

Anti-inflammatory Mechanism of Rhein in Treating Asthma Based on Network Pharmacology

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Research

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Abstract

Background: Network pharmacological methods were used to predict the anti-inflammatory targets and related pathways of rhein in the treatment of asthma, and to elucidate its mechanism of action. In addition, we validated the anti-inflammatory effects of rhein in HBE cells.

Methods: The corresponding targets of rhein were obtained from the TCMSP 2.3, and molecular docking was also performed. A network of predicted rhein targets was established and analysed with Cytoscape 3.7.1. The anti-inflammatory targets in the TTD database were searched to build a PPI network, which was merged with the ingredient-target network to screen anti-inflammatory targets associated with rhein. A network of anti-inflammatory rhein targets during the in vivo treatment of asthma was constructed to screen the anti-inflammatory targets related to asthma. KEGG enrichment analysis was performed with the Enrichr database and Cytoscape 3.7.1. The expression levels of proteins in the MAPK/NF- κ B signalling pathway were assessed by western blot analysis.

Results: Altogether, 17 targets were obtained. Epidermal active growth factor receptor (EGFR), E-selecting (E-SELE), macrophage migration inhibitory factor (MIF), and mitogen-activated protein kinase 14 (MAPK14) might be important anti-inflammatory targets of rhein during asthma treatment. We selected the MAPK signalling pathway to determine the anti-inflammatory effects of rhein.

Conclusion: The anti-inflammatory mechanism of the treatment of asthma with rhein may be related to MAPK14, EGFR, E-SELE and MIF as well as their signalling pathways. To prevent the exacerbation of asthma, instead of targeting a single pathway or a single target, all these targets and their signalling pathways should be controlled holistically. Rhein may reduce inflammation by inhibiting the MAPK/NF- κ B pathway.

Background

Asthma is an abbreviation of bronchial asthma. Asthma is a kind of chronic inflammatory disorder of the airways involving cells and corresponding components, and it is characterized by inflammation, hyperresponsiveness, stenosis and airway remodelling. Chronic inflammation is considered the hypostasis of asthma [1, 2].

Rhein is an effective monomer component, and it is separated and purified from traditional Chinese medicines, such as rhubarb. Rhein is a monanthraquinone 1,8-dihydroxyl anthraquinone derivative. Rhein has anti-inflammatory, antibacterial, antitumour and other effects [3, 4, 5, 6]. However, there are very few reports on the anti-inflammatory efficacy of rhein in the treatment of asthma.

Network pharmacology has become an emerging research method in recent years. It is considered a new model for the next generation of drug research. The construction of biomolecule networks, such as the drug-target-pathway, is the basis of network pharmacology [7, 8].

Network pharmacology can explore the efficacy of drugs from the component-target pathway, and the pathogenesis of diseases can also be reversed through drugs with known efficacy [9, 10]. Hence, we used network pharmacology to explore the anti-inflammatory mechanism of rhein in the treatment of asthma and to provide new ideas for treating asthma.

Materials And Methods

The TCMSP 2.3 (<https://tcmssp.com/tcmssp.php>), PubChem (<https://pubchem.ncbi.nlm.nih.gov>), TTD (<http://bidd.nus.edu.sg/group/cjttd/>), STITCH (<http://stitch.embl.de/>), DrugBank databases (<https://www.drugbank.ca/>) were used to search for the chemical composition of the drug and its targets. Genes related to diseases, gene-to-protein interactions, and protein-protein interaction information can be found in the GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) and String Databases (<http://string-db.org/cgi/input.pl>). We looked for signalling pathways related to biomolecules in the Enrichr (<http://amp.pharm.mssm.edu/Enrichr/>) and KEGG databases. Cytoscape 3.7.1 and systemsdock (<http://www.docksystem.com/>) were used for network construction and molecular docking respectively.

Prediction of the targets of rhein

The data about the three-dimensional chemical structure of rhein was searched and exported from the TSCM and PubChem databases. Then the data was imported into the Swiss target prediction database (<http://www.swisstargetprediction.ch/>), and reverse molecular docking was performed. Predictive targets were obtained by setting the target set to *Homo sapiens*. The results were used in further studies.

Molecular docking studies of rhein and its target proteins

The PDB-ID of the target proteins was queried in the PDB database (<http://www.rcsb.org/>). The data was imported into systemsDock for molecular docking studies. The docking score was used to determine the matching degree between rhein and the target. The score ranged from 0-10, and the greater the value was, the stronger the interaction was [11].

Construction of the rhein-target network

A drug-target interaction network of rhein and its potential targets were constructed by using Cytoscape 3.7.1.

Construction of the rhein-target protein interaction network

By using String Database, the rhein-target protein interaction network was constructed by setting the protein type to *Homo sapiens*, setting the minimum interaction threshold to medium confidence, and keeping the remaining parameters silent.

Anti-inflammatory target protein screening

In the TTD database, information on the anti-inflammatory target proteins was searched by using the keyword anti-inflammatory, nine anti-inflammatory target proteins of them were selected for subsequent use, and these target proteins were put into Cytoscape 3.7.1 to construct an anti-inflammatory target protein PPI network.

Screening of the anti-inflammatory targets of the rhein effect and construction of an anti-inflammatory target network

The rhein-target protein network was combined with the anti-inflammatory protein PPI network, and the results, which were called the anti-inflammatory targets of the rhein effect, were imported into the String Database to construct the network. Screening values above 0.7 indicated a high confidence of protein interactions.

Search for asthma-related genes in humans

In the NCBI Gene Database (<http://www.ncbi.nlm.nih.gov>), asthma and *Homo sapiens* are searchable keywords, and the asthma-related genes of a human being can be acquired.

Network of the anti-inflammatory targets of rhein in the treatment of asthma

The anti-inflammatory target genes of rhein and human asthma-related genes were imported into the String Database to construct the response network of the anti-inflammatory targets of rhein during the in vivo treatment of asthma and to screen the anti-inflammatory targets related to the incidence of asthma.

KEGG pathway enrichment of the target genes

The Enrichr was used to analyse the KEGG biological pathway enrichment of the target genes to predict the anti-inflammatory targets of rhein.

Drug and Reagents

Rhein (purity $\geq 99\%$) was provided by MedChemExpress (Shanghai China)Co.,Ltd. The HBE cell lines were obtained from the American Collection of Cell Culture (ATCC, USA). Cell culture reagents including fetal bovine serum (FBS) , RPMI-1640 medium, and antibiotic+amphiphilic solution, dimethyl sulfoxide (DMSO),ovalbumin (OVA, grade V) were supplied by Sigma-Aldrich Co. LLC. Lipopolysaccharide (LPS) was purchased from Solarbio Co.,Ltd, NF-kBp65(A2547), p38MAPK(A1401), p-p38MAPK(AP0297), β -Tubulin(AS014) were provided by ABclonal Tech (Wuhan China) Co.,Ltd.

Exploration of safe doses of rhein in HBE cells

Cell viability was evaluated by the CCK-8 assay. The drug dose was selected within a range that is nontoxic to cells, and this dose ensured comprehensive accurate results of the subsequent experiments.

Culture and Treatment of HBE cells

The HBE cell lines were previously obtained from the American Collection of Cell Culture (ATCC, USA). The cells were cultured in appropriate bottles with sterile RPMI-1640 medium (Sigma-Aldrich) supplemented with 1% antibiotic+amphiphilic solution (Sigma-Aldrich) and 10% foetal bovine serum (FBS, Sigma-Aldrich) and incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% atmospheric air for 24 hours. The culture medium was changed every 2 days. Cells were grown to 80% confluence prior to treatment. Rhein was dissolved in culture grade DMSO (final concentration < 0.1%) in serum-free media. To investigate the protective effect of rhein, primary cultured HBE cells were treated with 0.1, 0.5, and 1.0 µM of rhein for 2 hours and then treated with 100µg/ml LPS+ 1µg/ml OVA for 24 hours while the OVA+LPS group received 100µg/ml LPS + 1µg/ml OVA treatment alone. The inflammation model refers to the reference [12].

Cell Viability Assays

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) assay. HBE cells (2×10⁵ cells in 200 µl of RPMI medium+2% FBS per well in a 96-well plate) were treated with rhein at concentrations of 0.1, 0.5 and 1.0µM. After 24 hours, the supernatant was removed, and 100 µl of the medium was added with CCK-8 solution to form crystals of formazan in the viable cells. The plate was incubated for 4 hours. The wells were immediately analysed in a spectrophotometer at a wavelength of 450 nm. Cell viability (%) was expressed as a percentage relative to the untreated control cells.

Western Blot Analysis

To observe the inflammation prevention effects of rhein on inflammation induced HBE cells, we treated cell samples with rhein (0.1 to 1µM) and induced inflammation by applying LPS (1µg/mL)+OVA (0.1mg/ml). MAPK/NF-κB activation, which plays an important role in inflammatory responses, was analyzed by western blot analysis to evaluate the effects of rhein in treating on HBE cells. Cells were lysed with RIPA lysis buffer including protease inhibitor (Solarbio, Beijing, China). Total protein concentration of obtained extract was quantified with a BCATM protein assay kit (Pierce, Appleton, WI, USA). After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Temecula, CA, USA). Afterwards, the membrane was blocked with 5% milk for 1 hour, and then incubated with primary antibodies against NF-κBp65(A2547), p38MAPK(A1401), p-p38MAPK(AP0297), β-Tubulin(AS014) (all purchased from ABclonal) overnight at 4°C. The primary antibodies were diluted with 5% milk at a dilution of 1:1000. Subsequently, the primary antibodies were probed with the secondary antibody HRP Goat Anti-Rabbit IgG (H+L) (AS014) (1:3000; purchased from ABclonal Tec.) for 1 hour at room temperature. Subsequently, the signals of protein bands were captured with Image Lab™ Software (Bio-Rad).

Statistical analyses

Descriptive statistics were performed using Prism 7.0 software. The measurement data is expressed as the mean ± S.E.M. Comparisons among groups were performed by one-way ANOVA. Comparisons of two

groups were performed by t-test. $P < 0.05$ was considered statistically significant.

Results

Rhein and its predicted targets

Seventeen predicted targets of rhein were searched in STITCH, DrugBank databases. A drug-target interaction network of rhein and its potential targets were constructed by using Cytoscape 3.7.1 (**Fig. 1**).

Rhein-target network results

The rhein-target protein interaction networks were constructed by using String Database (**Fig. 2**).

The reverse docking scores of rhein and its related targets.

The PDB-IDs were imported into systemsDock for molecular docking studies. The docking score was above 5, verified the reliability of the predicted targets (**Table 1**).

Network of rhein anti-inflammatory targets during treatment of asthma

The Cytoscape 3.7.1 merge function was used to combine the rhein-predicted target network with the anti-inflammatory target PPI network in the TTD, considering the overlapping results and a high confidence interval above 0.7, rhein anti-inflammatory targets, including mitogen-activated protein kinase (MAPK14), receptor tyrosine-protein kinase erbB-2 (ERBB2), tumour necrosis factor receptor superfamily member 1A (TNFRSF1A), and epidermal growth factor receptor (EGFR) were identified (**Fig. 3**).

KEGG pathway enrichment of the target genes

According to the KEGG pathway enrichment of the target genes, the anti-inflammatory target proteins of rhein associated with asthma were involved in 87 signalling pathways. The Enrichr analysis results were sorted in descending order of the combined score. The top ten pathways included the MAPK signalling pathway, hepatitis C signalling pathway, epithelial cell signalling during *Helicobacter pylori* infection, immune signalling pathway, and proteoglycans in cancer signalling pathway and et al (**Fig. 4**).

The cytotoxicity of rhein on HBE cells

To examine the cytotoxicity of rhein on HBE cells, the cells were treated with various concentrations of rhein (0.1 to 1 μ M) in RPMI medium and fetal bovine serum (FBS) for 24 hours. There was no observed cytotoxicity of rhein in the HBE cells when the final concentration of rhein was less than 40 μ M (**Fig.5**).

Inflammation prevention effects of rhein on HBE cells via MAPK/NF- κ B pathway

The results showed that rhein treatment dose dependently inhibited phosphorylation of MAPKp38 and significantly reduced the expression of NF- κ B of p65 in the HBE-cell activated MAPK/NF- κ B pathway by

LPS+OVA recombinant. Rhein treatment is associated with the MAPK/NF- κ B pathway, and rhein would prevent inflammation (Fig.6).

Discussion

It has been reported that rhein has anti-inflammatory activities [13, 14]. The results of this study show the anti-inflammatory effects of rhein. Youdong Xu et al. explained the anti-inflammatory effects of rhein based on its molecular mechanism [15]. MAPK14, EGFR, EERB2, TNFRSF1A, etc., are the main targets of the anti-inflammatory effects of rhein. Rhein can directly act on EGFR, MAPK14, EERB2, and TNFRSF1A and can also indirectly act on other targets to exert its anti-inflammatory effects [16,17,18,19].

EGFR is an epidermal growth factor receptor that is widely distributed in epithelial tissues and plays an important regulatory role in the development of respiratory inflammation [20,21,22]. EGFR inhibitors can effectively inhibit acute inflammation of the rat respiratory tract caused by exogenous zinc ions [23]. EGFR inhibitors reduce the symptoms of allergic asthma caused by dust mites by reducing the production of pro-inflammatory factors, such as IL-6 and IL-8 [24,25]. Thus, it is speculated that rhein interacts with EGFR, blocking the binding of EGFR to pro-inflammatory cytokines, to exert an anti-inflammatory effect.

MAPK14, which is also called p38 α , is one of the four p38 MAP kinases in mammals. It plays an important role in the cellular cascade triggered by pro-inflammatory cytokines or extracellular stimuli and is a key signalling molecule in the lung inflammation induced by *S. pneumonia* [26, 27]. Inflammatory factors, such as IL-1 β , IL-6 and TNF- α , can positively regulate signalling pathways, like ERK and nuclear factor- κ B (NF- κ B), by activating the p38 MAPK signalling pathway and cascading to amplify the inflammatory response [28, 29]. The transcriptional cascade regulated by P38 MAPKs leads to the production of pro-inflammatory factors, such as TNF- α and IL-1 β , which in turn leads to the activation of enzymes involved in inflammation [30]. It is speculated that rhein exerts its anti-inflammatory effect by inhibiting the release of pro-inflammatory factors, such as TNF- α and IL-1 β , by inhibiting MAPK14.

TNFRSF1A is a type 1 TNF receptor that mediates inflammatory responses mainly by activating NF- κ B, p38, and ERK1/2 to induce IL-6 and IL-8 synthesis and apoptosis [31, 32]. It is thus concluded that rhein interacts with TNFRSF1A to reduce the secretion of anti-inflammatory factors, such as IL-6, and exert an anti-inflammatory effect.

EERB2, which is called HER2, NEU and CD340, is a 185-kDa cell membrane receptor encoded by the proto-oncogene *erbB-2* and is a member of the EGFR family. EERB2 can induce IL-6 autocrine activity, which in turn affects the JAK-STAT pathway- or NF- κ B pathway-mediated inflammatory responses [33]. It is speculated that rhein interacts with EERB2 and may exert anti-inflammatory effects by regulating the secretion of IL-6 [34, 35, 36].

In this study, the anti-inflammatory mechanism of rhein was confirmed by network pharmacology. The main targets of the anti-inflammatory effect of rhein and the related signalling pathways were predicted.

Rhein exerted an anti-inflammatory effect by acting on multiple targets. However, network pharmacology research is based on network modelling, database resource development and software application. The network model has certain differences from the in vivo environment.

In this study, we revealed a signalling nexus involving rhein, MAPK/NF κ B pathway and inflammation in the HBE cells as shown in the summary diagram(**Fig.7**). We have demonstrated that rhein can reduce the expression of phosphorylation-p38 and p65 induced by LPS+OVA via the MAPK/NF- κ B pathway in HBE cells, and further reduce the inflammatory responses [37,38]. In the future, the anti-inflammatory effects of rhein need to be verified by further animal experiments.

Conclusion

In summary, it is the first time for us to investigate the mechanism of rhein in treating asthma by its anti-inflammatory effect. Network pharmacologic analysis indicated that rhein may not only have the anti-inflammatory function by regulating MAPK14, EGFR, ESRB2 and TNFRSF1A, but also have the effect of treating asthma by regulating the MAPK/NF- κ B signaling pathway. Additionally, the expression of MAPK/NF- κ B pathway proteins was validated in HBE cell lines by western blot. All these experimental results provided further ideas into the molecular mechanisms and pathways associated with treatment of asthma by rhein. Meanwhile, it also provides a new direction for the treatment of asthma.

Abbreviations

CCK-8: Cell Counting Kit-8; EGFR: Epidermal active growth factor receptor; E-SELE: E-selecting; HBE: human bronchial epithelial; IL-1 β : Interleukin-1 β ; IL-6: Interleukin-6; IL-8: Interleukin-8; LPS: Lipopolysaccharide; MAPK14: mitogen-activated protein kinase 14; MIF: macrophage migration inhibitory factor; OVA: Ovalbumin; TNF- α : Tumor Necrosis Factor- α .

Declarations

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Supplementary Table SII.

Authors' contributions

All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee. Written informed consent was obtained from individual or guardian participants.

Patient consent for publication

Not applicable

Publication of clinical datasets

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table1. The reverse docking score of rhein and related targets

| Targets | Rhein |
|---------|-------|
| CREB1 | 8.785 |
| ERK1 | 8.476 |
| SRC | 8.254 |
| RasH | 8.015 |
| PAK1 | 8.012 |
| HRas | 7.506 |
| PDK1 | 7.408 |
| Grb2 | 7.085 |
| MP2K1 | 6.972 |
| Myc | 6.446 |
| EGFR | 6.032 |
| MEK1 | 5.643 |

Figures

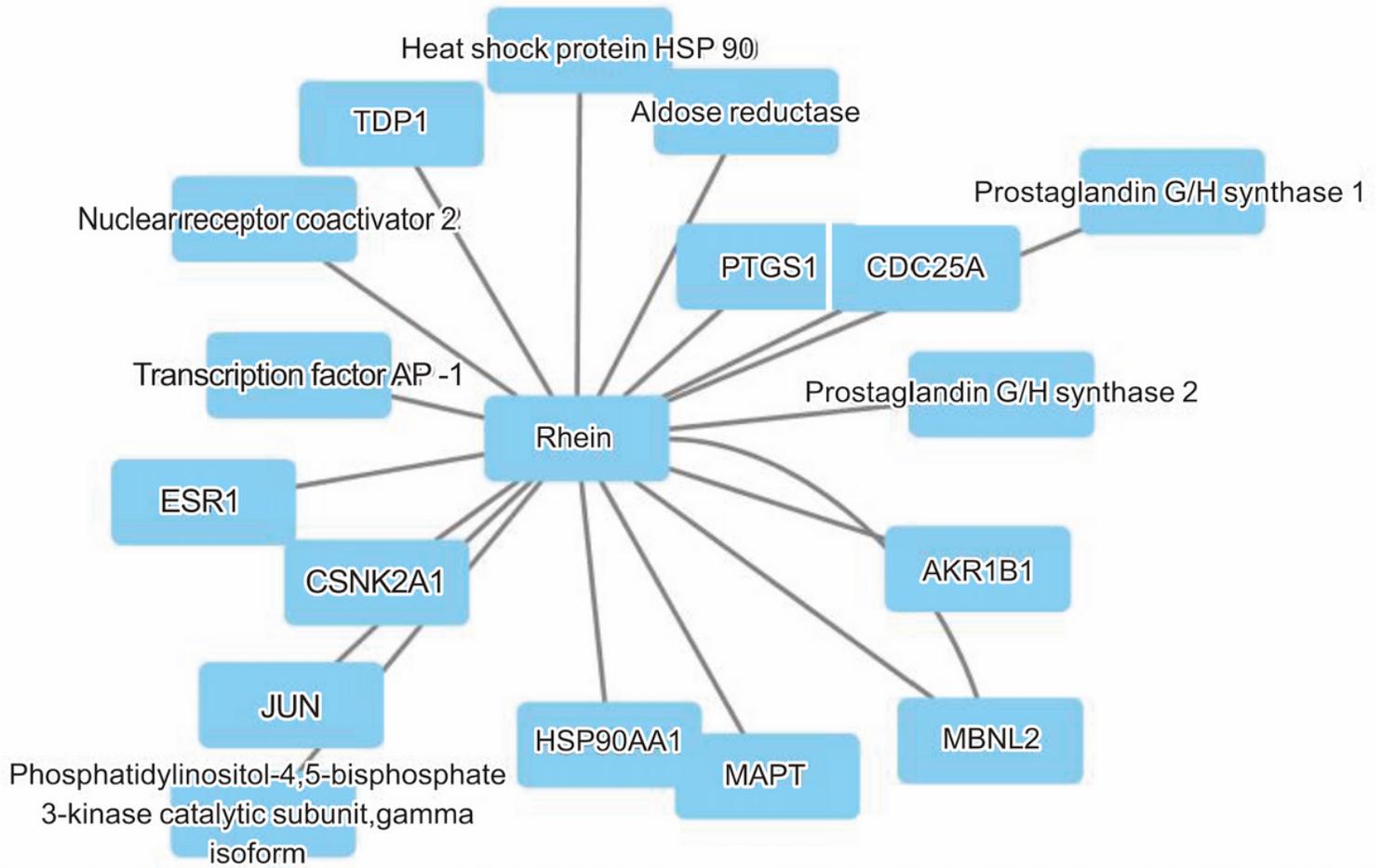


Figure 1

Rhein and its predicted targets Seventeen predicted targets of rhein were found in the STICH,Drugbank databases.

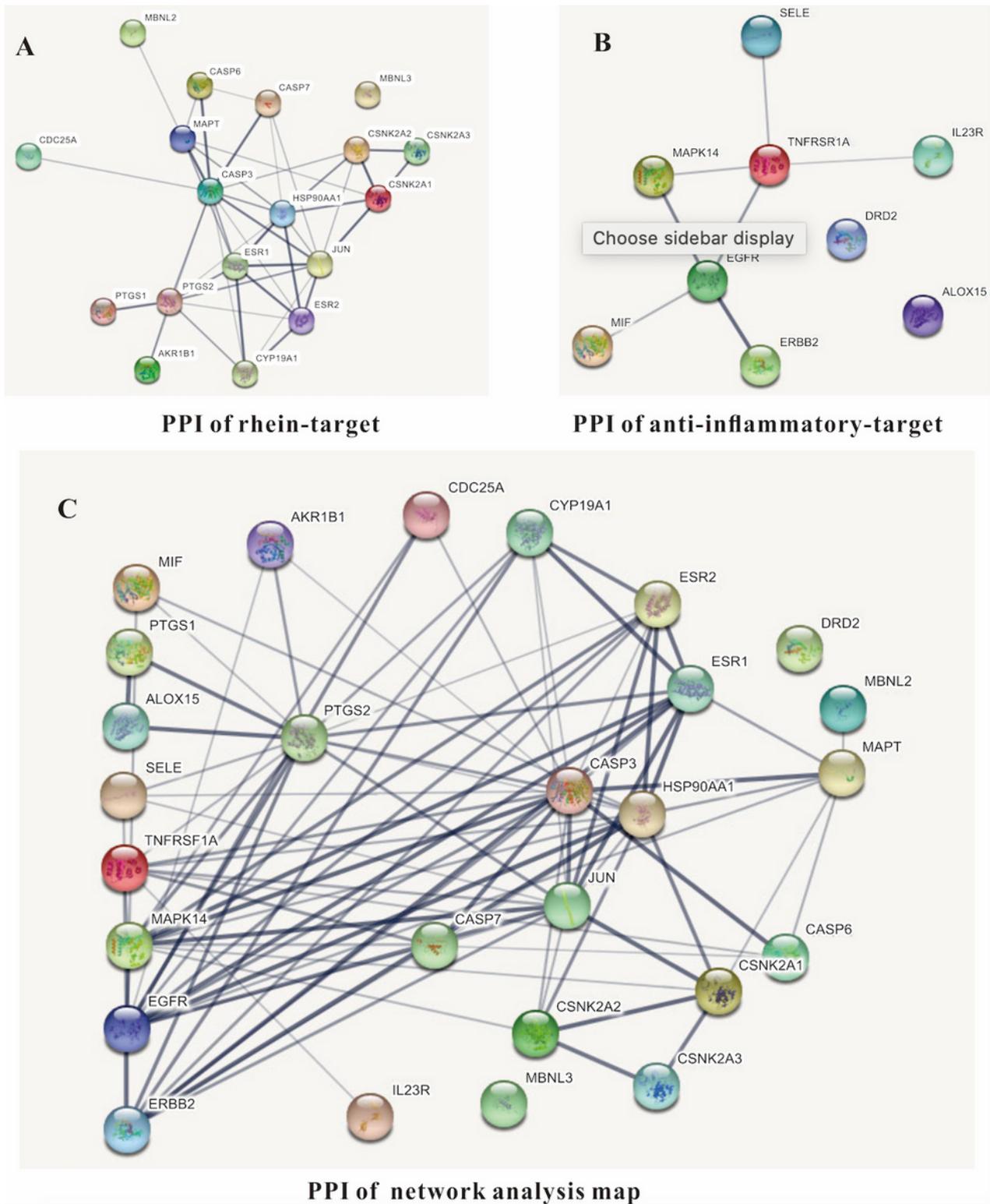


Figure 2

Rhein-target network results (a) The protein protein interaction network of rhein-target proteins was constructed in the String database. (b) Nine anti-inflammatory proteins were identified in the TTD, and in the PPI network, there were nine interacting targets, constituting seven interacting relationships. (c) Network analysis map of the interactions between rhein-target proteins and anti-inflammatory proteins. There were 27 nodes, 92 edges, and the local clustering coefficient was 0.713.

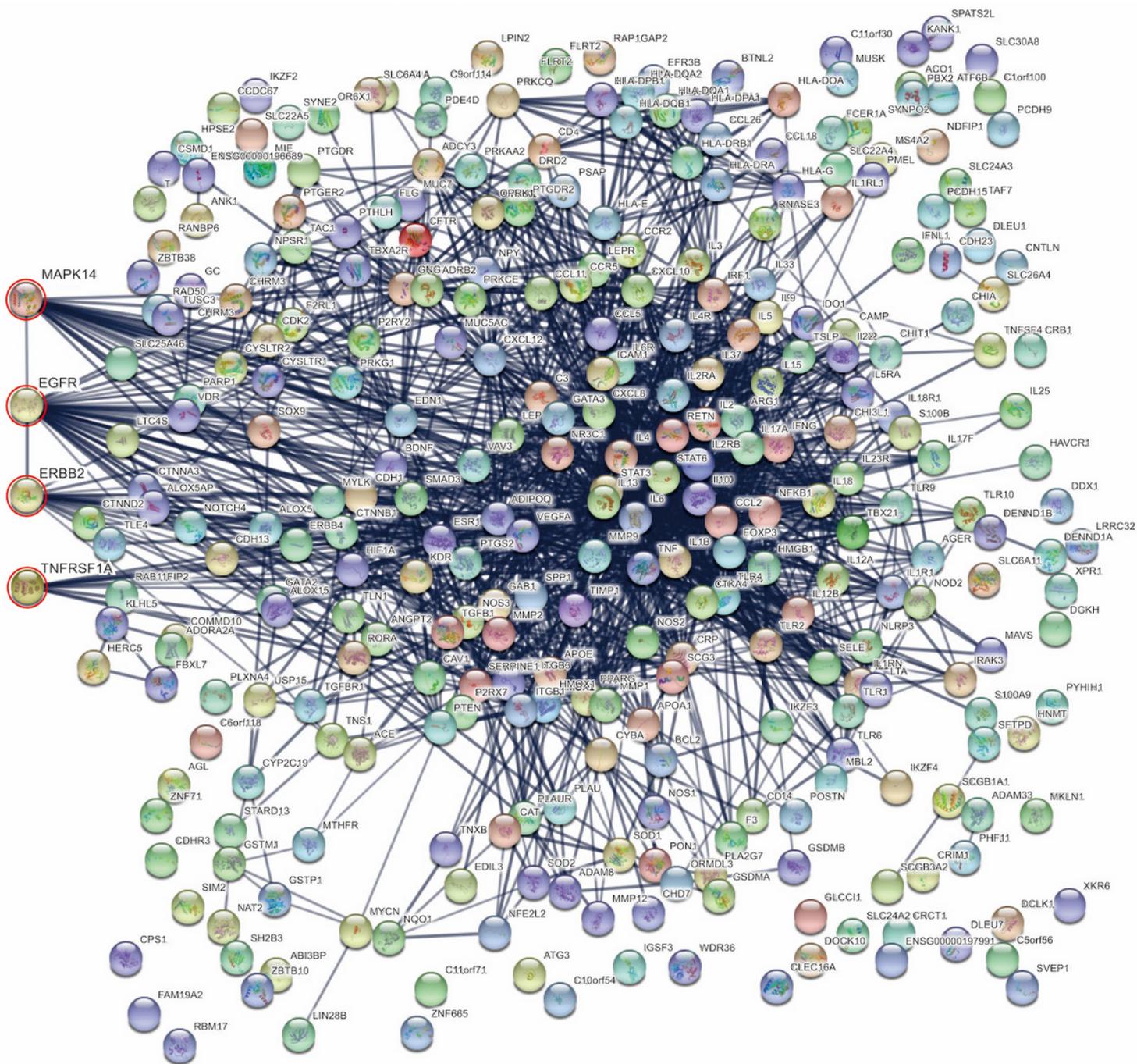


Figure 3

Network of rhein anti-inflammatory targets during treatment of asthma. The Cytoscape 3.7.1 merge function was used to combine the rhein-predicted target network with the anti-inflammatory target PPI network in the TTD, considering the overlapping results and a high confidence interval above 0.7. Rhein anti-inflammatory targets, including mitogen-activated protein kinase (MAPK14), receptor tyrosine-protein kinase erbB-2 (ERBB2), tumour necrosis factor receptor superfamily member 1A (TNFRSF1A), and epidermal growth factor receptor (EGFR) were identified.



Enriched Terms

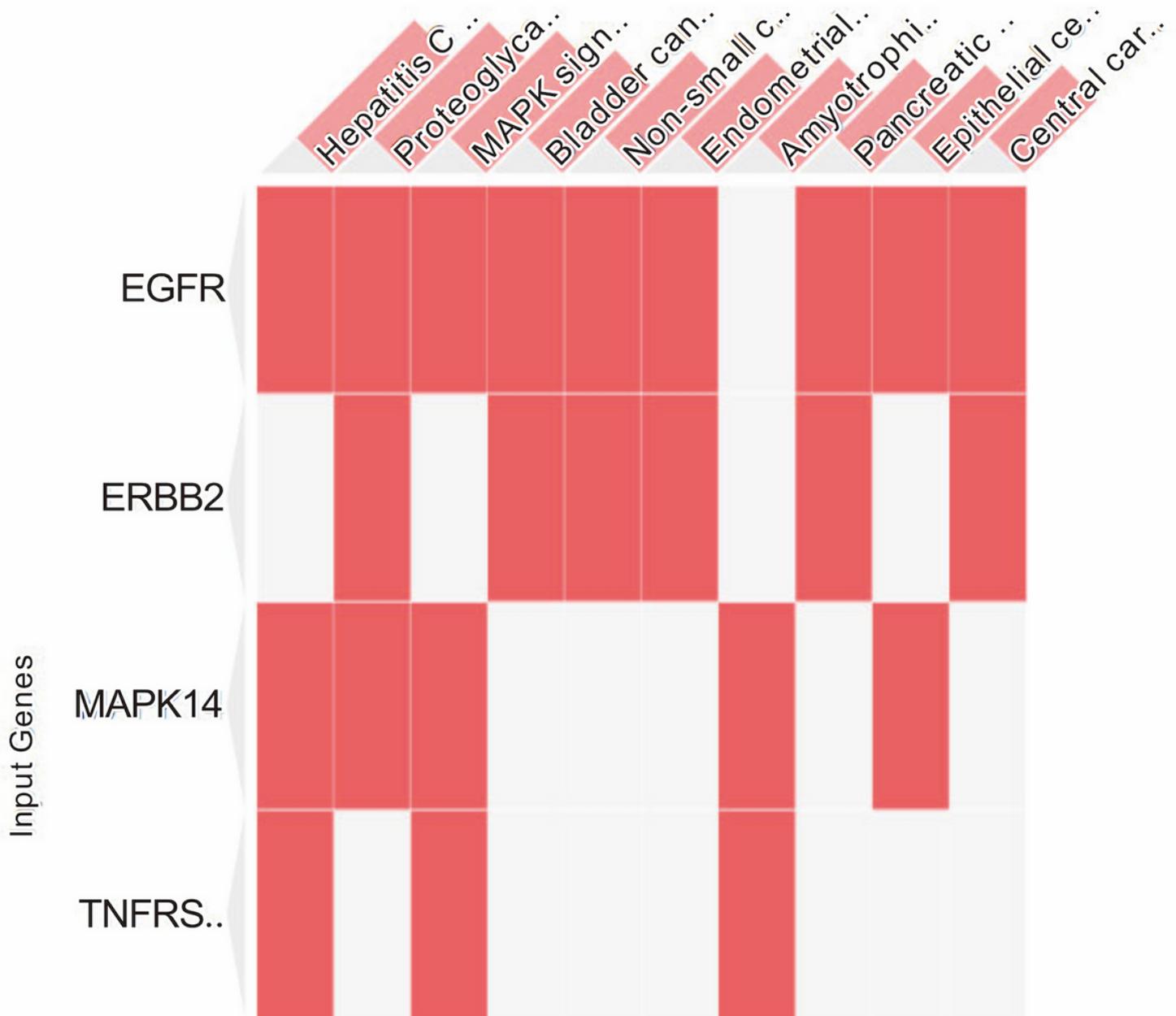


Figure 4

KEGG pathway enrichment of the target genes The top ten signalling pathways: MAPK signalling pathway; hepatitis C signalling pathway; epithelial cell signalling during Helicobacter pylori infection; proteoglycans in cancer signalling pathway; bladder cancer signalling pathway; non-small cell lung cancer signalling pathway; endometrial cancer signalling pathway; pancreatic cancer signalling pathway; central carbon metabolism in cancer signalling pathway; and amyotrophic lateral sclerosis (ALS) signalling pathway.

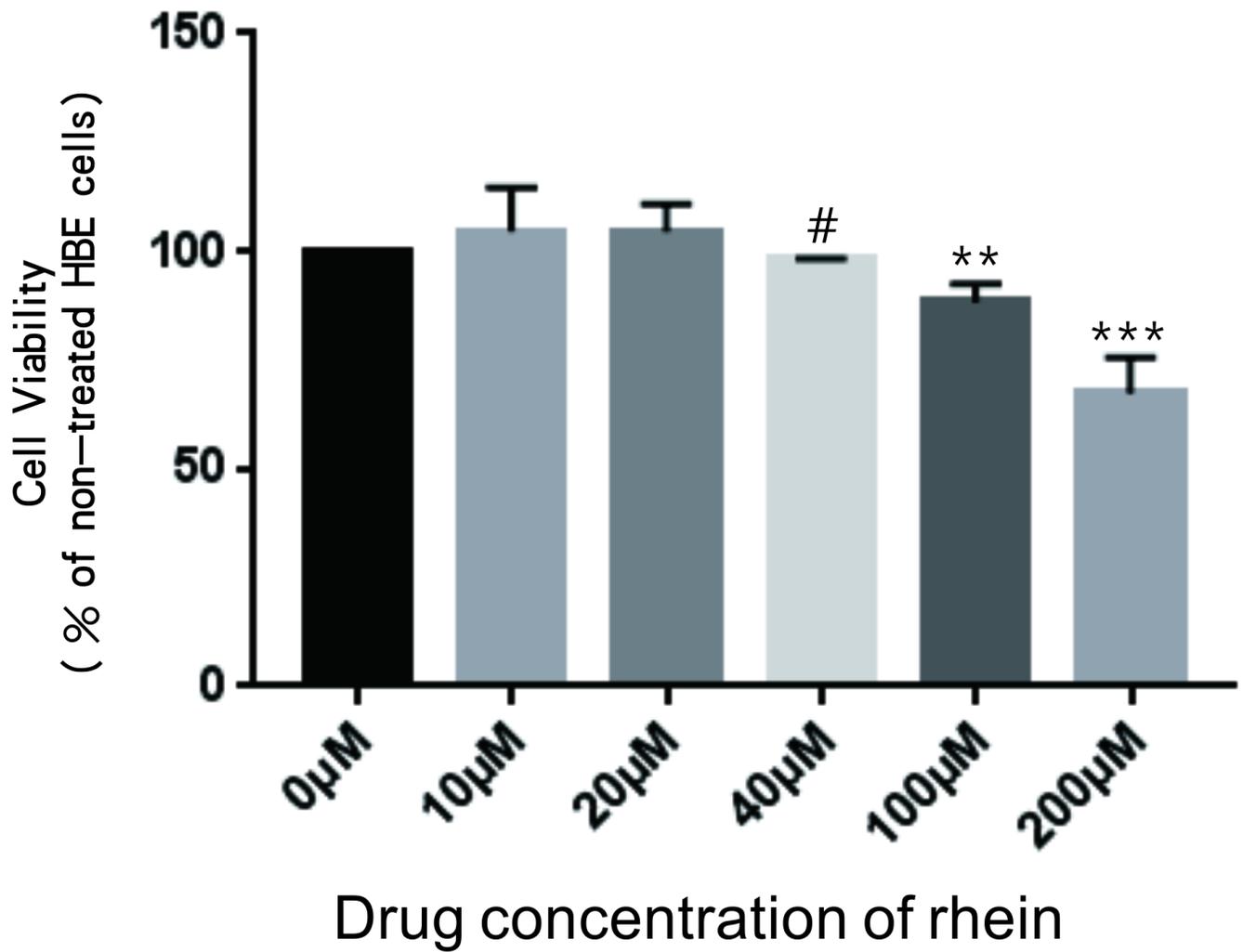


Figure 5

The cytotoxicity of rhein on HBE cells Cell viability assay of rhein on HBE cells. The cytotoxicity of the rhein on HBE cells. Statistical significance was determined by unpaired t test compared to non-treated group. There was no cytotoxicity of the rhein in HBE cells when the final concentration of rhein was less than 40 µM. #p>0.05 vs. 0uM ,**p < 0.05 vs. 0uM; ***p < 0.01 vs. 0uM.

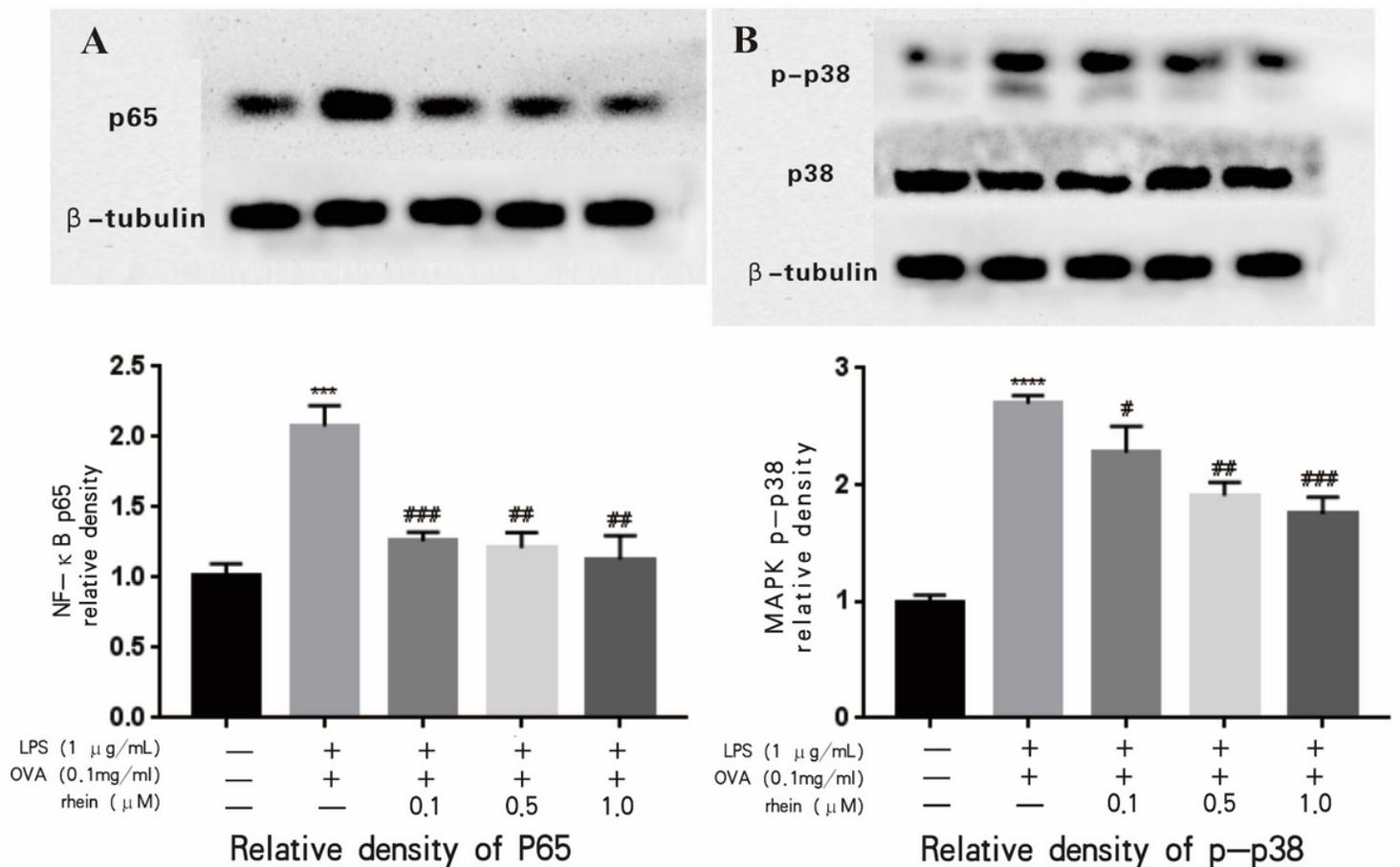


Figure 6

Inflammation prevention effects of rhein on HBE cells via MAPK/NF-κB pathway Preventing inflammation effect of rhein in LPS+OVA-induced inflamed HBE cells via inhibiting MAPK/NF-κB activity. Cells were pretreated with rhein (0.1μM, 0.5μM, 1μM) and stimulated inflammation with LPS (1μg/mL)+OVA(0.1mg/ml) for 24h. (a) The protein expression of NF-κB activity using at treated doses (0.1μM, 0.5μM, 1μM) and (b) the protein expression of the MAPK signaling pathway. Ratio of each protein expression was measured by Image J. Statistical significance was determined by unpaired t test compared to OVA+LPS-induced inflamed HBE cells. Data represent the mean±SD of at least three independent experiments. Each experiment was performed in triplicate. *p<0.05 compared to the non-LPS treated HBE cells group, #p<0.05 compared to only LPS-treated HBE cells.

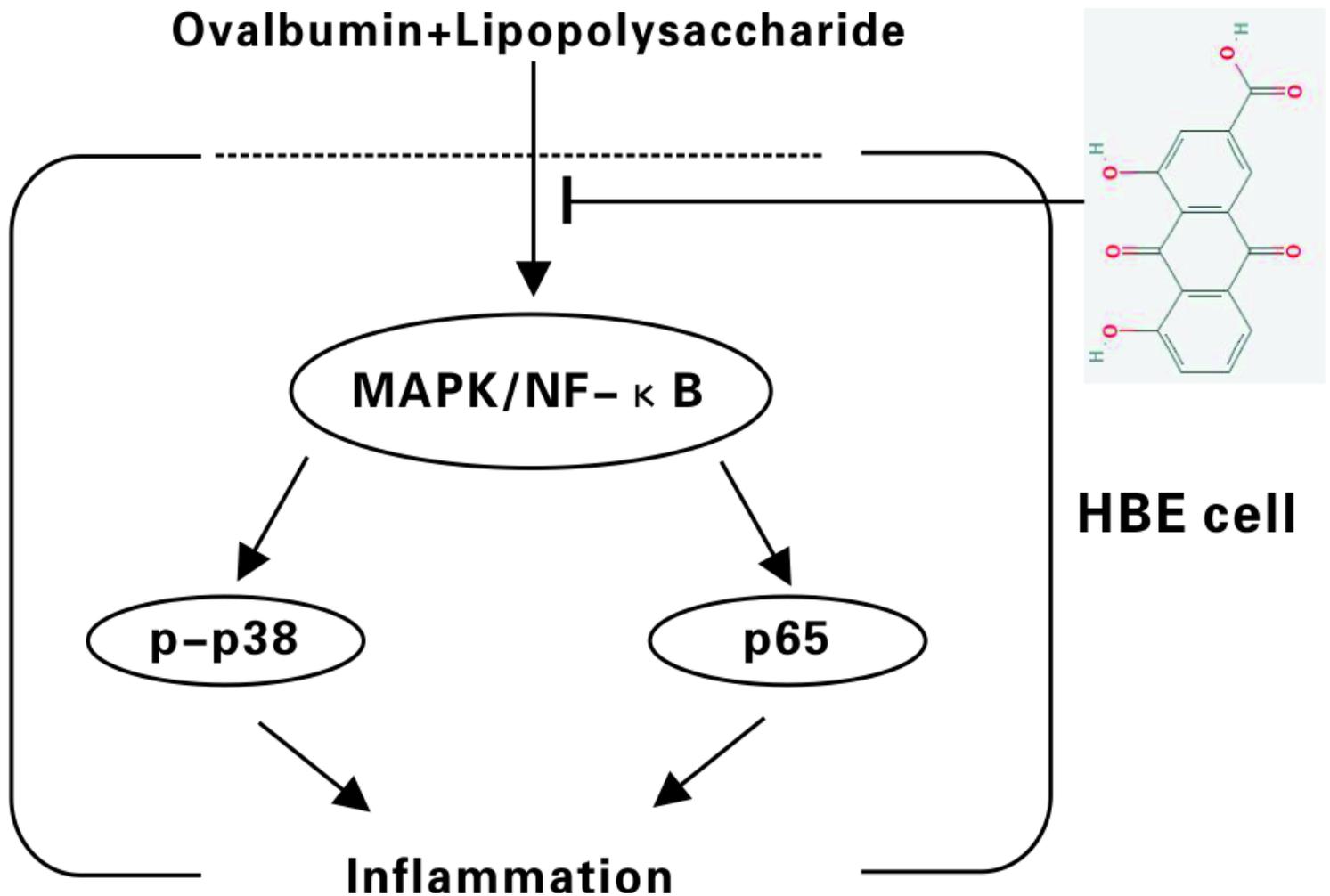


Figure 7

Flowchart of the mechanism of rhein alleviated inflammation A schematic diagram depicting the mechanism of rhein alleviated inflammation in LPS+OVA induced HBE cells. LPS+OVA initiates the inflammatory response cascade through MAPK/NF-κB pathway mediated down-regulation p-p38 and p65, and further reducing inflammatory response.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTableSII.docx](#)