocation was concealed in sealed, opaque envelopes. Treatment groups: Vehicle, MTX alone, Luteolin alone, and MTX + Luteolin. Each mouse was housed individually under specific pathogen-free conditions. Sample size was determined by G*Power 3.1 (effect size d = 1.5 from pilot data, α = 0.05, power = 0.80). MTX was administered intraperitoneally at 1 mg/kg twice weekly, as previously described [22]. The two weekly injections were spaced 3.5 days apart (Monday morning and Thursday afternoon), and luteolin was administered via oral gavage at 50 mg/kg daily. Vehicle mice received appropriate solvents (PBS for MTX, 0.5% carboxymethylcellulose for luteolin). Treatment continued for 3 weeks (Day 25–46). Mice were monitored daily for signs of arthritis from Day 25 (arthritis onset) to Day 46 (end of treatment). Arthritis severity in daily monitoring was assessed using a 0–4 scale per paw (0 = normal, 1 = mild swelling and erythema, 2 = moderate swelling, 3 = pronounced swelling, 4 = severe swelling with joint rigidity), as detailed in Section 2.14. The sum of the four paws gave a maximum score of 16 per mouse, and body weight was measured weekly throughout the study as an indicator of systemic toxicity. At study end, mice were euthanized by cervical dislocation under deep isoflurane anesthesia (5% induction, 2% maintenance). All animal procedures were approved by the Institutional Animal Care and Use Committee of Kangtai Medical Laboratory Services Hebei Co., Ltd. (Approval No.: MDL2024-10-13-01). Kangtai Medical Laboratory Services Hebei Co., Ltd. is a certified contract research organization (CRO) that provides animal facility services under its own IACUC, which is accredited by the Hebei Provincial Association for Laboratory Animal Science. The animal experiments described in this study were conducted at the Kangtai facility. The IACUC of the authors' home institutions (Guangzhou University of Chinese Medicine and Hebei University) has reviewed and accepted the ethical approval from Kangtai as equivalent to their own standards. The collaborative arrangement and ethical oversight are fully compliant with institutional policies.

2.14 Arthritis Assessment and Tissue Analysis

Arthritis severity was evaluated throughout the study by clinical scoring and measurement of paw swelling. Both hind paws were measured, and the mean thickness was used for analysis (forepaws were not measured due to predominant hind-limb involvement in the CIA model). Clinical arthritis scores were assessed using a standard scoring system [23] (0–4 per paw; maximum score 16 per mouse), where 0 = normal, 1 = mild swelling and erythema, 2 = moderate swelling, 3 = pronounced swelling, and 4 = severe swelling and joint rigidity/deformity. Scoring and paw measurements were performed by two independent investigators blinded to treatment groups. Paw thickness was measured using digital calipers.

At the end of the treatment period (Day 46), mice were euthanized and hind limbs were collected for histological analysis. Joint tissues were fixed in 4% paraformaldehyde, decalcified in 10% EDTA, embedded in paraffin, and sectioned at 5 μ m. Sections were stained with hematoxylin and eosin (H&E) and Safranin O–Fast Green to evaluate synovial inflammation, cartilage integrity, and bone erosion. For H&E staining, sections were deparaffinized in xylene, rehydrated through graded ethanol, stained with hematoxylin (Cat# HHS32, Sigma-Aldrich, St. Louis, MO, USA) for 5 min, rinsed, differentiated in 0.3% acid alcohol, blued in 0.2% ammonia water, and counterstained with eosin (Cat# HT110116, Sigma-Aldrich, St. Louis, MO, USA) for 2 min. For Safranin O–Fast Green staining, sections were stained with 0.02% Fast Green (Cat# F7258, Sigma-Aldrich, St. Louis, MO, USA) for 5 min, rinsed, then stained with 0.1% Safranin O (Cat# S8884, Sigma-Aldrich, St. Louis, MO, USA) for 10 min. All slides were dehydrated, cleared, and mounted with resinous mounting medium.

The ankle joints from both hind limbs were dissected, embedded, and sectioned. For each mouse, the histopathological scores from the left and right ankle joints were averaged to obtain a single value per mouse. Histopathological scoring was performed by two independent investigators blinded to the treatment groups, and discrepancies were resolved by consensus. Inflammation, cartilage damage, and bone erosion were each scored on a 0–3 scale as follows: Inflammatory score: 0 = no inflammation, 1 = mild perivascular infiltration, 2 = moderate synovial infiltration, 3 = severe diffuse infiltration; Cartilage damage score: 0 = intact cartilage, 1 = mild proteoglycan loss, 2 = moderate fibrillation, 3 = severe erosion; Bone erosion score: 0 = no erosion, 1 = mild cortical erosion, 2 = moderate trabecular damage, 3 = severe bone destruction. The final histopathological score for each joint was calculated as the sum of the three subscores.

2.15 Serum Cytokine Measurement

At the end of the treatment period, blood samples were collected from mice and allowed to clot at room temperature, followed by centrifugation at 3000 $\times$ g for 10 min to obtain serum. Serum IL-6 levels were measured using a mouse IL-6 ELISA kit (Cat# PI326, Beyotime, Shanghai, China) according to the manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader, and IL-6 concentrations were calculated from a standard curve.

2.16 Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD) of at least three independent experiments or biological replicates. Sample size calculation was performed using G*Power 3.1 software, with a power of 80% and an alpha level of 0.05. For *in vitro* experiments, each condition was tested in triplicate wells and repeated at least three times independently ($n = 3$ biological replicates). For the CIA mouse model, sample size was determined using G*Power 3.1 software (two-tailed *t*-test, effect size $d = 1.5$ based on pilot data, $\alpha = 0.05$, power = 0.80), which indicated 6–7 mice per group. To account for potential variability and animal loss (<20%), we enrolled 8 mice per group. All animals completed the study without attrition.

Prior to hypothesis testing, homogeneity of variance was assessed using Levene's test, and data were log-transformed when variance heterogeneity was detected. For comparisons between two groups, the unpaired Student's *t*-test was used. For comparisons among three or more groups, one-way analysis of variance (ANOVA) was applied, followed by Tukey's honest significant difference (HSD) post-hoc test for multiple comparisons, with *p*-value adjusted for multiple testing. For time-course data in the CIA model, arthritis score trajectories were analyzed using repeated-measures ANOVA with Greenhouse-Geisser correction when the sphericity assumption was violated. A *p*-value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 9.3 (GraphPad Software, Inc., La Jolla, CA, USA). Given the multiple endpoints assessed (clinical scores, histology, serum cytokines, and organ function markers), all measurements were performed under blinded conditions. The consistency of findings was confirmed by the main experiment (*n* = 8 per group). Repeated-measures ANOVA was used for longitudinal arthritis scores to reduce the risk of Type I error due to multiple time points.

3 Results

3.1 Target Prediction for Luteolin and Rheumatoid Arthritis

To define the potential therapeutic landscape of luteolin in rheumatoid arthritis (RA), we first compiled luteolin-related targets and RA-associated genes from multiple public databases. A total of 9, 4, 72, 79, and 104 putative drug-target genes for luteolin were retrieved from ChEMBL, DrugBank, ETCM, SEA, and Swiss Target Prediction databases, respectively. After merging and removing duplicates, 166 unique luteolin-associated targets were identified. For rheumatoid arthritis (RA), 543 and 7326 RA-related genes were collected from OMIM and GeneCards, respectively, resulting in 7707 unique RA-associated genes after deduplication.

3.2 Common Targets and Protein-Protein Interaction Network

To identify candidate targets through which luteolin may exert anti-RA effects, we next examined the overlap between luteolin-associated targets and RA-related genes and mapped their protein interactions. Intersection of the two datasets yielded 102 overlapping targets, which represent potential therapeutic targets of luteolin against RA (Fig. 1A). These common targets were used to construct a PPI network via STRING, comprising 106 nodes and 10,302 edges (Fig. 1B). Topological analysis identified ACTB, AKT1, DHFR, EGFR, and STAT3 as hub proteins based on high degree values. Among them, ACTB, AKT1, and EGFR ranked highest and are known to play central roles in RA-related inflammatory signaling. Among the hub targets, ACTB showed the highest degree centrality. However, as a ubiquitously expressed cytoskeletal protein frequently used as a loading control, its high connectivity likely reflects non-specific physical interactions rather than pathway-specific functional relevance. Therefore, ACTB was not prioritized for further experimental validation. In contrast, DHFR was of particular interest given its established role as the direct pharmacological target of MTX. Notably, the DHFR promoter contains tandem Sp1 binding sites, and Sp1 is a well-known transcriptional activator of DHFR [24], prompting us to examine whether luteolin modulates the Sp1-DHFR axis.

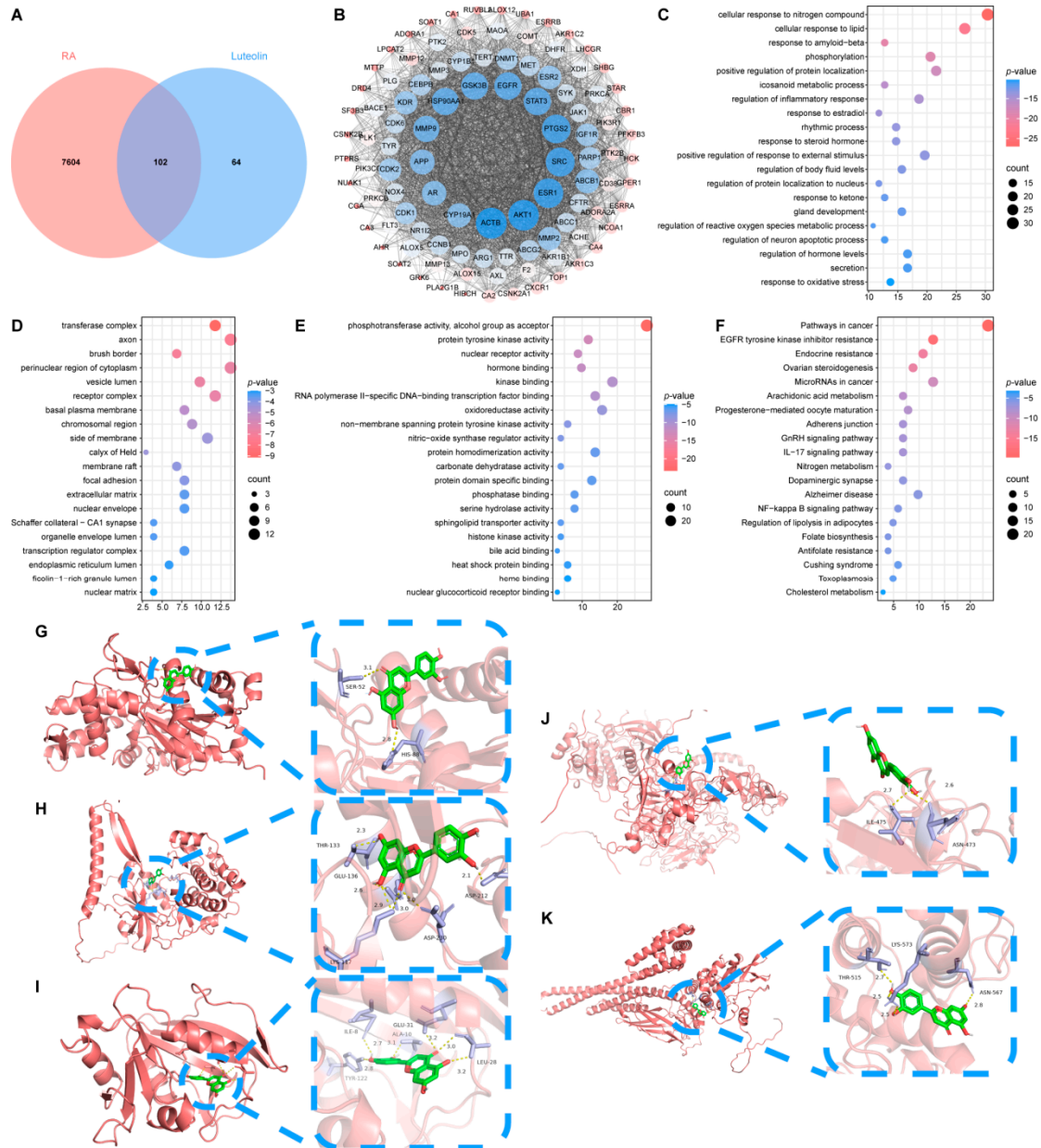


Figure 1: Network pharmacology analysis of luteolin against rheumatoid arthritis (RA). (A) Venn diagram illustrating the overlapping targets between luteolin and RA. (B) Protein-protein interaction (PPI) network of the common targets. Core hub targets are highlighted. (C–E) Gene Ontology (GO) enrichment analysis of the common targets in terms of biological processes (C), cellular components (D), and molecular functions (E). The top 20 enriched terms are shown. (F) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the common targets. The top 20 enriched pathways are displayed. (G–K) Molecular docking models showing the binding poses of luteolin with the core target proteins: (G) ACTB, (H) AKT1, (I) DHFR, (J) EGFR, and (K) STAT3. Hydrogen bonds are indicated by blue dashed lines.

3.3 GO and KEGG Enrichment Analyses

Functional enrichment analyses indicated that the common luteolin-RA targets are involved in pathways related to cellular stress responses and kinase-driven signaling, supporting their potential roles in RA

pathogenesis. GO enrichment analysis revealed that the 102 common targets were significantly enriched in 316 biological processes (BP), 81 molecular functions (MF), and 117 cellular components (CC). The top enriched BP terms included “cellular response to nitrogen compound,” “cellular response to lipid,” and “response to amyloid-beta” (Fig. 1C). Key CC terms were “transferase complex, transferring phosphorus-containing groups,” “axon,” and “brush border” (Fig. 1D). Enriched MF terms involved “phosphotransferase activity, alcohol group as acceptor,” “protein tyrosine kinase activity,” and “nuclear receptor activity” (Fig. 1E).

KEGG pathway analysis showed significant enrichment in pathways such as “Pathways in cancer,” “EGFR tyrosine kinase inhibitor resistance,” “Endocrine resistance,” “Ovarian steroidogenesis,” and “MicroRNAs in cancer” (Fig. 1F).

3.4 Molecular Docking Validation

To validate whether luteolin could directly bind the key hub proteins identified in the network analysis, we performed molecular docking simulations. Molecular docking was performed to assess the binding affinity of luteolin to the five hub proteins. Luteolin exhibited high binding affinity to all targets (Fig. 1G–K), with docking scores of -7.2 kcal/mol for ACTB, -7.8 kcal/mol for AKT1, -8.3 kcal/mol for DHFR, -7.9 kcal/mol for EGFR, and -8.0 kcal/mol for STAT3. DHFR showed the strongest binding affinity. Interaction analysis indicated that luteolin primarily binds via non-covalent interactions, including conventional hydrogen bonds and carbon-hydrogen bonds. Notably, luteolin formed hydrogen bonds with DHFR residues ILE8 and GLU31, and established hydrophobic interactions with LEU28 and TYR122, which likely contributed to the high binding affinity (-8.3 kcal/mol) observed for the DHFR–luteolin complex.

3.5 Luteolin Sensitizes MTX-Resistant FLS to MTX-Induced Apoptosis by Reducing DHFR Expression

To experimentally confirm MTX resistance and test whether luteolin could restore MTX responsiveness, an MTX-resistant human FLS model was established and evaluated for viability and apoptosis under MTX and luteolin treatments. We generated RA-FLS cells by progressively exposing primary RA-FLS to increasing MTX concentrations over three months. RA-FLS displayed markedly higher MTX tolerance than parental, MTX-sensitive FLS. As shown in Fig. 2A,B, parental FLS demonstrated a dose-dependent decline in viability when exposed to MTX, whereas RA-FLS maintained significantly greater viability under the same MTX doses. For example, 100 nM MTX reduced parental FLS viability by ~60% ($***p < 0.001$ vs. 0 nM), whereas the same dose caused only a ~15% decrease in RA-FLS ($*p < 0.05$ vs. 0 nM), confirming the resistant phenotype (Fig. 2A,B). Dose-response curves from three independent MTT assays yielded IC_{50} values of 85.5 ± 12.3 nM for parental FLS and 326.0 ± 45.7 nM for RA-FLS/MTX, giving a resistance index of 3.81 (approximately 3.8-fold resistance). This confirms a stable MTX-resistant phenotype.

We next determined whether luteolin could functionally reverse resistance and enhance MTX-induced cytotoxicity (Fig. 2C–E). In RA-FLS, MTX (50 nM) alone had little effect on viability over 72 h. Luteolin (20 μ M) alone reduced viability by approximately 15–20% at 48 h and 72 h ($*p < 0.05$ vs. control); however, the combination of MTX + luteolin resulted in a significantly greater decrease in viability at 48 h and 72 h compared to either agent alone ($**p < 0.01$, $***p < 0.001$) (Fig. 2C). By 72 h, combined treatment lowered RA-FLS viability to ~60% of control, compared to ~80–90% with single-agent treatments ($**p$ -value < 0.001 vs. MTX alone or luteolin alone). In line with these findings, Flow cytometric Annexin V/PI analysis further revealed that combination treatment increased both early and late apoptotic populations in RA-FLS cells. Specifically, MTX + luteolin induced 7.14% Annexin V+/PI– early apoptosis and 8.87% Annexin V+/PI+ late apoptosis, totaling 16.01% apoptotic cells. In contrast, MTX alone induced 3.62% early apoptosis and 10.10%

late apoptosis (13.72% total), while luteolin alone induced 5.25% early apoptosis and 4.01% late apoptosis (9.26% total) (Fig. 2D,E, $p < 0.01$). Collectively, these results demonstrate that luteolin substantially enhances MTX's cytotoxic efficacy in resistant FLS by promoting both growth inhibition and apoptosis when used in combination.

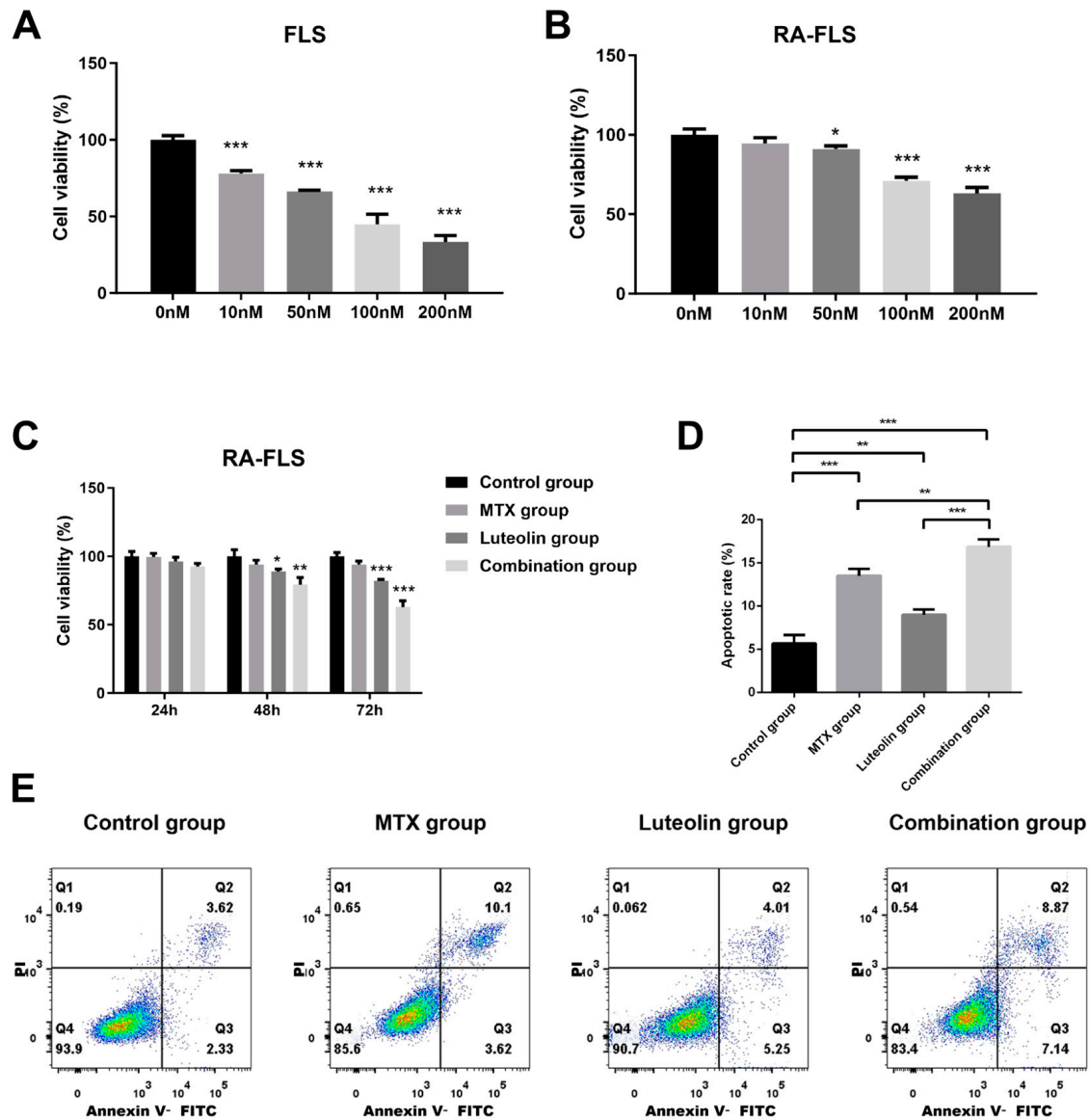


Figure 2: Luteolin sensitizes MTX-resistant FLS to MTX-induced apoptosis. (A,B) Dose-dependent inhibition of cell viability by MTX treatment (0–200 nM) in parental FLS (A) and MTX-resistant FLS (RA-FLS, B), assessed by MTT assay at 72 h. Luteolin significantly inhibited viability in both cell types, with greater sensitivity in parental FLS. (C) Time-course effects on viability of RA-FLS after treatment with MTX (0.5 μ M), luteolin (10 μ M), or combination thereof, demonstrating significant synergistic suppression with combination treatment. (D) Apoptotic rates determined by Annexin V/PI staining after 24 h of treatment. The combination group displayed significantly increased apoptosis compared to either agent alone. (E) Representative flow cytometric dot plots showing early (Annexin V+/PI–) and late (Annexin V+/PI+) apoptosis in different treatment conditions. Combination treatment markedly enhanced the apoptotic cell population. Data shown as mean $\pm$ SD from triplicate independent experiments. Statistical differences indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6 Luteolin Suppresses DHFR Overexpression in Resistant RA-FLS/MTX Cells, Reducing DHFR mRNA and Lowering Protein Levels

Because DHFR is the principal target of MTX and was predicted as a hub gene, we tested whether DHFR upregulation contributes to MTX resistance and whether luteolin can suppress DHFR expression. Basal DHFR expression was dramatically elevated in RA-FLS compared to parental cells (Fig. 3A,B). DHFR mRNA was approximately 15-fold higher in RA-FLS ($***p < 0.001$, Fig. 3A), and Western blot analysis confirmed markedly increased DHFR protein (Fig. 3B). To determine whether luteolin affects DHFR, we treated RA-FLS with MTX, luteolin, or both (Fig. 3C,D). Luteolin alone visibly reduced DHFR protein levels, while MTX alone exerted only a moderate effect. Notably, combined MTX + luteolin treatment nearly abolished DHFR expression, lowering it to ~20% of control ($***p < 0.001$ vs. control). Densitometric quantitation showed that either luteolin or MTX alone partially suppressed DHFR (Fig. 3C,D), but their combination was significantly more potent than either agent alone ($p < 0.01$). These findings suggest that luteolin can effectively reverse the aberrantly high DHFR levels in resistant FLS, potentially explaining its ability to resensitize cells to MTX.

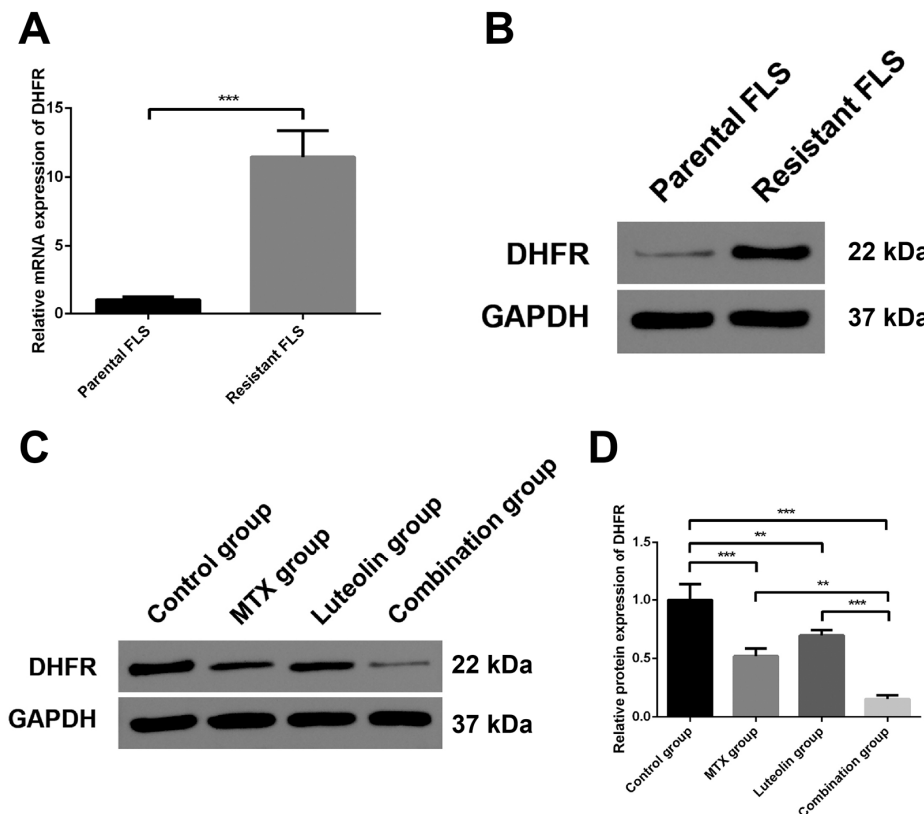


Figure 3: Luteolin suppresses dihydrofolate reductase (DHFR) expression in MTX-resistant synovial fibroblasts. (A) RT-qPCR analysis showing significantly elevated DHFR mRNA levels in MTX-resistant compared to parental FLS. (B) Western blot validation showing correspondingly increased DHFR protein expression in resistant cells. GAPDH used as loading control. Lane assignment (left to right): lane 1, Parental FLS; lane 2, MTX-resistant FLS (RA-FLS). (C,D) Effect of MTX, luteolin, and combination treatment on DHFR protein levels in resistant cells. Representative Western blot (Lane assignment (left to right): lane 1, Control (vehicle); lane 2, MTX alone; lane 3, luteolin alone; lane 4, MTX + luteolin (combination)). (C) and quantitative densitometric analysis, (D) showing significant luteolin-mediated suppression of DHFR, with the greatest effect seen in the combination treatment group. Data shown as mean $\pm$ SD from three independent experiments ($n = 3$ per group); $**p < 0.01$, $***p < 0.001$.

3.7 Luteolin Downregulates Sp1 to Repress DHFR Transcription, Reducing Sp1 and Attenuating Sp1-Driven DHFR Promoter Activation

To elucidate the upstream regulatory mechanism of DHFR suppression by luteolin, we focused on the transcription factor Sp1, a known activator of the GC-rich DHFR promoter. Overexpression of Sp1 in RA-FLS significantly elevated DHFR expression (~4-fold), enhanced viability (Fig. 4A–C). In contrast, silencing Sp1 or DHFR via siRNA reversed these effects, lowering DHFR mRNA/viability. Importantly, luteolin countered Sp1-driven resistance: in Sp1-overexpressing RA-FLS cells, luteolin reduced DHFR mRNA to near-baseline and abolished the survival advantage conferred by Sp1 (comparable to siDHFR, Fig. 4A–C). Representative Annexin V/PI profiles showed that transcription factor overexpression decreased apoptosis (1.00% early and 3.91% late apoptosis; 4.91% total), whereas siRNA knockdown markedly increased apoptosis (5.03% early and 11.90% late apoptosis; 16.93% total). Luteolin similarly restored apoptosis in factor-overexpressing cells (4.23% early and 11.00% late apoptosis; 15.23% total) (Fig. 4D).

We further confirmed that luteolin directly suppresses Sp1 expression and inhibits Sp1-dependent DHFR transcriptional activation. Luteolin alone lowered Sp1 mRNA to ~50% of control ($***p < 0.001$), and the combination of MTX + luteolin suppressed Sp1 to ~20% of control, whereas MTX alone had a more modest effect (Fig. 4E–H). Western blot analysis showed concordant suppression of Sp1 protein. In a DHFR promoter–luciferase assay, Sp1 overexpression increased promoter activity ~2.3-fold (Fig. 4F,G), and luteolin reduced this Sp1-induced transactivation by ~35% ($p < 0.01$). Together, these results indicate that luteolin inhibits Sp1-dependent DHFR transcription, thereby restoring MTX sensitivity in resistant FLS.

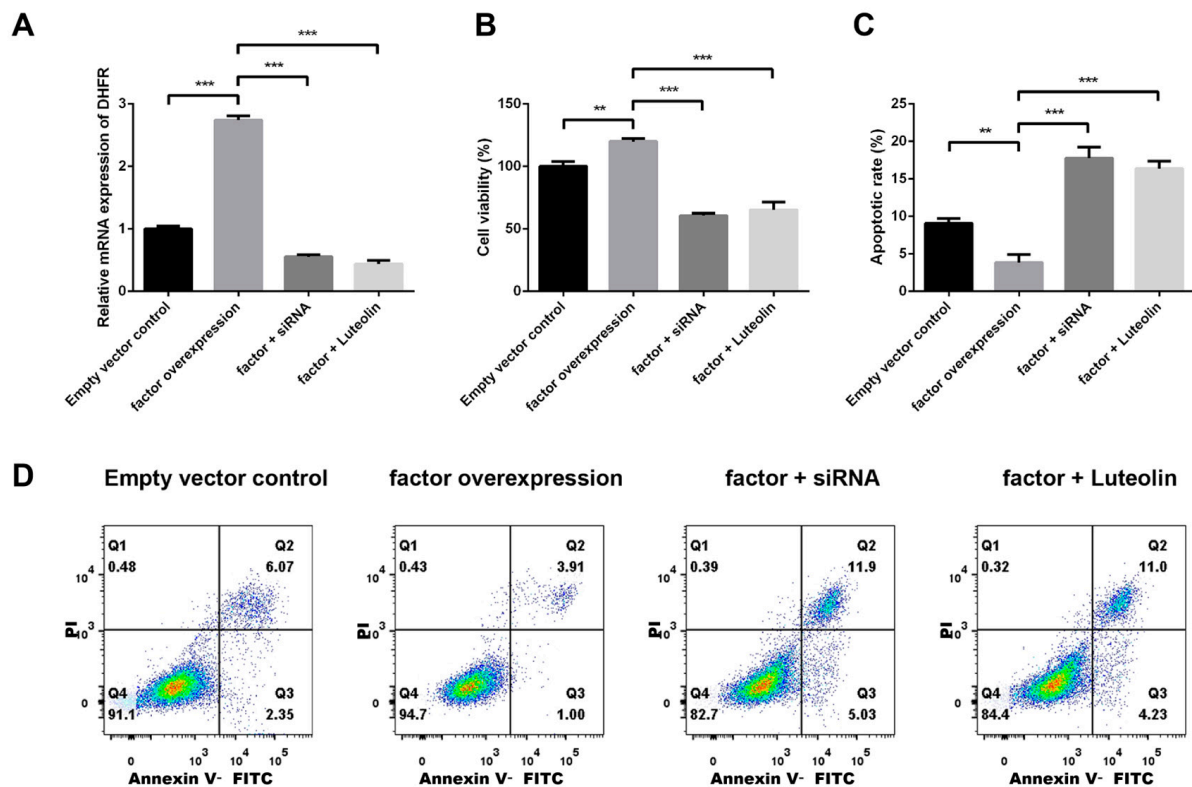


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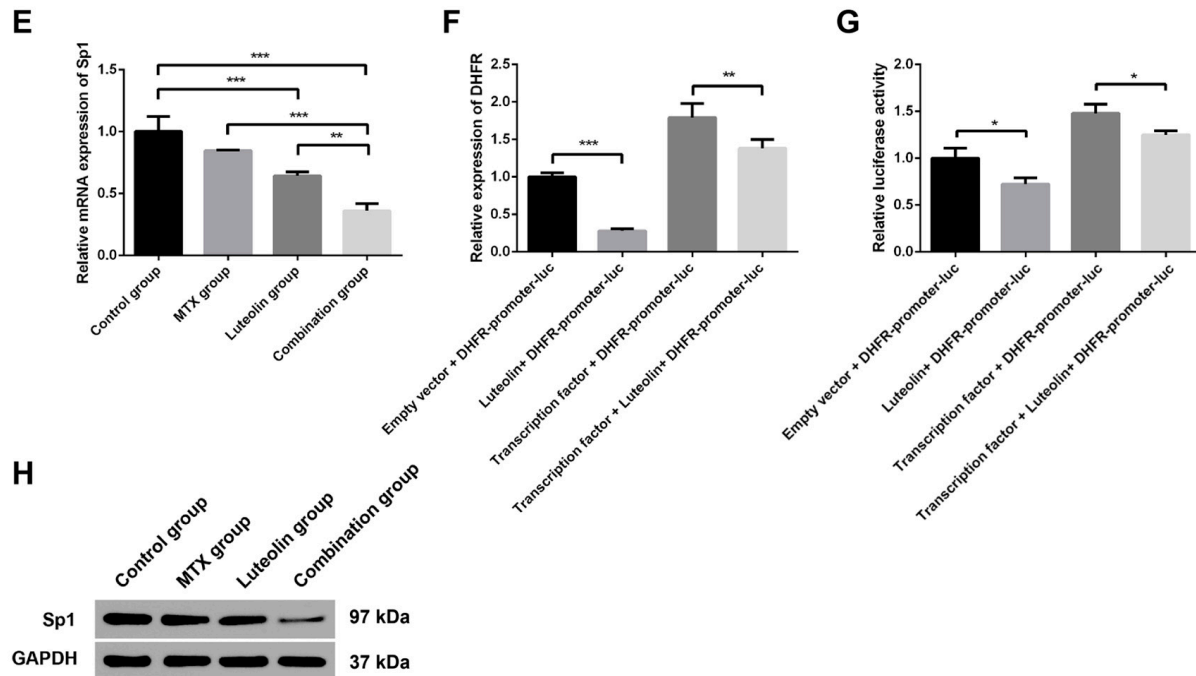


Figure 4: Luteolin inhibits Sp1-mediated transcription of the DHFR gene, attenuating MTX resistance.

(A) DHFR mRNA expression measured by RT-qPCR in MTX-resistant FLS treated with empty vector, transcription factor (Sp1) overexpression plasmid, Sp1-siRNA, or luteolin. Luteolin significantly suppressed Sp1-induced DHFR transcription. (B,C) Cell viability (B) and apoptotic rates (C) in MTX-resistant FLS under the same conditions, demonstrating luteolin's ability to significantly reverse Sp1-induced cell survival and resistance. (D) Flow cytometric dot plots illustrating enhanced apoptosis with luteolin and siRNA-mediated Sp1 inhibition compared to the Sp1 overexpression group. (E) Relative expression levels of Sp1 mRNA assessed by RT-qPCR. Treatment with luteolin notably reduced Sp1 expression, especially in combination with MTX. (F,G) Luciferase reporter assays using DHFR promoter-driven constructs. Treatment with luteolin substantially decreased Sp1-mediated activation of the DHFR promoter compared with controls, confirming transcriptional regulation. (H) Representative Western blot showing significantly reduced Sp1 protein expression after luteolin treatment in resistant cells. GAPDH served as an internal control. Lane assignment (left to right): lane 1, Control (vehicle); lane 2, MTX alone; lane 3, luteolin alone; lane 4, MTX + luteolin (combination). Data shown as mean $\pm$ SD from three independent experiments ($n = 3$ per group); significant differences indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.8 Luteolin Inhibits P38 MAPK/STAT3 Signaling in Resistant RA-FLS/MTX Cells, Decreasing P-P38 and P-STAT3 and Thereby Limiting Sp1/DHFR Activation

Because p38 MAPK and STAT3 are known upstream regulators of stress-responsive transcription factors, we evaluated whether hyperactivated p38/STAT3 signaling contributes to Sp1-mediated DHFR upregulation in resistant FLS and whether luteolin suppresses this pathway. RA-FLS cells showed elevated phospho-p38 MAPK and phospho-STAT3, with no change in total p38 or STAT3 (Fig. 5A–E). Luteolin treatment significantly reduced these phosphorylated forms, indicating inhibition of p38 and STAT3 activation. Densitometric analysis across three experiments showed a ~50% decrease in p-p38 and a ~40% decrease in p-STAT3 following luteolin.

Pharmacological pathway inhibition further supported these findings. RA-FLS/MTX treated with the p38 inhibitor SB203580 (10 μ M) exhibited a ~30% reduction in DHFR mRNA (Fig. 5F,G). These data suggest that p38 (and to a lesser degree STAT3) signaling helps maintain DHFR mRNA expression in

resistant FLS by enhancing Sp1 activity. Cell viability was significantly decreased by the combination of p38 or STAT3 inhibitor with luteolin compared to either agent alone (Fig. 5H). Annexin V/PI staining revealed that combined inhibitor + luteolin treatment produced the strongest apoptotic response, with 8.15% early apoptosis and 12.50% late apoptosis, totaling 20.65% apoptotic cells, compared with 7.88% in controls (Fig. 5I,J). ChIP-qPCR analysis revealed that treatment with either the p38 inhibitor or luteolin significantly reduced the binding of Sp1 to the DHFR promoter compared with the control group, and the combination of both agents resulted in an even more pronounced reduction (Fig. 5K). To verify that luteolin specifically represses DHFR transcription through Sp1-binding sites, we introduced mutations into the two canonical Sp1 recognition sequences (positions –63 and –28) of the DHFR promoter reporter. This mutant construct (DHFR-mut) was then tested alongside the wild-type reporter (DHFR-wt) in dual-luciferase assays. Overexpression of Sp1 strongly increased luciferase activity driven by DHFR-wt. In contrast, the same Sp1 overexpression failed to elevate luciferase expression from DHFR-mut, indicating that disruption of Sp1 binding completely abolished the transcriptional response (Fig. 5L). Collectively, these data demonstrate that Sp1 occupancy at the DHFR promoter is markedly enhanced under the tested conditions.

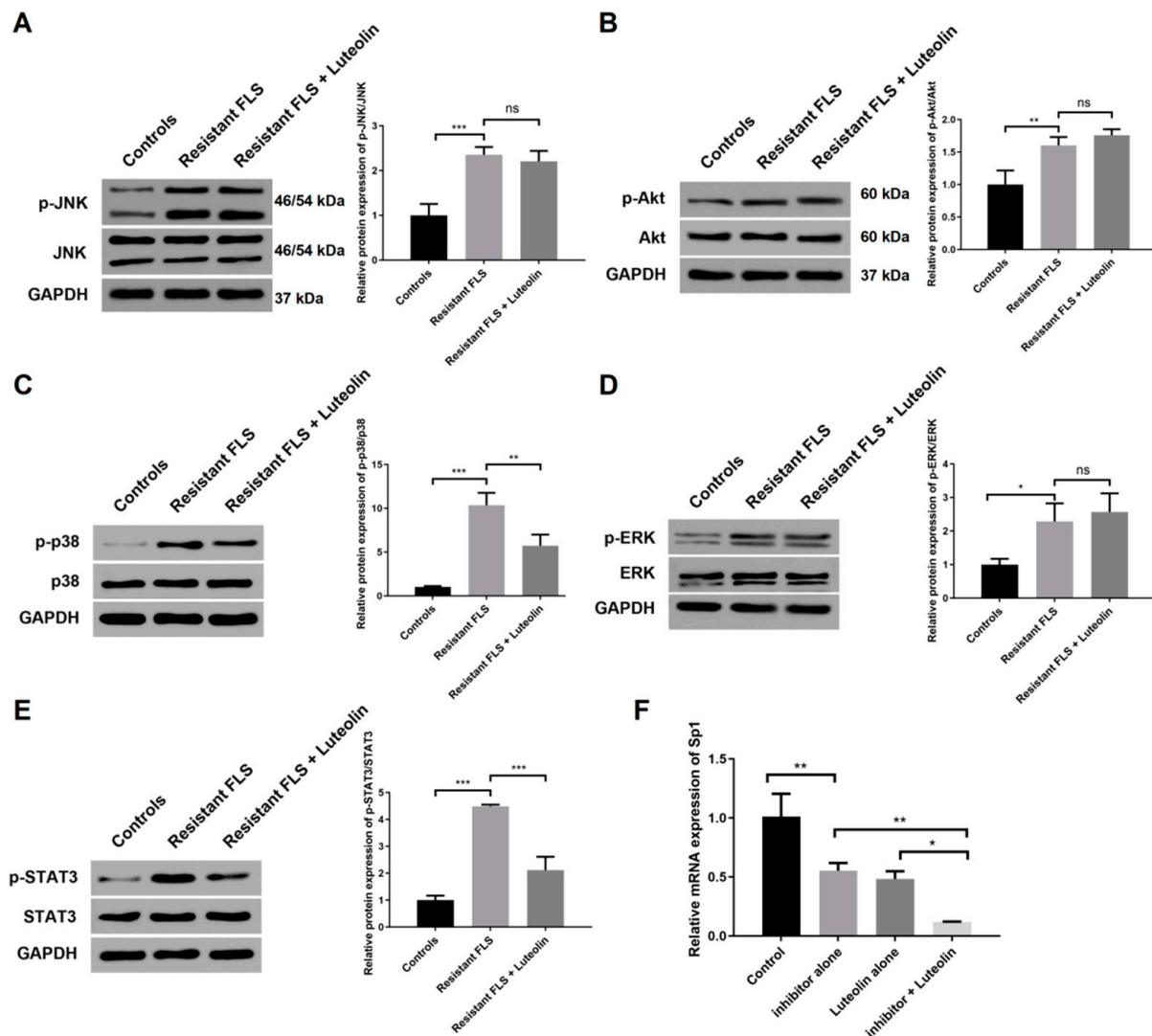


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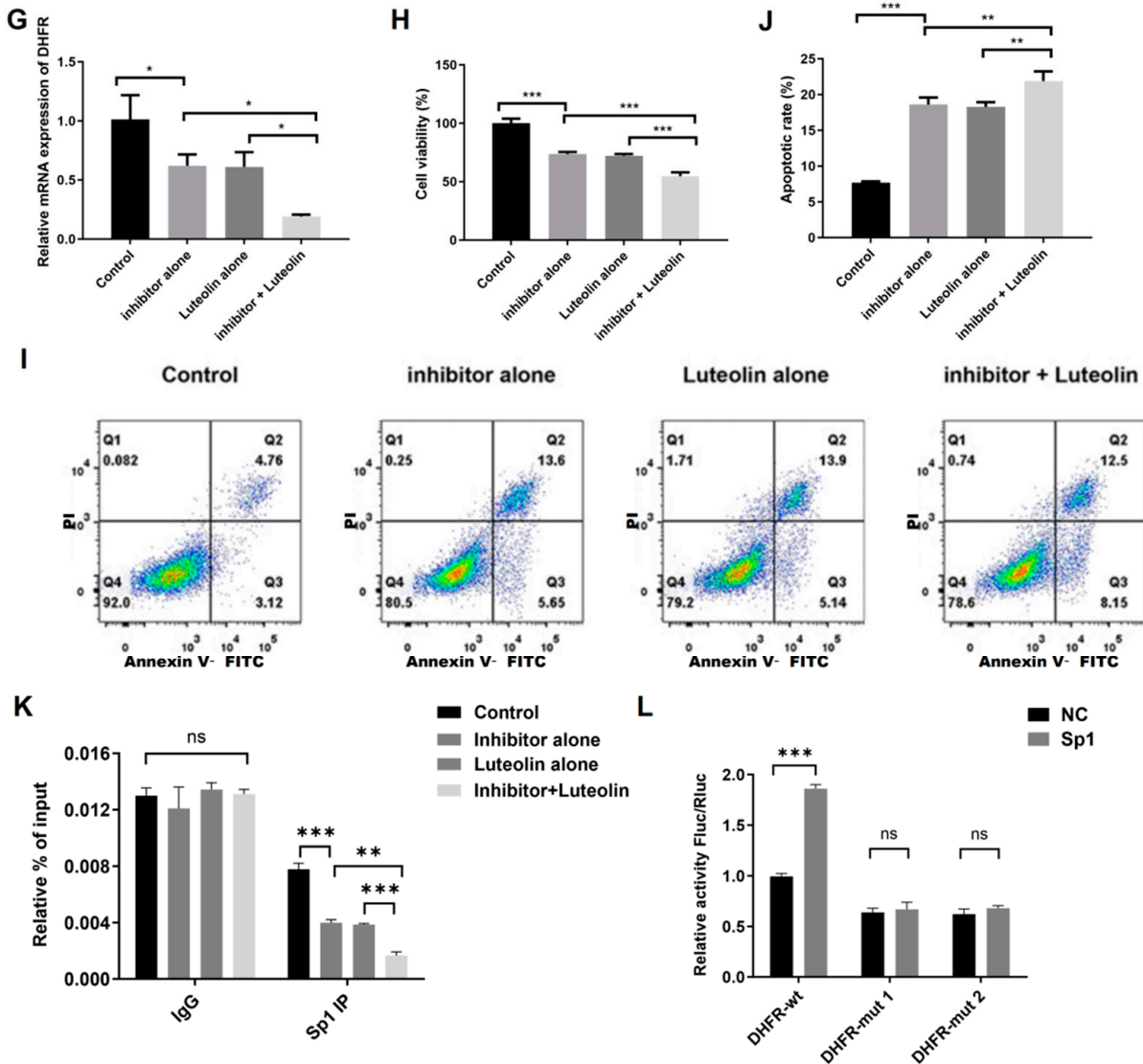


Figure 5: Luteolin inhibits DHFR transcription and restores MTX sensitivity through suppression of p38 MAPK and STAT3 signaling pathways. (A–E) Representative Western blots showing signaling proteins in controls, resistant FLS (RA-FLS), and resistant FLS + luteolin groups. Lane assignment for each blot (left to right): lane 1, Controls; lane 2, Resistant FLS; lane 3, Resistant FLS + luteolin. GAPDH was used as a loading control. Targets: p-JNK/JNK (A), p-Akt/Akt (B), p-p38/p38 (C), p-ERK/ERK (D), p-STAT3/STAT3 (E). (F,G) RT-qPCR measurement of Sp1 (F) and DHFR (G) mRNA expression in resistant cells after inhibition of p38 MAPK/STAT3 pathways and/or luteolin treatment. Combined luteolin and inhibitor treatment profoundly suppressed Sp1 and DHFR expression. (H) Cell viability after treatment with p38/STAT3 inhibitor, luteolin, or their combinations. (I) Representative flow cytometry plots illustrating apoptosis induction in resistant cells treated with luteolin alone, pathway inhibitors alone, and their combination. Combined treatments yielded maximum apoptotic cell populations. (J) Quantitative bar graph showing percentages of early (Annexin V+/PI–) and late (Annexin V+/PI+) apoptotic cells. (K) ChIP-qPCR analysis of Sp1 binding to the DHFR promoter. (L) Dual-luciferase reporter assays in 293T cells assessing the impact of Sp1 overexpression on wild-type DHFR promoter activity compared to that of two Sp1-binding site mutants: DHFR-mut1 (at –63) and DHFR-mut2 (at –48). Data shown as mean ± SD from three independent experiments (n = 3 per group). Statistical significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, “ns” denotes not significant ($p \geq 0.05$).

3.9 Luteolin Synergizes with MTX in CIA Mice, Reducing Serum IL-6 and Arthritis Scores While Alleviating MTX-Associated Hepatic and Renal Injury Markers

To determine whether luteolin can enhance MTX efficacy *in vivo*, we evaluated combination therapy in the CIA mouse model and assessed inflammation, disease severity, and systemic toxicity. CIA mice developed pronounced synovial inflammation, high serum IL-6, and joint damage, mirroring features of human RA. Both MTX and luteolin monotherapy partially mitigated disease, reducing serum IL-6 from ~150 pg/mL to ~80–90 pg/mL ($***p < 0.001$ vs. CIA control) (Fig. 6A), and lowering arthritis index scores from ~4.5 to ~2–3 (Fig. 6B). The combination treatment showed improved control of disease progression over time, as reflected by reduced arthritis scores (Fig. 6C) and paw swelling (Fig. 6D). Weekly arthritis scores (mean $\pm$ SD) indicated that the MTX + luteolin group exhibited a progressive decline in clinical severity, with a mean arthritis score of 0.67 ± 0.52 at week 4, compared with 1.50 ± 0.55 in the MTX-alone group and 1.50 ± 0.55 in the luteolin-alone group. Consistently, arthritis scores in the CIA control group remained high throughout the study (3.83 ± 0.41 at week 4). Strikingly, the combination of MTX + luteolin reduced IL-6 to (~50 pg/mL), which was significantly lower than the CIA control ($***p < 0.001$) and also lower than either monotherapy, but remained above the control group level. Clinical arthritis severity was greatly diminished (score < 1 , $***p < 0.001$ vs. monotherapies). This combined regimen also led to marked histological improvements, including preserved cartilage architecture and minimal synovitis (Fig. 6E).

We also measured serum biomarkers of liver and kidney function (ALT, AST, BUN, creatinine). Compared with CIA controls, all treatment groups showed reduced levels of these markers (Fig. 6F–I). Notably, the MTX + luteolin group exhibited the lowest levels, although the MTX dose used (1 mg/kg twice weekly) is not known to be hepatotoxic in mice ($***p < 0.001$). Therefore, these reductions likely reflect the overall improvement of systemic inflammation rather than direct organ-protective effects against MTX toxicity. Taken together, these *in vivo* findings demonstrate that luteolin synergizes with MTX to reduce inflammatory damage and joint destruction while sparing hepatic and renal function. Hence, luteolin may serve as a beneficial adjunct to MTX, not only amplifying its therapeutic impact but also potentially mitigating its toxicity.

Overall, the CIA experiments provide proof-of-concept that luteolin can enhance MTX efficacy in a clinically relevant RA model. These convergent data indicate that luteolin's action persists in the complex inflammatory environment of arthritis and may translate to improved outcomes in RA management.

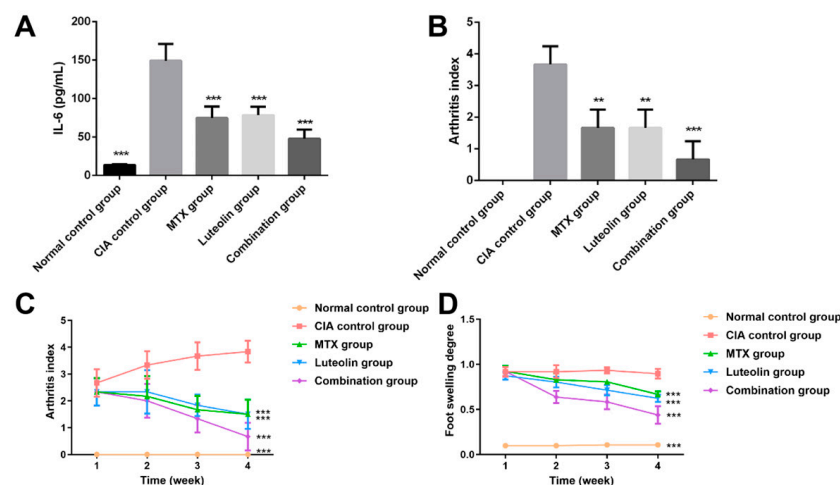


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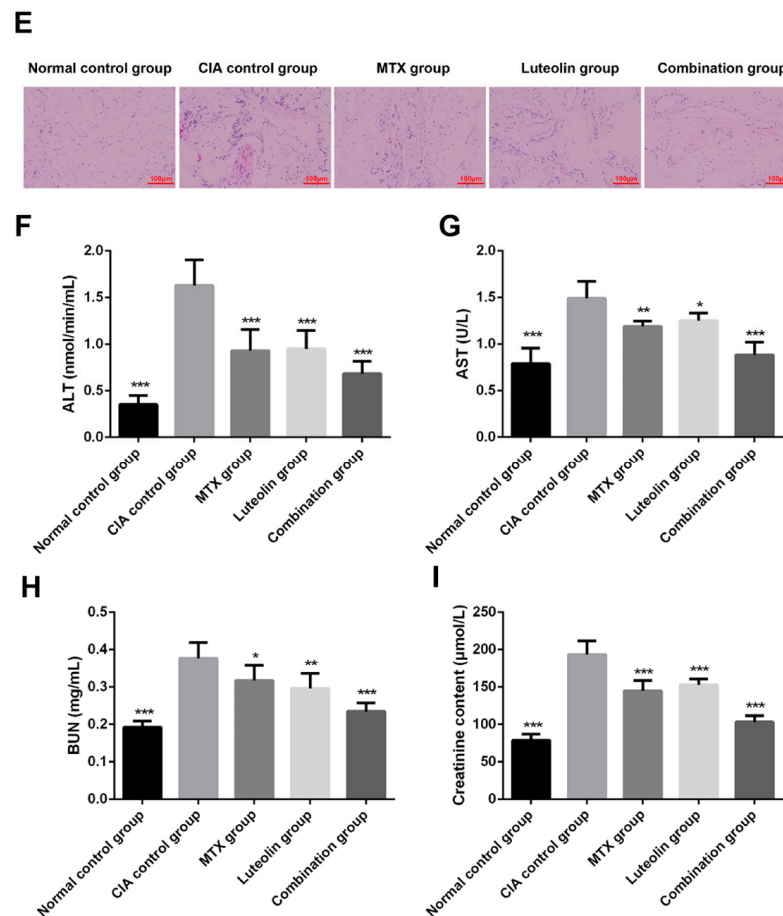


Figure 6: Luteolin enhances the therapeutic efficacy of methotrexate (MTX) in CIA mice. (A) Serum IL-6 concentrations in CIA mice treated with MTX, luteolin, or a combination thereof. Combination treatment significantly reduced serum IL-6 compared to either treatment alone. (B) Arthritis index scores at the study endpoint (day 46). Combination therapy markedly lowered arthritis severity relative to monotherapies. (C) Time-dependent changes in arthritis index over four weeks of treatment. Combination treatment rapidly and consistently attenuated disease progression from the second week onward. (D) Foot swelling measurements recorded weekly, showing significantly decreased paw edema in the MTX and luteolin combination group. (E) Representative histopathological images of ankle joint sections stained with hematoxylin-eosin. MTX + luteolin treatment notably reduced synovial inflammation and hyperplasia compared to single treatments. Scale bar = 100 μ m. (F–I) Serum levels of alanine aminotransferase (ALT, F), aspartate aminotransferase (AST, G), blood urea nitrogen (BUN, H), and creatinine (I). The combination therapy group showed the lowest levels among all treated groups. Given the low MTX dose used, these reductions likely reflect decreased systemic inflammation rather than direct hepatoprotective or renoprotective effects. Data shown as mean $\pm$ SD from three independent experiments ($n = 8$ per group); statistical significance indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus CIA control group.

4 Discussion

In this study, we demonstrate that luteolin reverses MTX resistance in rheumatoid arthritis synovial fibroblasts through a dual mechanism: suppressing Sp1-dependent DHFR transcription and inhibiting p38 MAPK/STAT3 signaling. Network pharmacology predicted DHFR as a key target of luteolin, and molecular docking confirmed high binding affinity. Using an MTX-resistant cell model (RA-FLS/MTX), we found that luteolin reduces DHFR expression by blocking Sp1 binding to the DHFR promoter and

attenuates p38/STAT3 survival signals, thereby resensitizing cells to MTX. In the collagen-induced arthritis mouse model, co-administration of luteolin and MTX significantly improved arthritis scores and synovial pathology compared to MTX alone. These findings identify a novel mechanism by which a natural flavonoid overcomes antifolate resistance and support the potential use of luteolin as an adjunctive therapy for MTX-refractory RA.

4.1 Comparison with Previous Studies and Novelty of This Work

Unlike previous studies that primarily focused on luteolin's anti-inflammatory and anti-proliferative effects in RA [25], our work addresses the clinically relevant issue of MTX refractoriness and provides evidence that luteolin can resensitize MTX-resistant synovial fibroblasts to MTX. Mechanistically, our findings support a model in which luteolin suppresses DHFR transcription by reducing Sp1-mediated promoter activation, together with inhibition of pro-survival signaling (p38 MAPK/STAT3) that may reinforce Sp1 activity. Therefore, luteolin may represent an adjunct approach that extends beyond inflammation control by targeting a DHFR/Sp1-centered resistance axis in MTX-refractory RA.

4.2 Role of DHFR Overexpression in Methotrexate Resistance

Our work reinforces the concept that DHFR upregulation is a central mechanism of MTX resistance, even in non-malignant cells like synovial fibroblasts. DHFR is the classical target of MTX; increased DHFR gene copy number or expression can allow cells to maintain folate metabolism in the presence of MTX [26]. Amplification of DHFR has been widely studied in cancer cell drug resistance. Consistent with this, both the mRNA levels (Fig. 2A) and protein levels (Fig. 2B) of DHFR were increased in the RA-FLS/MTX samples. This aligns with previous studies where RA patient lymphocytes on long-term MTX showed elevated DHFR protein without gene amplification [26]. Those patients did not uniformly exhibit clinical resistance, suggesting DHFR elevation might be an early compensatory change. In a controlled setting, our results demonstrate that DHFR overexpression in FLS correlates with and likely mediates a substantial loss of MTX efficacy. By knocking down DHFR (Fig. 4) or inhibiting its expression with luteolin, we were able to partly reverse resistance. Thus, targeting DHFR expression is a viable approach to overcome MTX resistance. It is worth noting that DHFR is not the only mechanism—our resistant FLS also showed pathway changes (p38, STAT3) and potentially other metabolic adaptations. However, DHFR is a bottleneck target: restoring MTX's ability to inhibit DHFR can re-engage its downstream anti-proliferative effects (e.g., accumulation of polyglutamated MTX and AICAR transformylase inhibition leading to adenosine release [27]). The pronounced effect of luteolin in restoring MTX-induced apoptosis in resistant cells underscores how critical DHFR levels are for MTX's cytotoxic action.

4.3 Sp1 as a Regulator of DHFR in RA-FLS

Although our network pharmacology analysis did not directly identify Sp1, the DHFR promoter is known to contain multiple Sp1 binding sites that drive its basal transcription [28,29]. Given that Sp1 is a master regulator of DHFR, we hypothesized that luteolin might suppress DHFR expression by inhibiting Sp1. Indeed, we provide direct evidence that Sp1 contributes to DHFR transcription in MTX-resistant synovial fibroblasts. The human DHFR promoter is known to be Sp1-responsive [30], and earlier studies in tumor cells showed that increased Sp1 activity can drive DHFR gene expression and MTX resistance [31]. Noé et al. reported that treating cells with a phorbol ester (TPA) increased MTX resistance via an Sp1-dependent rise in DHFR, and that mithramycin (an Sp1 inhibitor) could reverse this resistance [32]. Our data extend these findings to RA-FLS, indicating that in an inflammatory context, Sp1 may become aberrantly active and

induce DHFR. We observed basal Sp1 binding on the DHFR promoter in resistant FLS, and Sp1 inhibition (mithramycin or luteolin) suppressed DHFR promoter activity. These results suggest that transcriptional upregulation of DHFR in RA-FLS/MTX is at least partly Sp1-driven. Sp1 itself is ubiquitous and regulates many genes (housekeeping and inducible). It can be activated by post-translational modifications (phosphorylation, acetylation) in response to signaling pathways. Previous studies have identified multiple signaling pathways that regulate Sp1 transcriptional activity, including MAPKs (ERK, p38, JNK), PI3K/Akt, PKC, and CDKs, as well as acetylation by p300 and HDACs [33]. These pathways can modulate Sp1 through phosphorylation, acetylation, nuclear translocation, or protein stability [34]. Among these, p38 MAPK has been reported to directly phosphorylate Sp1 at Thr⁴⁵³ and Thr⁷³⁹, enhancing its binding to GC-rich promoters. STAT3, while not a direct Sp1 kinase, can cooperate with Sp1 on certain gene promoters or be activated by upstream signals (e.g., IL-6) that also affect Sp1 [35]. In our MTX-resistant RA-FLS model, we observed selective hyperactivation of p38 MAPK and STAT3, but not ERK, JNK, or Akt. Luteolin treatment inhibited both p-p38 and p-STAT3, and pharmacological inhibition of p38 (SB203580) or STAT3 (stattic) reduced Sp1-dependent DHFR expression [36,37]. Thus, our data support a model in which luteolin targets the p38/STAT3 axis to suppress Sp1-driven DHFR transcription, while other known Sp1-regulatory pathways (e.g., Akt, PKC) do not appear to play a major role in this specific resistance context. In RA, FLS are exposed to inflammatory cytokines (TNF- α , IL-1 β , IL-6, etc.) that trigger signaling cascades and may increase Sp1's activity or nuclear accumulation [38]. Indeed, Sp1 has been shown to mediate the effect of TNF- α on certain FLS genes (e.g., some matrix metalloproteinases) [39]. Our results imply that chronic MTX exposure (and possibly the cellular stress and cytokine milieu in RA) might amplify Sp1 activity, thereby elevating DHFR as a survival adaptation. Targeting Sp1 pharmacologically has been considered in cancer but is limited by toxicity (mithramycin is an older chemotherapy with significant side effects) [40]. Luteolin's ability to inhibit Sp1 without overt toxicity (at least in our models) is therefore notable.

4.4 Luteolin's Multi-Target Mechanism—Impact on MAPK and STAT3

Luteolin is known to be a multi-target molecule, which likely explains its efficacy in our system. We found that luteolin dampened the p38 MAPK and STAT3 pathways in resistant FLS. p38 MAPK is a critical pathway in RA-FLS, regulating the production of cytokines and MMPs. Consistent with recent reports that natural products such as anemarsaponin B alleviate inflammatory injuries via MAPK modulation [41], our data show that luteolin also suppresses p38 MAPK activation in MTX-resistant FLS. Interestingly, p38 can phosphorylate Sp1 on certain residues, enhancing Sp1's transcriptional activity in some contexts [42]. By inhibiting p38, luteolin might reduce such activating modifications on Sp1, thereby impairing Sp1's binding to the DHFR promoter. Additionally, p38 and JNK signaling contribute to AP-1 and NF- κ B activation; while our focus was on Sp1, a broader suppression of these pathways by luteolin would diminish the pro-inflammatory state of FLS [43]. STAT3 is another key transcription factor downstream of IL-6 and other cytokines in RA. High STAT3 activity in RA-FLS and T cells promotes survival and proliferation [44]. We observed elevated STAT3 activation in RA-FLS/MTX, which could be due to autocrine IL-6 signaling under stress. Luteolin's reduction of STAT3 phosphorylation (possibly by inhibiting upstream JAKs or cytokine production) could remove growth signals and make cells more susceptible to MTX. *In vivo*, we saw that MTX alone did not fully suppress p-STAT3 in the synovium, whereas luteolin co-therapy did. This indicates luteolin can fill gaps in MTX's mechanism by targeting inflammatory signaling. Prior studies have documented luteolin's inhibition of IL-6 and TNF- α production by synovial cells [45], which would also feed into less STAT3 activation. Therefore, luteolin's effect in overcoming MTX resistance is two-fold: (1) directly lowering the target (DHFR) through Sp1 interference, and (2) broadly attenuating survival pathways

(like MAPK/STAT3) that make FLS refractory to apoptosis. This multi-pronged action is advantageous in a complex disease like RA.

4.5 Implications for Therapy—Luteolin as an Adjunct to MTX

Our *in vivo* results in CIA mice provide encouraging evidence that adding luteolin can enhance MTX therapy in an RA model. Not only was arthritis severity significantly reduced, but the combination was associated with biochemical changes (lower synovial DHFR, p-STAT3) consistent with our mechanistic hypothesis. These findings resonate with a recent study on another flavonoid, nobiletin, which also improved MTX response in CIA mice by inhibiting a different resistance mechanism (P-glycoprotein-mediated drug efflux) [46]. Natural compounds like luteolin and nobiletin are appealing as adjuncts because of their relative safety and ability to target multiple pathways. Luteolin is abundant in foods (e.g., celery, parsley) and has shown low toxicity in animal studies even at high doses [45]. In fact, a co-ultramicrosonized formulation of luteolin with palmitoylethanolamide has been tested in rodent arthritis models, with results suggesting reduced inflammation and pain [47]. While bioavailability of luteolin can be a concern (it is moderately absorbed orally and undergoes metabolism), it is important to relate the concentrations used in our *in vitro* experiments to those achievable in humans. In this study, luteolin was used at 2.5–20 μM , with 10 μM being the typical concentration for mechanistic studies. However, reported peak plasma concentrations of free (unconjugated) luteolin after oral administration in humans are generally below 1 μM . Thus, the effective *in vitro* concentrations may exceed the levels that can be achieved systemically with conventional oral dosing in patients. Nevertheless, several points warrant consideration. First, in our CIA mouse model, oral luteolin at 50 mg/kg/day produced therapeutic effects, and rodent pharmacokinetic studies suggest that luteolin can accumulate in synovial tissue to levels higher than those in plasma. Second, novel delivery approaches (nanoparticles, liposomes) are being explored to enhance tissue targeting. If luteolin or its derivatives can be effectively delivered to the joint, they may help overcome partial responses to MTX. This could be especially relevant for patients who cannot tolerate escalation to biologics or targeted synthetic DMARDs due to cost or contraindications.

4.6 Limitations and Future Directions

This study has several limitations: (1) the MTX-resistant RA-FLS model was established *in vitro*, which may not fully recapitulate the *in vivo* microenvironment of RA; (2) the sample size of CIA mice was relatively small ($n = 8$ per group), and larger-scale studies are needed to confirm the findings; (3) the pharmacokinetic properties of luteolin in RA patients remain unclear, and clinical trials are required to evaluate its bioavailability and safety.

Although the MTX dose used in our CIA model (1 mg/kg twice weekly) is lower than typical hepatotoxic doses in mice, we observed that adding luteolin further reduced serum ALT, AST, BUN, and creatinine compared with MTX alone. However, these reductions may be secondary to better control of systemic inflammation in the combination group rather than direct hepatic or renal protection. Future studies using higher MTX doses or dedicated toxicity models are needed to evaluate potential organ-protective effects of luteolin.

Furthermore, while our data using Sp1 knockdown and pharmacological inhibition support an Sp1-dependent mechanism, future studies using a DHFR promoter with mutated Sp1 binding sites would provide additional validation.

In conclusion, we have shown that luteolin effectively mitigates methotrexate resistance in RA synovial fibroblasts by targeting the transcriptional control of DHFR through Sp1 inhibition and by blunting critical

survival pathways. These findings broaden our understanding of how natural compounds can modulate drug resistance mechanisms in inflammatory diseases. Incorporating such agents could enhance the efficacy of existing RA treatments, potentially allowing patients to regain responsiveness to first-line therapies like MTX. Further research is justified to translate these findings into clinical strategies for overcoming drug resistance in rheumatoid arthritis.

5 Conclusion

Luteolin reverses MTX resistance in RA by dual mechanisms: inhibiting Sp1-mediated DHFR transcription and suppressing p38 MAPK/STAT3 signaling. *In vitro* and *in vivo* studies validate luteolin as a promising adjunct to MTX therapy, providing a translational strategy to overcome antifolate resistance in RA patients.

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Availability of Data and Materials: The data supporting the findings of this study are available within the article. The public databases used in this study (ChEMBL, DrugBank, ETCM, SEA, SwissTargetPrediction, UniProt, GeneCards, OMIM, STRING, and DAVID) are openly accessible at the URLs provided in the Methods section.

Ethics Approval: All animal experiments were approved by the Institutional Animal Care and Use Committee of Kangtai Medical Laboratory Services Hebei Co., Ltd. (Approval No.: MDL2024-10-13-01) and were conducted by this company. Kangtai is a certified contract research organization (CRO) that provides animal facility services under its own IACUC, which is accredited by the Hebei Provincial Association for Laboratory Animal Science. Although Kangtai is not affiliated with the authors' institutions, the authors' home institutions (Guangzhou University of Chinese Medicine and Hebei University) have reviewed and accepted this ethical approval as equivalent to their own standards. The collaborative arrangement is fully compliant with institutional policies. The animal procedures followed the ARRIVE guidelines and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. di Matteo A, Bathon JM, Emery P. Rheumatoid arthritis. *Lancet*. 2023;402(10416):2019–33. [[CrossRef](#)].
2. Zack SR, Alzoubi O, Satoeya N, Singh KP, Deen S, Nijim W, et al. Another Notch in the belt of rheumatoid arthritis. *Arthritis Rheumatol*. 2024;76(10):1475–87. [[CrossRef](#)].
3. Gravallese EM, Firestein GS. Rheumatoid arthritis—Common origins, divergent mechanisms. *N Engl J Med*. 2023;388(6):529–42. [[CrossRef](#)].
4. Friedman B, Cronstein B. Methotrexate mechanism in treatment of rheumatoid arthritis. *Jt Bone Spine*. 2019;86(3):301–7. [[CrossRef](#)].
5. Liu R, Song Y, Li C, Zhang Z, Xue Z, Huang Q, et al. The naturally occurring flavonoid nobiletin reverses methotrexate resistance via inhibition of P-glycoprotein synthesis. *J Biol Chem*. 2022;298(4):101756. [[CrossRef](#)].
6. Blume SW, Snyder RC, Ray R, Thomas S, Koller CA, Miller DM. Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene *in vitro* and *in vivo*. *J Clin Investig*. 1991;88(5):1613–21. [[CrossRef](#)].

7. Zhang CY, Feng YX, Yu Y, Sun WJ, Bai J, Chen F, et al. The molecular mechanism of resistance to methotrexate in mouse methotrexate-resistant cells by cancer drug resistance and metabolism SuperArray. *Basic Clin Pharmacol Toxicol.* 2006;99(2):141–5. [\[CrossRef\]](#).
8. Rodenhuis S, Kremer JM, Bertino JR. Increase of dihydrofolate reductase in peripheral blood lymphocytes of rheumatoid arthritis patients treated with low-dose oral methotrexate. *Arthritis Rheum.* 1987;30(4):369–74. [\[CrossRef\]](#).
9. Abali EE, Skacel NE, Celikkaya H, Hsieh YC. Chapter 9 regulation of human dihydrofolate reductase activity and expression. *Vitam Horm.* 2008;79:267–92. [\[CrossRef\]](#).
10. Scionti I, Michelacci F, Pasello M, Hattinger CM, Alberghini M, Manara MC, et al. Clinical impact of the methotrexate resistance-associated genes C-MYC and dihydrofolate reductase (DHFR) in high-grade osteosarcoma. *Ann Oncol.* 2008;19(8):1500–8. [\[CrossRef\]](#).
11. Vizcaíno C, Mansilla S, Portugal J. Sp1 transcription factor: A long-standing target in cancer chemotherapy. *Pharmacol Ther.* 2015;152:111–24. [\[CrossRef\]](#).
12. Hou Y, Wu J, Huang Q, Guo L. Luteolin inhibits proliferation and affects the function of stimulated rat synovial fibroblasts. *Cell Biol Int.* 2009;33(2):135–47. [\[CrossRef\]](#).
13. Guan Y, Zhao X, Liu W, Wang Y. Galuteolin suppresses proliferation and inflammation in TNF- α -induced RA-FLS cells by activating HMOX1 to regulate IKK β /NF- κ B pathway. *J Orthop Surg Res.* 2020;15(1):484. [\[CrossRef\]](#).
14. Ji M, Ryu HJ, Hong JH. Signalling and putative therapeutic molecules on the regulation of synoviocyte signalling in rheumatoid arthritis. *Bone Joint Res.* 2021;10(4):285–97. [\[CrossRef\]](#).
15. Liu X, Wang Z, Qian H, Tao W, Zhang Y, Hu C, et al. Natural medicines of targeted rheumatoid arthritis and its action mechanism. *Front Immunol.* 2022;13:945129. [\[CrossRef\]](#).
16. Aziz N, Kim MY, Cho JY. Anti-inflammatory effects of luteolin: A review of *in vitro*, *in vivo*, and *in silico* studies. *J Ethnopharmacol.* 2018;225:342–58. [\[CrossRef\]](#).
17. Luan J, Hu Z, Cheng J, Zhang R, Yang P, Guo H, et al. Applicability and implementation of the collagen-induced arthritis mouse model, including protocols (Review). *Exp Ther Med.* 2021;22(3):939. [\[CrossRef\]](#).
18. Zhou W, Zhang H, Wang X, Kang J, Guo W, Zhou L, et al. Network pharmacology to unveil the mechanism of Moluodan in the treatment of chronic atrophic gastritis. *Phytomedicine.* 2022;95:153837. [\[CrossRef\]](#).
19. Song S, Wen F, Gu S, Gu P, Huang W, Ruan S, et al. Network pharmacology study and experimental validation of Yiqi Huayu decoction inducing ferroptosis in gastric cancer. *Front Oncol.* 2022;12:820059. [\[CrossRef\]](#).
20. Yu Z, Yin Y, Wang X, Zhou G, Shang Y, Zhang Y, et al. Network pharmacology and experimental evaluation of *Lonicera japonica* Flos in rheumatoid arthritis. *Biotechnol Appl Biochem.* 2026;73(2):611–22. [\[CrossRef\]](#).
21. Li M, Cui Y, Wu X, Yang X, Huang C, Yu L, et al. Integrating network pharmacology to investigate the mechanism of quercetin's action through AKT inhibition in co-expressed genes associated with polycystic ovary syndrome and endometrial cancer. *Int J Biol Macromol.* 2025;297:139468. [\[CrossRef\]](#).
22. Park EM, Park MK, Lee DG, Baek SY, Woo JW, Kwok SK, et al. Reciprocal regulation of TH17 and regulatory T cells by methotrexate and its therapeutic effects in collagen-induced arthritis (CIA). *J Rheum Dis.* 2013;20(2):94. [\[CrossRef\]](#).
23. Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. *Nat Protoc.* 2007;2(5):1269–75. [\[CrossRef\]](#).
24. Ding J, Fayyaz AI, Ding Y, Liang D, Luo M. Role of specificity protein 1 (SP1) in cardiovascular diseases: Pathological mechanisms and therapeutic potentials. *Biomolecules.* 2024;14(7):807. [\[CrossRef\]](#).
25. Xiao B, Li J, Qiao Z, Yang S, Kwan HY, Jiang T, et al. Therapeutic effects of *Siegesbeckia orientalis* L. and its active compound luteolin in rheumatoid arthritis: Network pharmacology, molecular docking and experimental validation. *J Ethnopharmacol.* 2023;317:116852. [\[CrossRef\]](#).
26. Meng XN, Ma JF, Liu YH, Li SQ, Wang X, Zhu J, et al. Dynamic genomic changes in methotrexate-resistant human cancer cell lines beyond *DHFR* amplification suggest potential new targets for preventing drug resistance. *Br J Cancer.* 2024;130(11):1819–27. [\[CrossRef\]](#).
27. Cronstein BN, Aune TM. Methotrexate and its mechanisms of action in inflammatory arthritis. *Nat Rev Rheumatol.* 2020;16(3):145–54. [\[CrossRef\]](#).
28. Schilling LJ, Farnham PJ. Transcriptional regulation of the dihydrofolate reductase/rep-3 locus. *Crit Rev Eukaryot Gene Expr.* 1994;4(1):19–53. [\[CrossRef\]](#).

29. Wells J, Held P, Illenye S, Heintz NH. Protein-DNA interactions at the major and minor promoters of the divergently transcribed *dhfr* and *rep3* genes during the Chinese hamster ovary cell cycle. *Mol Cell Biol.* 1996;16(2):634–47. [\[CrossRef\]](#).
30. Tarnowski M, Paradowska-Gorycka A, Dąbrowska-Zamojcin E, Czerewaty M, Śluczanowska-Głąbowska S, Pawlik A. The effect of gene polymorphisms on patient responses to rheumatoid arthritis therapy. *Expert Opin Drug Metab Toxicol.* 2016;12(1):41–55. [\[CrossRef\]](#).
31. Blits M, Jansen G, Assaraf YG, van de Wiel MA, Lems WF, Nurmohamed MT, et al. Methotrexate normalizes up-regulated folate pathway genes in rheumatoid arthritis. *Arthritis Rheum.* 2013;65(11):2791–802. [\[CrossRef\]](#).
32. Sramek M, Neradil J, Veselska R. Much more than you expected: The non-DHFR-mediated effects of methotrexate. *Biochim Biophys Acta Gen Subj.* 2017;1861(3):499–503. [\[CrossRef\]](#).
33. Ivanenko KA, Prassolov VS, Khabusheva ER. Transcription factor Sp1 in the expression of genes encoding components of MAPK JAK/STAT and PI3K/Akt signaling pathways. *Mol Biol.* 2022;56(5):832–47. [\[CrossRef\]](#).
34. Sun X, Xiao C, Wang X, Wu S, Yang Z, Sui B, et al. Role of post-translational modifications of Sp1 in cancer: State of the art. *Front Cell Dev Biol.* 2024;12:1412461. [\[CrossRef\]](#).
35. Chen J, Mao C, Han N, Zhou Q, Feng C, Sun X, et al. Post-translational modifications of Stat3: The state of the art. *Cell Signal.* 2025;135:112048. [\[CrossRef\]](#).
36. Singh Tuli H, Rath P, Chauhan A, Sak K, Aggarwal D, Choudhary R, et al. Luteolin, a potent anticancer compound: From chemistry to cellular interactions and synergetic perspectives. *Cancers.* 2022;14(21):5373. [\[CrossRef\]](#).
37. Mahwish, Imran M, Naeem H, Hussain M, Alsagaby SA, Al Abdulmonem W, et al. Antioxidative and anticancer potential of luteolin: A comprehensive approach against wide range of human malignancies. *Food Sci Nutr.* 2025;13(1):e4682. [\[CrossRef\]](#).
38. Shen C, Peng C, Zhang S, Li R, Liu S, Kuang Y, et al. Eukaryotic translation initiation factor 6-mediated ribosome biogenesis promotes synovial aggression and inflammation by increasing the translation of SP1 in rheumatoid arthritis. *Int Immunopharmacol.* 2024;142(Pt B):113164. [\[CrossRef\]](#).
39. Kim B, Kim HY, Yoon BR, Yeo J, Jung JI, Yu KS, et al. Cytoplasmic zinc promotes IL-1 β production by monocytes and macrophages through mTORC1-induced glycolysis in rheumatoid arthritis. *Sci Signal.* 2022;15(716):eabi7400. [\[CrossRef\]](#).
40. Wang X, Cui P, Deng Y, Zhang B, Gao Z, Li T, et al. Function of *SP1* in tumors and focused treatment approaches for immune evasion (Review). *Oncol Lett.* 2025;30(4):483. [\[CrossRef\]](#).
41. Hu Y, Ren Z, Zhao Z, Huang Y, Huang W, Liu J, et al. Anemarsaponin B mitigates acute pancreatitis damage in mice through apoptosis reduction and MAPK pathway modulation. *BIOCELL.* 2024;48(5):745–58. [\[CrossRef\]](#).
42. Singh SP, Dosch AR, Mehra S, De Castro Silva I, Bianchi A, Garrido VT, et al. Tumor cell-intrinsic p38 MAPK signaling promotes IL1 α -mediated stromal inflammation and therapeutic resistance in pancreatic cancer. *Cancer Res.* 2024;84(8):1320–32. [\[CrossRef\]](#).
43. Noé V, Alemany C, Nicolás M, Ciudad CJ. Sp1 involvement in the 4 β -phorbol 12-myristate 13-acetate (TPA)-mediated increase in resistance to methotrexate in Chinese hamster ovary cells. *Eur J Biochem.* 2001;268(11):3163–73. [\[CrossRef\]](#).
44. Novak-Kotzer H, Capera J, Jainarayanan A, Elanchezhian M, Valvo S, Mayya V, et al. STAT3 phosphorylation in the rheumatoid arthritis immunological synapse. *J Autoimmun.* 2025;155:103456. [\[CrossRef\]](#).
45. Zhu M, Sun Y, Su Y, Guan W, Wang Y, Han J, et al. Luteolin: A promising multifunctional natural flavonoid for human diseases. *Phytother Res.* 2024;38(7):3417–43. [\[CrossRef\]](#).
46. Singh DP, Bhargavan B, Chhunchha B, Kubo E, Kumar A, Fatma N. Transcriptional protein Sp1 regulates LEDGF transcription by directly interacting with its *Cis*-elements in GC-rich region of TATA-less gene promoter. *PLoS One.* 2012;7(5):e37012. [\[CrossRef\]](#).
47. Cordaro M, Cuzzocrea S, Crupi R. An update of palmitoylethanolamide and luteolin effects in preclinical and clinical studies of neuroinflammatory events. *Antioxidants.* 2020;9(3):216. [\[CrossRef\]](#).