

Investigation of Pharmacological Mechanism of Natural Product Using Pathway Fingerprints Similarity Based on “Drug-Target-Pathway” Heterogenous Network

Feifei Guo (✉ ffguo@icmm.ac.cn)

China Academy of Chinese Medical Sciences Institute of Chinese Materia Medica

<https://orcid.org/0000-0003-3624-4704>

Chunhong Jiang

Shantou University Medical College <https://orcid.org/0000-0001-5522-2389>

Yujie Xi

Tianjin University of Traditional Chinese Medicine <https://orcid.org/0000-0002-0269-868X>

Dan Wang

Beijing Proteome Research Center <https://orcid.org/0000-0001-8494-9149>

Yi Zhang

China Academy of Chinese Medical Sciences

Ning Xie

State Key Laboratory of Innovative Natural Medicine and TCM Injections, Ganzhou, China

<https://orcid.org/0000-0003-0231-2588>

Yi Guan

Shantou University Medical College <https://orcid.org/0000-0001-6057-9243>

Fangbo Zhang

China Academy of Chinese Medical Sciences <https://orcid.org/0000-0001-5426-0324>

Hongjun Yang

China Academy of Chinese Medical Sciences <https://orcid.org/0000-0001-5501-3288>

Research article

Keywords: Natural products, andrographolide derivative, pathway fingerprints, “Drug-Target-Pathway” network, anti-inflammatory

Posted Date: March 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-273452/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Investigation of Pharmacological Mechanism of Natural Product Using Pathway Fingerprints Similarity Based on “Drug-Target-Pathway” Heterogenous Network

Feifei Guo^{1,*}, Chunhong Jiang^{2,*}, Yujie Xi^{1,3}, Dan Wang⁴, Yi Zhang¹, Ning Xie⁵, Yi Guan², Fangbo Zhang^{1,#}, Hongjun Yang^{1,6#}

1 Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China.

2 Joint Institute of Virology (Shantou University and The University of Hong Kong), Shantou University Medical College, Shantou, China.

3 Tianjin University of Traditional Chinese Medicine, Tianjin, China.

4 State Key Laboratory of Proteomics, Beijing Proteome Research Center, National Center for Protein Sciences-Beijing (PHOENIX Center), Beijing Institute of Lifeomics, Beijing, China.

5 State Key Laboratory of Innovative Natural Medicine and TCM Injections, Ganzhou, China.

6 China Academy of Chinese Medical Sciences, Beijing, China

*These authors contribute equally.

#Corresponding authors

Hongjun Yang, email: hjyang@icmm.ac.cn

Fangbo Zhang, email: fbzhang@icmm.ac.cn

Abstract

Natural products from traditional medicine inherit bioactivity from their source herbs. However, the pharmacological mechanism of natural products is often unclear and studied insufficiently. Pathway fingerprint similarity based on “drug-target-pathway” heterogeneous network provides new insight into Mechanism of Action (MoA) for natural products compared with reference drugs, which are selected approved drugs with similar bioactivity. Natural products with similar pathway fingerprints may have similar MoA to approved drugs. In our study, XYPI, an andrographolide derivative, had similar anti-inflammatory activity to Glucocorticoids (GCs) and Nonsteroidal Anti-inflammatory Drugs (NSAIDs), and GCs and NSAIDs have completely different MoA. Based on similarity evaluation, XYPI has similar pathway fingerprints as NSAIDs, but has similar target profile with GCs. The expression pattern of genes in LPS-activated macrophages after XYPI treatment is similar to that after NSAID but not GC treatment, and this experimental result is consistent with the computational prediction based on pathway fingerprints. These results imply that the pathway fingerprints of drugs have potential for drug similarity evaluation. This study used XYPI as an example to propose a new approach for investigating the pharmacological mechanism of natural products using pathway fingerprint similarity based on a “drug-target-pathway” heterogeneous network.

Keywords: Natural products, andrographolide derivative, pathway fingerprints, “Drug-Target-Pathway” network, anti-inflammatory

Introduction

Traditional Chinese medicine (TCM) plays an essential role in healthcare in China. Many useful natural drugs are derived from plant sources of TCMs. Moreover, more than half of approved drugs have been derived from natural products in the past twenty years[1]. One significant drug developed from traditional medicinal plants was artemisinin, which was first discovered by Dr. Y. Tu, who won the Nobel Prize for her contribution to malaria treatment. Natural products have complex and diverse structures that can provide lead compound libraries for drug discovery. Natural products from TCM also have highly selective and specific pharmacological activity based on the mechanisms of action of the original herb. In addition, many drugs are discovered via the application of natural product chemistry.

Computational methods are applied to select natural products with high bioactivity. Compounds that perform well in in silico predictions can be used as promising starting materials for experimental work. Virtual screening has a high success rate for activity prediction. Various computational methods have been used to predict the bioactivity of natural products, including quantitative structure-activity relationship (QSAR) models[2], pharmacophore models[3], and molecular docking[4]. In silico methods represent valuable filtering tools in the search for new

activities of natural products.

In addition to structure-based viral screening methods, a pathway-based approach was also proposed to predict the bioactivity of natural products. 'Pathway fingerprints' were proposed to describe the profiles of significant therapeutic functions influenced by compounds. Compounds sharing similar pharmacological effects may bind to the same group of targets (i.e., protein family), which synergize to counter the redundancy of biological networks. Moreover, drugs without common targets can also exert similar therapeutic effects on the same disease because different targets participate in the same pathway, which is closely associated with the pathological process[5]. Pathway-based similarity evaluation of compounds has been applied in bioactivity prediction. In our previous study, active compounds from TCM were predicted by pathway-based similarity evaluation and validated by a pharmacological activity test[6]. Pathway fingerprints have also been applied for drug discovery[5, 6] and repositioning[7].

Natural products from traditional medicine always have bioactivities similar to those of the original plant. However, the exact mechanism of action (MoA) of a natural product is often unclear. In contrast, approved drugs have a more explicit MoA. Comparison with approved drugs can help to explore the exact MoA of a natural product. As an example, andrographolide is the main active ingredient of *Andrographis paniculate*, which is used as an herbal medicine in both traditional Indian and Chinese medicine (where it is known as kalmegh and chuanxinlian, respectively)[8]. *A. paniculata* extracts exhibit anti-inflammatory activity that is commonly attributed to andrographolide[9]. Xiyanning injection (XYPI) is a CFDA-approved drug that consists of andrographolide derivatives, including water-soluble sulfonated andrographolide[10]. The effect of XYPI on sepsis and acute lung injury has been confirmed by many studies [10–13]. As a natural product, XYPI has been reported to have multiple types of pharmacological activity, including antimicrobial, antiviral and anti-inflammatory effects.

In the clinic, Glucocorticoids (GCs) and Nonsteroidal Anti-inflammatory Drugs (NSAIDs) both have anti-inflammatory effects. The anti-inflammatory effect of NSAIDs occurs via inhibition of cyclooxygenase (COX) in vivo activity and reduced biosynthesis of local tissue prostaglandin (PG)[14]. The main mechanism underlying the anti-inflammatory effect of glucocorticoids is a genetic effect, which involves binding to the glucocorticoid receptor (GR) on the nuclear membrane[15]. After activation, nuclear factors bind to the specific promoter sequence of the glucocorticoid response element, which affects gene transcription, causing transcription to increase or decrease accordingly and affecting inflammatory genes to exert anti-inflammatory effects. Therefore, our study attempts to confirm the mechanism of XYPI (andrographolide derivatives) using both NSAIDs and GCs as a reference. To reflect the common characteristics of glucocorticoids, we selected six commonly used GCs for subsequent analysis, namely, prednisolone, methylprednisolone, bexamethasone, prednisolone, cortisone and hydrocortisone[15]. The NSAIDs aspirin, ibuprofen, naproxen, ketoprofen, magnesium salicylate, salsalate, and diflunisal were chosen as controls to analyze the similarities and differences between XYPI and NSAIDs[14].

We proposed an approach using pathway fingerprint similarity based on a “drug-target-pathway” heterogeneous network to explore the mechanisms of natural drugs XYPI using NSAIDs and GCs as reference agents with anti-inflammatory activity. As a semistructured representation method, the heterogeneous information network is an effective tool for integrating information, which can fuse more types of objects and their complex interactions via multiple social network platforms. In this study, a heterogeneous network similarity algorithm used for social networks was applied to investigate the pathway fingerprint similarity between drugs in the heterogeneous "drug-target-pathway" network.

Methods

Target prediction of XYPI, NSAIDs and GCs

Three types of targets were used to construct the target profile for the drug. STITCH provides the target based on literature mining[16], PubChem provides the target based on bioactivity tests in bioassays[17, 18] and BATMAN-TCM predicts drug targets based on structure and function annotation[19]. For a query compound-target interaction, a combined confidence score (CS) was applied to comprehensively evaluate the probability of a target from the three types of resources, which are calculated as follows:

$$CS = 1 - (1 - P_{stitch}) \times (1 - P_{pubchem}) \times (1 - P_{batman})$$

P_{stitch} represents the probabilities of drug-target interaction from STITCH, $P_{pubchem}$ represents the probabilities of drug-target interaction from PubChem Bioassay, and P_{batman} represents the probabilities from BATMAN-TCM. In subsequent analysis, the targets with $CS > 0.4$ were selected as potential targets with higher reliability for analysis.

Hierarchical clustering of drugs based on target similarity

The target similarity between two drugs was measured based on comprehensive targets of the compounds. To eliminate the bias caused by the target number, we random pick 50 targets each time to calculate the $s_{a,b}$ 100 time as a correction. For example, T_a represents 50 randomly selected targets from the target space of drug a , and T_b represents 50 randomly selected targets from the target space of drug b . The similarity score $s_{a,b}$ for this random was calculated as follows:

$$s_{a,b} = \frac{T_a \cup T_b}{T_a \cap T_b}$$

And the final similarity score S was calculated as follows:

$$S = \frac{\sum_{i=1}^{100} S_{a,b}}{100}$$

The hierarchical clustering of N compounds was executed by the R package hClust[20] based on the target similarity matrix of compounds.

Hierarchical clustering of drugs based on similarity of pathway fingerprints from a “drug-target-pathway” heterogeneous network

The pathway fingerprint similarity of two drugs was measured based on a “drug-target-pathway” heterogeneous network using the PathSim method, which has been applied to recommendation systems in social networks[21]. The metapath is a path defined in the network mode that links two types of objects which is an concept in heterogeneous information network analysis. The metapath not only characterizes the semantic relationship between objects but also extracts feature information between objects. In the “drug-target-pathway” heterogeneous network, the metapath “drug-target-pathway-target-drug” of two drugs was considered to describe the linkage between two drugs. In this instance, pathway fingerprints of compounds were described using GO biological process terms of targets of drugs. Under the metapath framework, PathSim was developed to find peer objects in the network (e.g., find drugs with similar GO function descriptions) and to measure the similarity of peer objects based on metapaths. The “drug-target-pathway” heterogeneous network consists of drug-target interactions and target-pathway relations from the Gene Ontology database[22] (only biological process, downloaded on 2018.4.3). To eliminate the bias caused by the imbalanced target numbers of two drugs, we developed an optimized PathSim algorithm, in which the similarity score of two drugs was calculated based on only 50 random picked targets for each drug from the target space of drug. The ultimate similarity score of two drugs was the average score of similarity scores of 100 random times. The hierarchical clustering of N compounds was executed by the R package hClust[20] based on the pathway fingerprints similarity matrix of compounds.

LPS-induced RAW264.7 mouse macrophage inflammation model

Chemicals and reagents

Trypsin-EDTA digestion solution (Beijing Solable Technology Co., Ltd.), dimethyl sulfoxide (DMSO), LPS (CST), fetal bovine serum (Gibco, New Zealand), a penicillin-streptomycin double antibody (Beijing Solable Technology Co., Ltd.), DMEM culture medium (Gibco, New Zealand), and a mouse

IL-6 ELISA kit (CUSABIO, Wuhan, China) were used. The RAW264.7 mouse macrophage cell line was purchased from the Cell Resources Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and frozen in liquid nitrogen for use. A ZD-420 electric thermostatic water bath, a Multiskan Ascent microplate reader (Thermo Electron, United States), a BS224S electronic balance (Serdulis in Germany), a Napco5410 carbon dioxide incubator (American NAPCO), a DMIL inverted microscope (Germany LEICA), an optical microscope (Olympus), a program storage box, an ultraclean platform were also used.

Cell culture and experimental procedure

Normal RAW264.7 cells were cultured in DMEM culture medium containing a final concentration of 10% fetal bovine serum and 100,000 U/L penicillin and placed in a cell incubator with 5% CO₂ and a temperature of 37°C. The medium was changed once every 48-72 h according to the cell growth. After the cells had grown to 70%~80% confluence, they were digested with 0.25% trypsin-EDTA and centrifuged to separate the cells. The cells were then subcultured by passage once every 6 days and frozen in liquid nitrogen for later use. The total number of inoculated cells was 2×10⁴ per well, and the plates were placed in a cell incubator containing 5% CO₂ at 37°C. After 24 hours of adherence, the cell culture solution was discarded, and 100 μl of DMEM culture medium containing a final concentration of 0.5 to 5 μg/ml LPS was added for inflammatory stimulation. After 48 hours of treatment, the cell supernatant in each group of wells was collected. Interleukin (IL)-6 concentrations were determined using Duo Set ELISA Kits (CUSABIO, Wuhan, China). The experiment was conducted following the manufacturer's instructions. And the morphological differences between cells in each group were observed with a microscope.

RAW264.7 cells (5 × 10⁵ cells/well in a 6-well plate) were pretreated with or without XYPI, ketoprofen, or prednisolone for 24 h and then incubated with LPS for 48 h. Cells lysates were prepared with RIPA lysis buffer and protease inhibitors (Solarbio, Beijing, China), and centrifuged at 14,000 g for 15 min at 4 °C. Then protein samples were completed for western assay.

The statistical significance of differences between two groups was determined by an unpaired Student's t-test. The results were considered statistically significant when the p value was less than 5%. ELISA kits were used to detect the levels of the inflammatory factor IL-6 in the cell supernatant. Data are expressed as the mean using a t-test, with P<0.05 indicating statistical significance.

RNA extraction, library construction and sequencing

Total RNA was extracted with TRIzol Reagent (Thermo Fisher, USA) according to the manufacturer's instructions, and RNA integrity was assessed using the RNA Nano 6000 Assay Kit and the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Three biological replicates were used. A total amount of 3 μg RNA per sample was applied for library construction. Briefly, sequencing libraries were generated by using the NEBNext® Ultra™ RNA Library Prep Kit for

Illumina® (NEB, USA) according to the instructions. First, poly-T oligo-bound magnetic beads were applied to purify mRNA before RNA fragmentation was carried out. Then, random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-) were used to synthesize first-strand cDNA, and DNA Polymerase I and RNase H were used to synthesize second-strand cDNA. After that, PCR was performed to enrich the cDNA template following adenylation of the 3' ends of the DNA fragments and ligation of the adapters. The PCR products were purified (AMPure XP system), and the Agilent Bioanalyzer 2100 system was used to evaluate library quality. The AcBot Cluster Generation System was applied for clustering of the index-coded samples by using TruSeq PE150 Cluster Kit v3-cBot-HS (Illumina). Then, the sequencing library was sequenced on an Illumina HiSeq 4000 platform, and 150 bp paired-end reads were generated. This whole experiment was conducted at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

The EdgeR package was used for RNA-SEQ differential gene expression analysis[23], and the resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). Gene with a $FDR \leq 0.1$ and $\text{fold change} \geq 1.5$ were assigned as differentially expressed.

The principal component analysis (PCA) method was used to visualize the clustering of samples, as it can intuitively observe the clustering of samples in the experimental group and the control group. PCA analysis and visualization was executed by the R package FactoMineR[24].

Results

Potential Target prediction for XYPI

Using "Xi yan ping Injection", "Andrographolide sulfonates" and "Andrographolide" as keywords to mine the medical literature abstracts from PubMed from 1950 to 2015, a total of 327 related genes mentioned in the literature were excavated. Combining 327 targets based on literature mining, 172 targets predicted by BATMAN-TCM, and 12 targets extracted from PubChem Bioassay, there were 491 potential targets of XYPI.

By integrating the confidence scores of targets from different resources (text mining, biological activity-related target data (PubChem), and structure-based target prediction (BATMAN-TCM)), 140 targets of XYPI with a combined confidence score greater than 0.4 were retained for subsequent analysis. Based on the functional enrichment of the integrated targets, XYPI may be involved in regulation of the inflammatory response, leukocyte differentiation, ROS metabolic process, etc. (**Figure 1A**). The above results indicate that research on XYPI should focus on immune and inflammation-related pathways.

XYPI has targets similar to those of GCs but pathway fingerprints similar to those of NSAIDs

There were 140 targets of XYPI (XYPI), 65 common targets of GCs (target for at least two GCs) and 161 common targets of NSAIDs (target for at least two NSAIDs) whose confidence scores were greater than 0.4. To investigate the similarity of XYPI with GCs and NSAIDs, a Venn diagram of XYPI, GCs and NSAIDs showed that there were 12 common targets of XYPI, GC and NSAIDs, and XYPI had common targets with both GC and NSAIDs (**Figure 1B**). To evaluate the target similarity between XYPI and different GCs and NSAIDs separately, unsupervised hierarchical clustering of XYPI, 6 glucocorticoids and 7 NSAIDs based on combined target similarity was applied. The clustering results showed that 7 glucocorticoids clustered with each other, as well as NSAIDs (**Figure 1C**), which indicates that GCs have a high similarity with each other at the target level, as do NSAIDs. However, GCs and NSAIDs have relatively different target spaces, which may be due to the different MoA by which they exert anti-inflammatory effects. XYPI clustered with glucocorticoids, which shows that the targets of XYPI are more similar to those of glucocorticoids than those of NSAIDs.

After investigating the similarity of the pathway fingerprints of XYPI, GCs and NSAIDs, the unsupervised clustering results showed that XYPI is more similar to NSAIDs, which was contrary to the previous target-based clustering results (**Figure 1D**). Although seven NSAIDs were separated into two clusters, 6 GCs were still clustered with each other based on pathway fingerprint similarity. This implies that GCs have similar targets and pathway fingerprints. However, NSAIDs may participate in different pathways, causing them to fall into two groups.

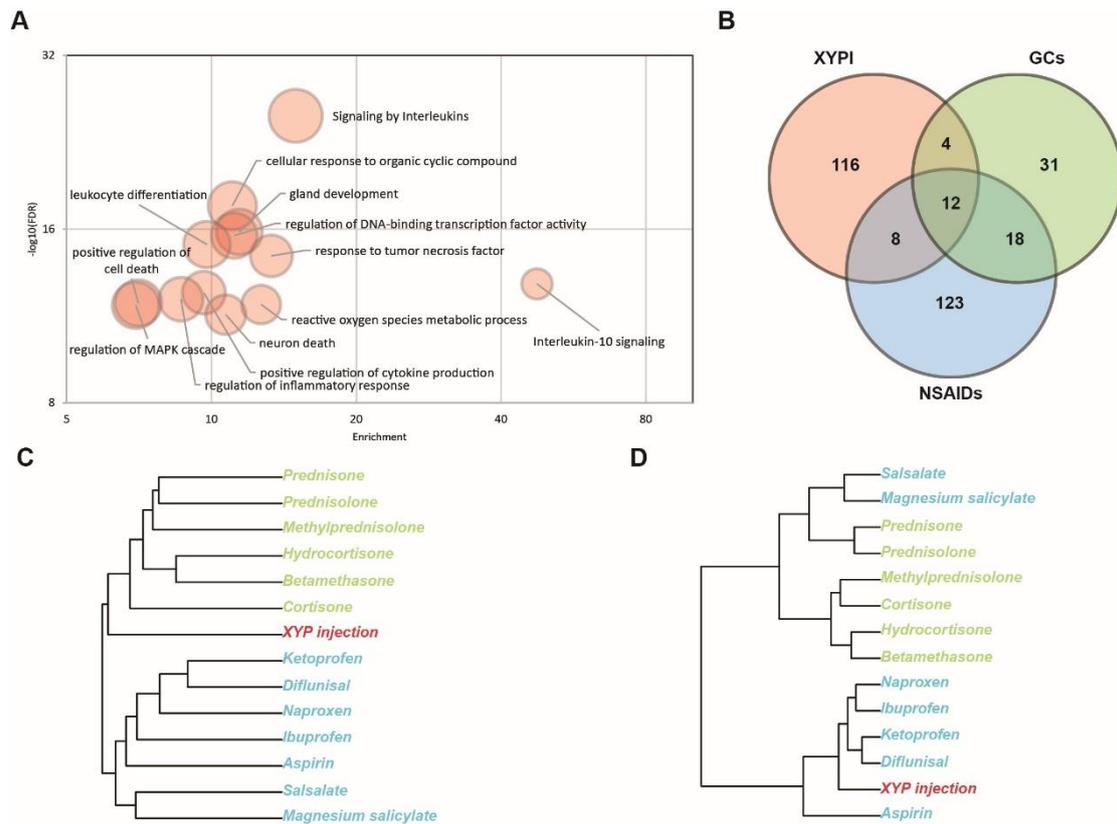


Figure 1 Comparison of targets of XYPI, GCs and NSAIDs.

(A) Bubble chart of functional enrichment of potential XYPI targets. The X axis is the enrichment score for each term using the whole genome as background, the Y axis is the negative log₁₀(FDR), and the node size is related to the targets matched in this term.

(B) Venn diagram of the drug targets of XYPI, GCs and NSAIDs.

(C&D) Hierarchical clustering of XYPI, GCs (green) and NSAIDs (blue) based on target similarity (C) and pathway fingerprint similarity (D).

XYPI has pathway fingerprints similar to those of NSAIDs in terms of immune and inflammatory pathways

To explore the role of XYPI in immune and inflammatory responses, a Venn diagram was generated to compare the differences between XYPI drug targets and genes in the GO terms “immune response” and “inflammatory response”. The results showed that 59/140 and 37/140 targets of XYPI also participate in immune and inflammatory responses, respectively. Similar results were obtained for GCs and NSAIDs (**Figure 2A**). This implies that most targets of XYPI, GCs and NSAIDs participate in immune and inflammatory pathways. Next, hierarchical clustering of XYPI, GCs and NSAIDs based on pathway fingerprints showed that XYPI was most similar to two NSAIDs (aspirin and ketoprofen), if the pathways were restricted to inflammation-related pathways (**Figure 2B**). In addition, ketoprofen and ibuprofen were restricted to immune pathways

(Figure 2C). Hierarchical clustering based on pathway fingerprints shows that most GCs and NSAIDs have similar pathway fingerprints, which indicates that GCs and NSAIDs have distinct target pathways in terms of inflammation and immunity. These results indicate that XYPI is more similar to NSAIDs in terms of immunity and inflammation.

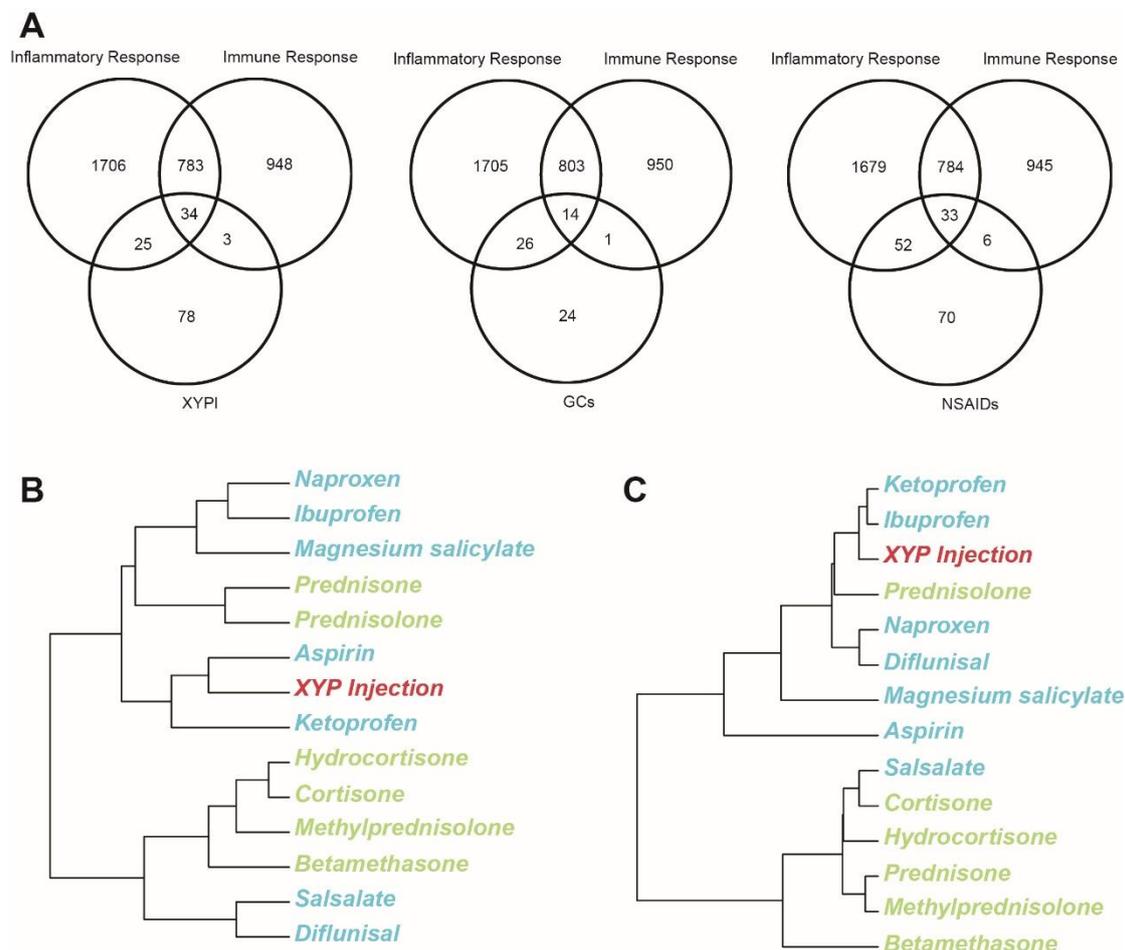


Figure 2 Comparison of targets and pathways of XYPI, GCs and NSAIDs.

(A) Venn diagram of the drug targets of XYPI, GCs, and NSAIDs with genes in inflammatory and immune pathways.

(B&C) Hierarchical clustering of XYPI, GCs (green) and NSAIDs (blue) based on pathway fingerprint similarity restricted to inflammatory (B) and immune (C) pathways.

Shared pathways between XYPI and NSAIDs based on a “drug-target-pathway” heterogeneous network

To further illustrate the anti-inflammatory characteristics of XYPI, we identified the shared pathways of XYPI and NSAIDs (ketoprofen). The results showed that these targets are involved in the inflammatory response, especially regulation of the acute inflammatory response and the production of cytokines (**Figure 3A**). In the above process, XYPI tends to affect molecules related

to positive regulation of the inflammatory response and molecules involved in cytokine production, which include proinflammatory proteins such as NFKBIA, FABP4, IL2, and CCL4 (Figure 3A).

To investigate the immune effect of XYPI, we selected prednisolone as representative of GCs which are most similar to XYPI in terms of immune response, to identify the shared pathways of XYPI and GCs (Figure 3B). The results show that GCs are more inclined to negatively regulate cytokine production and interferon gamma-mediated signaling pathways and simultaneously participate more in the function of acquired immunity. XYPI is more likely to be involved in leukocyte migration, the complement receptor signal transduction pathway, the LPS-mediated signaling pathway and the innate immune response. Among these factors, ICAM1, MMP9, PTPN6, and SRC are regulated by XYPI and participate in the process of leukocyte migration. In addition, XYPI participates in the LPS-mediated MAPK signaling pathway to affect immune function.

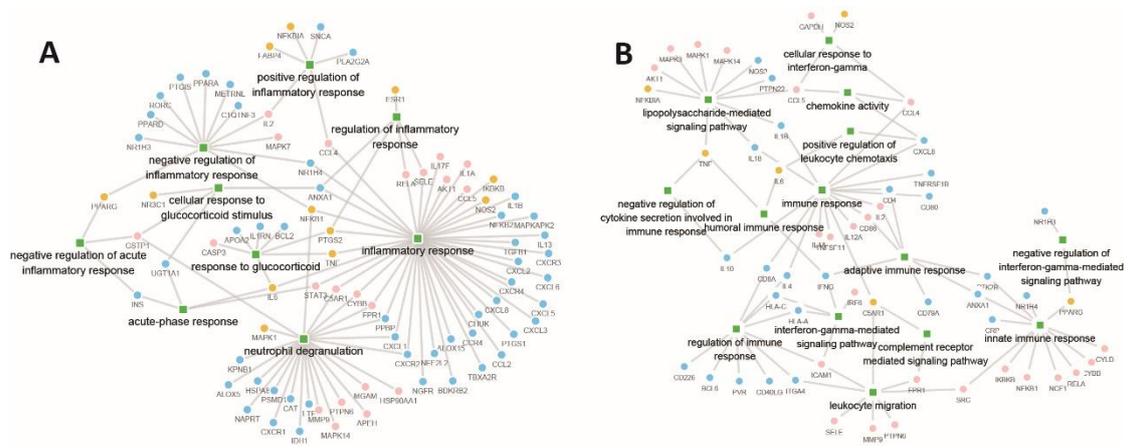


Figure 3 Comparison of XYPI and anti-inflammatory drugs in terms of inflammatory and immune pathways.

(A) “drug-target-pathway” heterogeneous network of XYPI and ketoprofen s in terms of inflammatory response

(B) “drug-target-pathway” heterogeneous network of XYPI and prednisolone in terms of immune response

Transcriptome of XYPI against LPS-activated murine macrophage model exhibiting its anti-inflammatory effect

To compare the pharmacological effects of XYPI, an LPS-activated murine macrophage model was used. Prednisolone and ketoprofen were selected as representative GCs and NSAIDs, respectively, because of their high similarity with XYPI based on pathway fingerprints. In LPS-activated macrophages, transcription of the cytokine IL6 was increased after 48h of stimulation (Figure 4A). Pretreatment with low- and high-dose XYPI (low dose: 0.02 mg/ml, high dose: 0.04 mg/ml)

strongly decreased cytokine expression in LPS-activated murine macrophages after **24h** of exposure. Pretreatment with ketoprofen and prednisolone (low dose: 1 μ M, high dose: 2 μ M) also reversed the upregulation of IL6. The results show that preadministration of XYPI has a significant anti-inflammatory effect by reducing IL-6 in activated macrophages, and this effect is similar to that of ketoprofen and prednisolone.

Transcriptome analysis based on RNA-seq was performed to detect the gene expression profile of LPS-activated macrophages after XYPI treatment, and ketoprofen and prednisolone were used as positive controls. A volcano plot of differentially expressed genes showed that the model group had a total of 117 differentially expressed genes compared with the control group, including 62 upregulated genes and 55 downregulated genes. Compared with the model group, the XYPI high-dose administration group had a total of 45 differentially expressed genes, including 23 upregulated genes and 22 downregulated genes. The ketoprofen high-dose group had 118 differentially expressed genes, including 55 upregulated genes and 73 downregulated genes. There were 883 differentially expressed genes in the prednisolone high-dose group, including 456 upregulated genes and 427 downregulated genes (**Figure 4B**). The results of PCA in **Figure 4C** show that the distance between the XYPI group and the control group was very small, indicating that after XYPI preadministration, the gene expression pattern of RAW263.4 cells was similar to that of the control group. As a positive control drug, ketoprofen had a gene expression pattern relatively similar to that of the control group. Although prednisolone has an anti-inflammatory effect, the gene expression pattern after treatment with prednisolone is very different from that of the control group and the group treated with XYPI and ketoprofen. The above results imply that XYPI may have a similar anti-inflammatory mechanism to ketoprofen, but it is quite different from the anti-inflammatory mechanism of prednisolone. A heatmap of hierarchical clustering was performed on the differentially expressed genes in each group, revealing a total of 7 distinct gene expression patterns, and functional enrichment analysis was performed for each group of genes (**Figure 4D**), which showed that high-dose XYPI pretreatment reversed the downregulation of genes related to the response to LPS and the immune and inflammatory response and recovered the inflammatory response by suppressing the positive regulation of cytokine and NO biosynthesis processes and T cell proliferation (Figure 4D). Compared with that of the model group, the response to LPS and IL1 was significantly upregulated after prednisolone treatment, and DNA replication, the cell cycle and immune and inflammatory responses were suppressed.

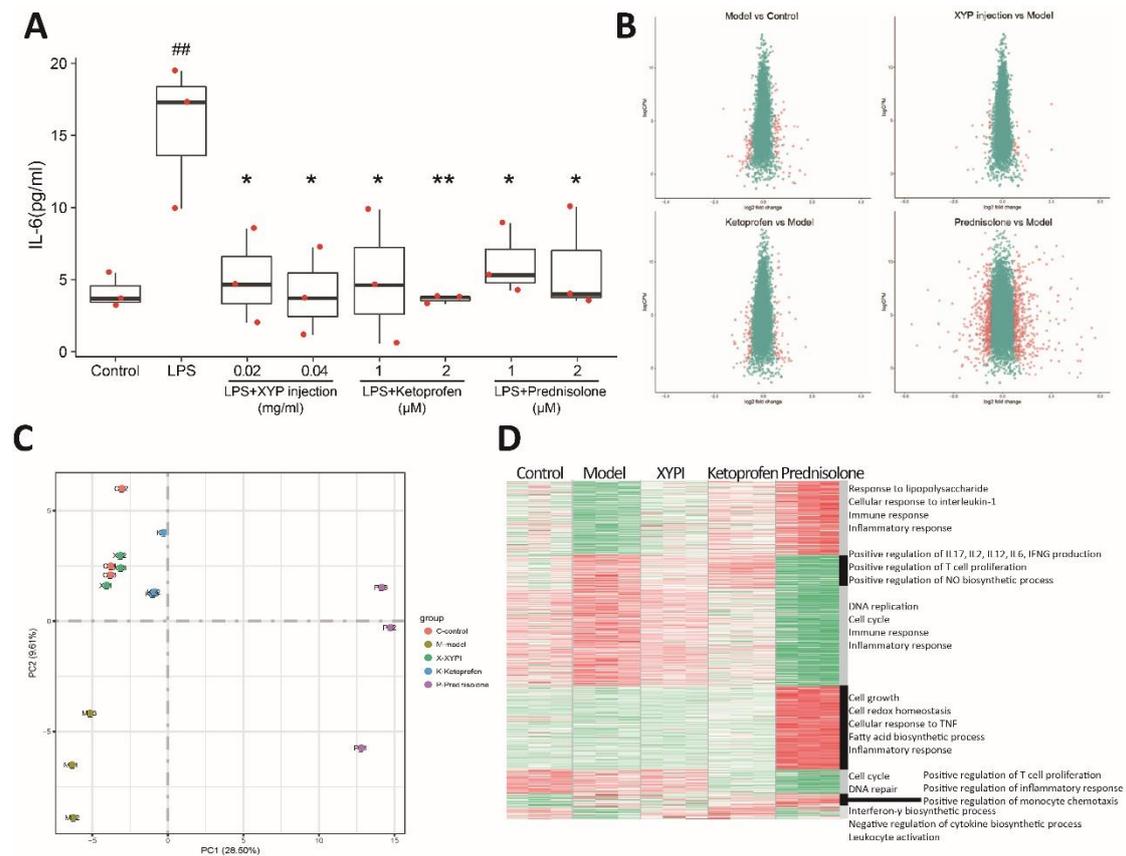


Figure 4 Transcriptome of XYPI pretreatment in an LPS-activated macrophage cell model.

(A) Comparison of the anti-inflammatory effects of XYPI with those of ketoprofen and prednisolone. The effect of the three agents on expression of the inflammatory factor IL-6 induced by LPS in the supernatant of RAW264.7 cells after 24 hours of pre-administration. * indicates the administration group vs the model group $p < 0.05$, ** $p < 0.01$. # indicates the model group vs the blank control group $p < 0.05$, ### $p < 0.01$.

(B-D) Volcano plot (B), PCA (C) and hierarchical clustering (D) of the transcriptome of the control group, model group (LPS-induced macrophage model), and drug group (pretreatment with high-dose XYPI, high-dose ketoprofen and high-dose prednisolone).

Expression of the shared pathways of XYPI, ketoprofen and prednisolone by integrating the transcriptome and a heterogeneous “drug-target-pathway network

To determine the specific pathways targeted by XYPI, the shared metapath ‘drug-target-pathway-target-drug’ of the heterogeneous network was extracted for XYPI and NSAIDs/GCs. In **Figure 3A and B**, a shared pathway from the “drug-target-pathway” heterogeneous network of XYPI and NSAIDs in terms of the inflammatory response and a similar

pathway of XYPI and GCs in terms of the immune response is shown. The expression of shared pathways after drug treatment was investigated based on transcriptome data for XYPI, ketoprofen and prednisolone.

A comparison of the inflammatory pathway for drug regulation between XYPI and ketoprofen (**Figure 5A**) showed that neutrophil degranulation and negative regulation of the inflammatory response were upregulated by XYPI (63.1% of genes upregulated in the former and 66.7% in the latter) and ketoprofen (47.2% of genes upregulated in the former and 66.7% in the latter). The 'cellular response to glucocorticoid stimulus' was upregulated by prednisolone (83.3% gene upregulated). The heatmap of differential gene expression in the XYPI, ketoprofen and prednisolone pathways mentioned above shows that most genes involved in neutrophil degranulation and negative regulation of the inflammatory response pathway were upregulated after XYPI and ketoprofen treatment but downregulated after XYPI treatment (**Figure 5B**). Genes related to 'cellular response to glucocorticoid stimulus' were obviously upregulated by prednisolone but downregulated by XYPI and ketoprofen (**Figure 5B**). This implied that prednisolone had an expression pattern distinct from those of XYPI and ketoprofen in terms of the inflammatory pathway.

A comparison of the immune pathways for drug regulation between XYPI and prednisolone (**Figure 5C**) showed that the innate (61/91 genes upregulated), adaptive (5/10 genes upregulated) and humoral (12/15 genes upregulated) immune responses were activated by XYPI but repressed by prednisolone (32/91, 4/10, and 3/15 genes upregulated, respectively). In addition, "cellular response to IFN- γ " was upregulated by XYPI (29/45 genes upregulated). The heatmap of differential gene expression in the XYPI, ketoprofen and prednisolone pathways mentioned above shows that most genes involved in innate, adaptive and humoral immune responses and cellular responses to IFN- γ were upregulated after XYPI and ketoprofen treatment but downregulated after XYPI treatment (**Figure 5D**). This implied that prednisolone repressed most aspects of the immune response.

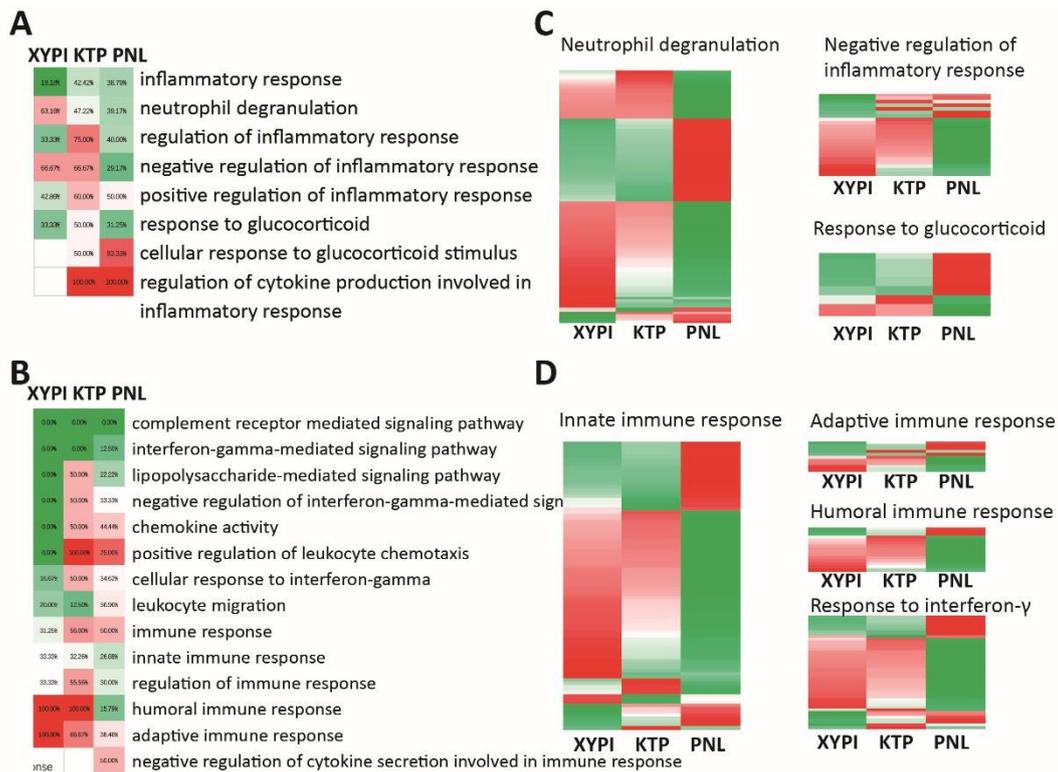


Figure 5 Expression of the shared pathways of XYPI, ketoprofen (KTP) and prednisolone (PNL)

(A&B) Pathway activation and repression after drug treatment in terms of inflammatory (A) and immune pathways (B).

(C&D) Heatmap of genes in the shared pathways after drug treatment in terms of the inflammatory (C) and immune pathways (D).

Discussion

As commonly used anti-inflammatory drugs in the clinic, GCs and NSAIDs were selected as controls to study the anti-inflammatory and immune effects of XYPI. XYPI injection has similar targets as GCs because XYPI clustered with GCs in hierarchical clustering based on target similarity. However, XYPI has similar pathway fingerprints as NSAIDs. The transcriptome of the LPS-activated macrophage model after drug treatment shows that the gene expression pattern of macrophages after XYPI treatment is much more similar to that of ketoprofen, with no repression of the immune response, than to prednisolone. XYPI and ketoprofen both participate in the positive regulation of the inflammatory response and cytokine production. GCs are more likely to negatively regulate the signaling pathways mediated by interferon γ and are more involved in the function of acquired immunity. The expression pattern of these pathways after XYPI treatment is diametrically opposite that of GCs. We propose that XYPI may have a similar anti-inflammatory mechanism as NSAIDs.

These results indicate that pathway fingerprints provide a new approach for drug discovery. Even for a single drug compound, the mechanism should be explored from a multitarget perspective. A drug's multiple targets (direct and indirect) interact with each other to shut down a cellular pathway, which may be an unintended pathway, demonstrating the potential for polypharmacy to impact complex diseases. In addition to target profiles, pathway fingerprints have also been used in polypharmacological studies to describe the function of drug therapy[5, 7, 25]. In this study, similarity analysis of pathway fingerprints and target profiles were both applied to investigate the MoA of natural products (XYPI). The experimental results confirmed that pathway fingerprints can be used to evaluate drug similarity and to predict MoA.

Natural products from traditional medicine inherit bioactivity from their source herbs. However, the pharmacological mechanism by which they protect against disease is often unclear and studied insufficiently. The prediction of MoA based on structures and target profiles has been performed extensively in drug discovery. However, the “drug-target-pathway” heterogeneous network provides new insight into drug MoA. Pathway fingerprints extracted from a “drug-target-pathway” heterogeneous network can describe the pathways affected by drugs, which are meaningful combinations of direct and indirect targets of drugs. The similarity evaluation of pathway fingerprints based on recommendation systems used in social networks is a technological transformation of social network technology to pharmacological research. Compared to novel compounds, natural products have more easily predictable bioactivities based on traditional use. Pathway fingerprint similarity provides new insight into natural products compared with reference drugs, which are selected approved drugs with similar bioactivity. Natural products with similar pathway fingerprints may have similar MoA to approved drugs. In our study, XYPI, an andrographolide derivative, had similar anti-inflammatory activity to GCs and NSAIDs. GCs and NSAIDs have completely different MoA. Based on pathway fingerprint similarity, XYPI has similar pathway fingerprints as NSAIDs. The expression pattern of genes in LPS-activated macrophages after XYPI treatment is similar to that after NSAID but not GC treatment. These results imply that the pathway fingerprints of drugs can provide new ideas for drug similarity investigations. Similarity evaluation for heterogeneous networks based on recommendation systems for social networks provides good reference methodologies for pathway fingerprints.

In summary, pathway fingerprints were applied to an XYPI MoA investigation. XYPI, GCs and NSAIDs have considerable anti-inflammatory effects. However, we found that XYPI has a similar target profile to GCs but similar pathway fingerprints to NSAIDs. Based on the experimental validation of the transcriptome, we found that the expression profile of ketoprofen is similar to that of XYPI, but prednisolone has a distinct profiling pattern, which indicates that the anti-inflammatory mechanism of XYPI may be different from that of GCs but similar to that of NSAIDs because XYPI does not have an immunosuppressive effect, unlike GCs. This experimental result is consistent with the computational prediction based on pathway fingerprints. This study used XYPI, an andrographolide derivative, as an example and proposed a new approach for investigating the pharmacological mechanism of natural products using pathway fingerprint similarity based on a “drug-target-pathway” heterogeneous network.

Competing interests

The authors declare that they have no competing interests.

Funding

The authors acknowledge support from the State Project for Essential Drug Research and Development of China (2019ZX09201005, 2019ZX09721001), the Fundamental Research Funds for the Central Public Welfare Research Institutes (ZZ13-YQ-048), and Key Research and Development Project of Shandong Province (2017CXGC1301).

Authors' contributions

F.G., C.H.J., N.X., Y.G. and H.J.Y. conceived the study and F.G., F.B.Z. and Y.J.X wrote the manuscript. F.G., W.Z., D.W. and C.H.J performed the data analysis. F.B.Z., W.Z. and Y.Z. launched the experiment study. All authors reviewed and approved the final manuscript.

Acknowledgments

Not applicable.

Reference

1. Cragg GM, Newman DJ (2013) Natural products: a continuing source of novel drug leads. *Biochim Biophys Acta* 1830:3670–3695. <https://doi.org/10.1016/j.bbagen.2013.02.008>
2. Gavernet L, Talevi A, Castro EA, Bruno-Blanch LE (2008) A Combined Virtual Screening 2D and 3D QSAR Methodology for the Selection of New Anticonvulsant Candidates from a Natural Product Library. *QSAR & Combinatorial Science* 27:1120–1129. <https://doi.org/10.1002/qsar.200730055>
3. Zhang H, Xu X, Chen L, et al (2011) Molecular determinants of magnolol targeting both RXR α and PPAR γ . *PLoS One* 6:e28253. <https://doi.org/10.1371/journal.pone.0028253>
4. Waszkowycz B, Clark DE, Gancia E (2011) Outstanding challenges in protein–ligand docking and structure-based virtual screening. *Wiley Interdisciplinary Reviews: Computational Molecular Science* 1:229–259. <https://doi.org/10.1002/wcms.18>
5. Ye H, Tang K, Yang L, et al (2012) Study of drug function based on similarity of pathway fingerprint. *Protein Cell* 3:132–139. <https://doi.org/10.1007/s13238-012-2011-z>

-
6. Guo F, Tang X, Zhang W, et al (2020) Exploration of the mechanism of traditional Chinese medicine by AI approach using unsupervised machine learning for cellular functional similarity of compounds in heterogeneous networks, XiaoErFuPi granules as an example. *Pharmacol Res* 160:105077. <https://doi.org/10.1016/j.phrs.2020.105077>
 7. Iwata M, Hirose L, Kohara H, et al (2018) Pathway-Based Drug Repositioning for Cancers: Computational Prediction and Experimental Validation. *J Med Chem* 61:9583–9595. <https://doi.org/10.1021/acs.jmedchem.8b01044>
 8. Low M, Khoo CS, Münch G, et al (2015) An in vitro study of anti-inflammatory activity of standardised *Andrographis paniculata* extracts and pure andrographolide. *BMC Complement Altern Med* 15:18. <https://doi.org/10.1186/s12906-015-0525-7>
 9. Akbar S (2011) *Andrographis paniculata*: a review of pharmacological activities and clinical effects. *Altern Med Rev* 16:66–77
 10. Li M, Yang X, Guan C, et al (2018) Andrographolide sulfonate reduces mortality in Enterovirus 71 infected mice by modulating immunity. *Int Immunopharmacol* 55:142–150. <https://doi.org/10.1016/j.intimp.2017.11.042>
 11. Banerjee M, Parai D, Chattopadhyay S, Mukherjee SK (2017) Andrographolide: antibacterial activity against common bacteria of human health concern and possible mechanism of action. *Folia Microbiol (Praha)* 62:237–244. <https://doi.org/10.1007/s12223-017-0496-9>
 12. Tan WSD, Liao W, Zhou S, Wong WSF (2017) Is there a future for andrographolide to be an anti-inflammatory drug? Deciphering its major mechanisms of action. *Biochem Pharmacol* 139:71–81. <https://doi.org/10.1016/j.bcp.2017.03.024>
 13. Lei X, Chen J, Ren J, et al (2015) Liver Damage Associated with *Polygonum multiflorum* Thunb.: A Systematic Review of Case Reports and Case Series. *Evid Based Complement Alternat Med* 2015:459749. <https://doi.org/10.1155/2015/459749>
 14. Bacchi S, Palumbo P, Sponta A, Coppolino MF (2012) Clinical pharmacology of non-steroidal anti-inflammatory drugs: a review. *Antiinflamm Antiallergy Agents Med Chem* 11:52–64. <https://doi.org/10.2174/187152312803476255>
 15. Vandewalle J, Luybaert A, De Bosscher K, Libert C (2018) Therapeutic Mechanisms of Glucocorticoids. *Trends Endocrinol Metab* 29:42–54. <https://doi.org/10.1016/j.tem.2017.10.010>
 16. Kuhn M, Szklarczyk D, Pletscher-Frankild S, et al (2014) STITCH 4: integration of protein-chemical interactions with user data. *Nucleic Acids Res* 42:D401–407. <https://doi.org/10.1093/nar/gkt1207>
 17. Wang Y, Bryant SH, Cheng T, et al (2017) PubChem BioAssay: 2017 update. *Nucleic Acids Res* 45:D955–D963. <https://doi.org/10.1093/nar/gkw1118>

-
18. Wang Y, Cheng T, Bryant SH (2017) PubChem BioAssay: A Decade's Development toward Open High-Throughput Screening Data Sharing. *SLAS Discov* 22:655–666. <https://doi.org/10.1177/2472555216685069>
 19. Liu Z, Guo F, Wang Y, et al (2016) BATMAN-TCM: a Bioinformatics Analysis Tool for Molecular mechANism of Traditional Chinese Medicine. *Scientific Reports* 6:21146. <https://doi.org/10.1038/srep21146>
 20. hclust function | R Documentation. <https://www.rdocumentation.org/packages/stats/versions/3.6.1/topics/hclust>. Accessed 22 Oct 2019
 21. Sun Y, Han J, Yan X, et al (2011) Pathsim: Meta path-based top-k similarity search in heterogeneous information networks. In: *In VLDB' 11*
 22. The Gene Ontology Consortium (2017) Expansion of the Gene Ontology knowledgebase and resources. *Nucleic Acids Res* 45:D331–D338. <https://doi.org/10.1093/nar/gkw1108>
 23. Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139–140. <https://doi.org/10.1093/bioinformatics/btp616>
 24. Lê S, Josse J, Husson F (2008) FactoMineR: An R Package for Multivariate Analysis. *Journal of Statistical Software* 025:
 25. Guo F, Tang X, Zhang W, et al (2020) Exploration of the mechanism of traditional Chinese medicine by AI approach using unsupervised machine learning for cellular functional similarity of compounds in heterogeneous networks, XiaoErFuPi granules as an example. *Pharmacol Res* 160:105077. <https://doi.org/10.1016/j.phrs.2020.105077>

Figures

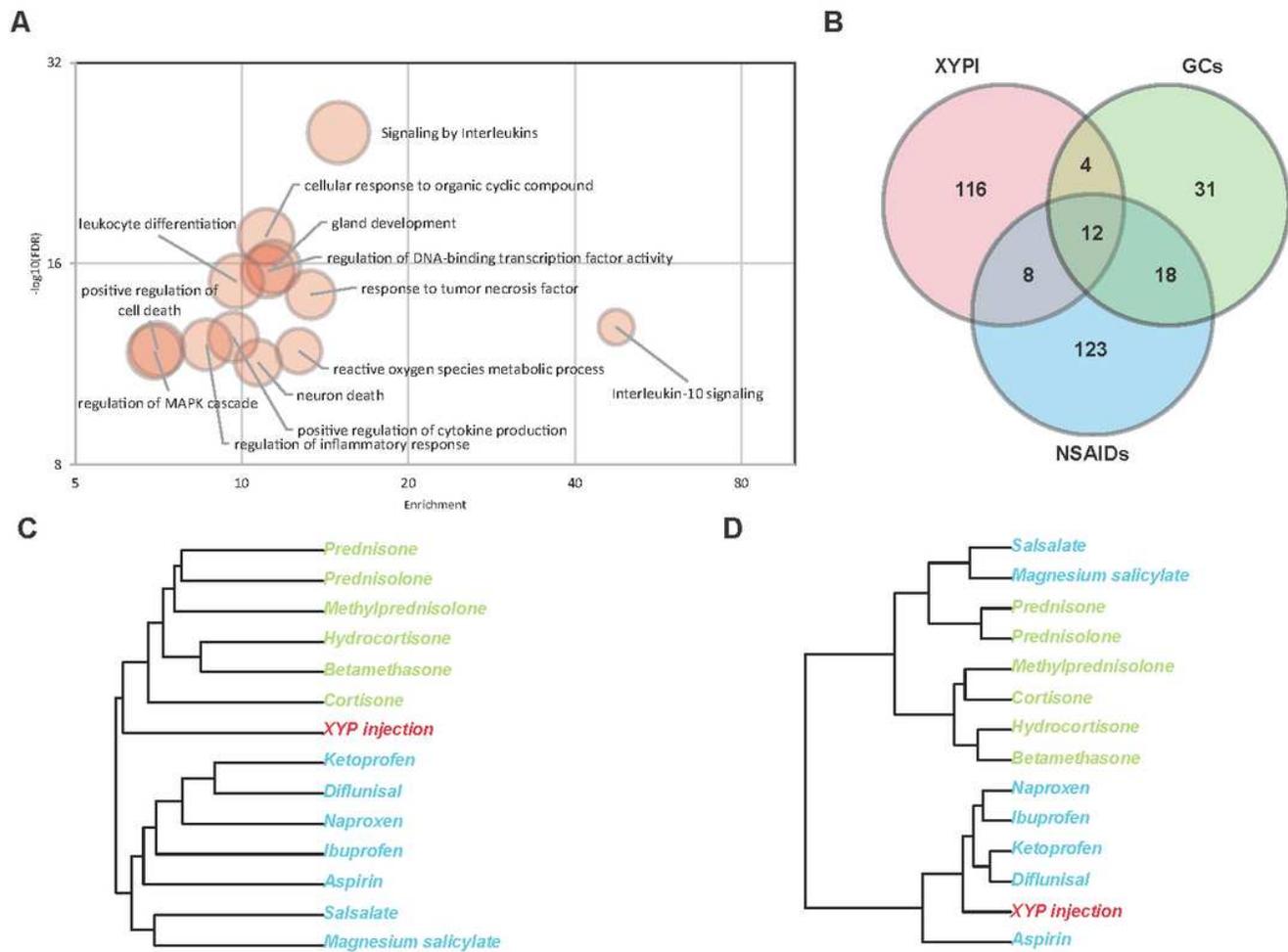


Figure 1

Comparison of targets of XYPI, GCs and NSAIDs. (A) Bubble chart of functional enrichment of potential XYPI targets. The X axis is the enrichment score for each term using the whole genome as background, the Y axis is the negative $\log_{10}(\text{FDR})$, and the node size is related to the targets matched in this term. (B) Venn diagram of the drug targets of XYPI, GCs and NSAIDs. (C&D) Hierarchical clustering of XYPI, GCs (green) and NSAIDs (blue) based on target similarity (C) and pathway fingerprint similarity (D).

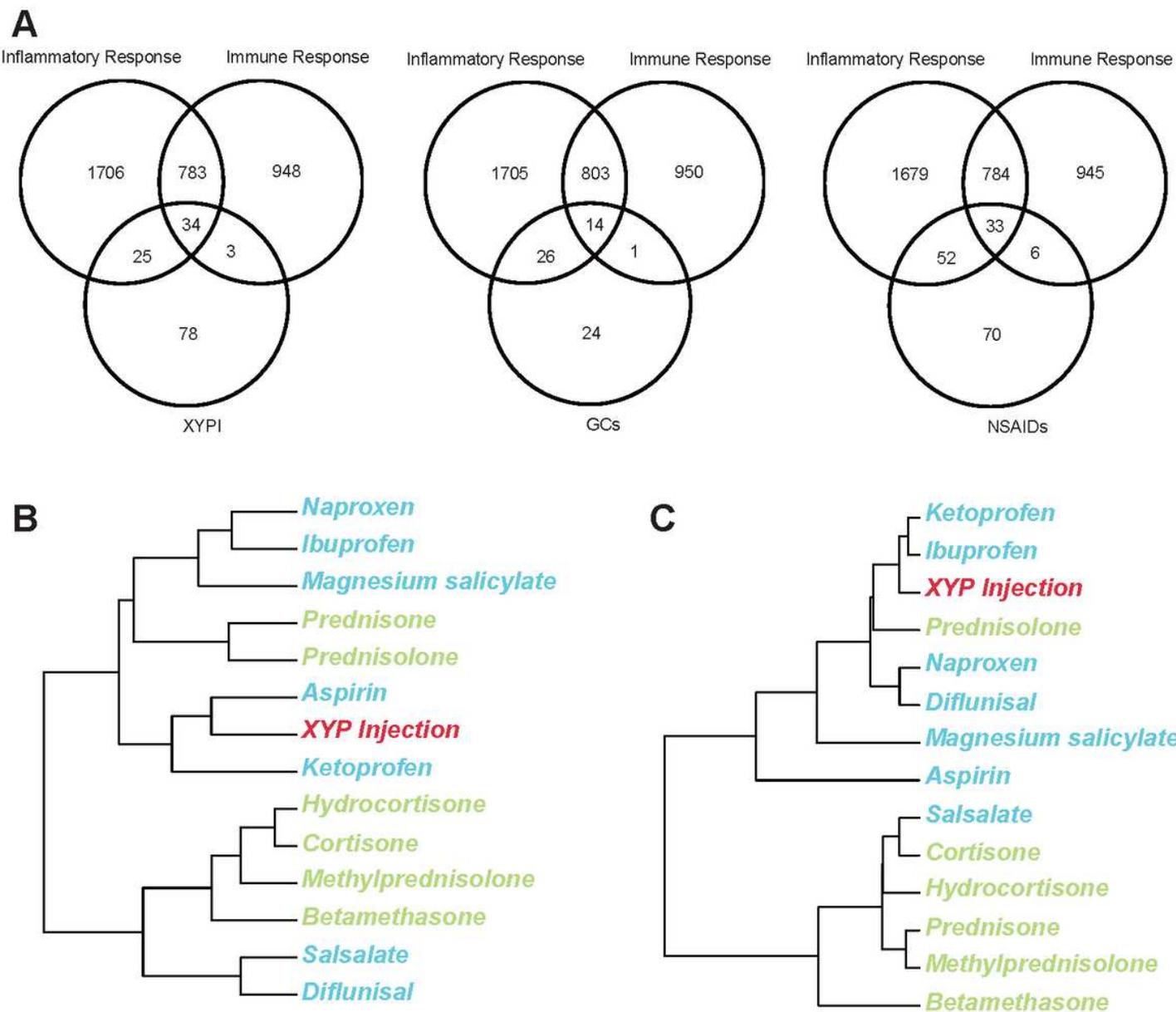


Figure 2

Comparison of targets and pathways of XYPI, GCs and NSAIDs. (A) Venn diagram of the drug targets of XYPI, GCs, and NSAIDs with genes in inflammatory and immune pathways. (B&C) Hierarchical clustering of XYPI, GCs (green) and NSAIDs (blue) based on pathway fingerprint similarity restricted to inflammatory (B) and immune (C) pathways.

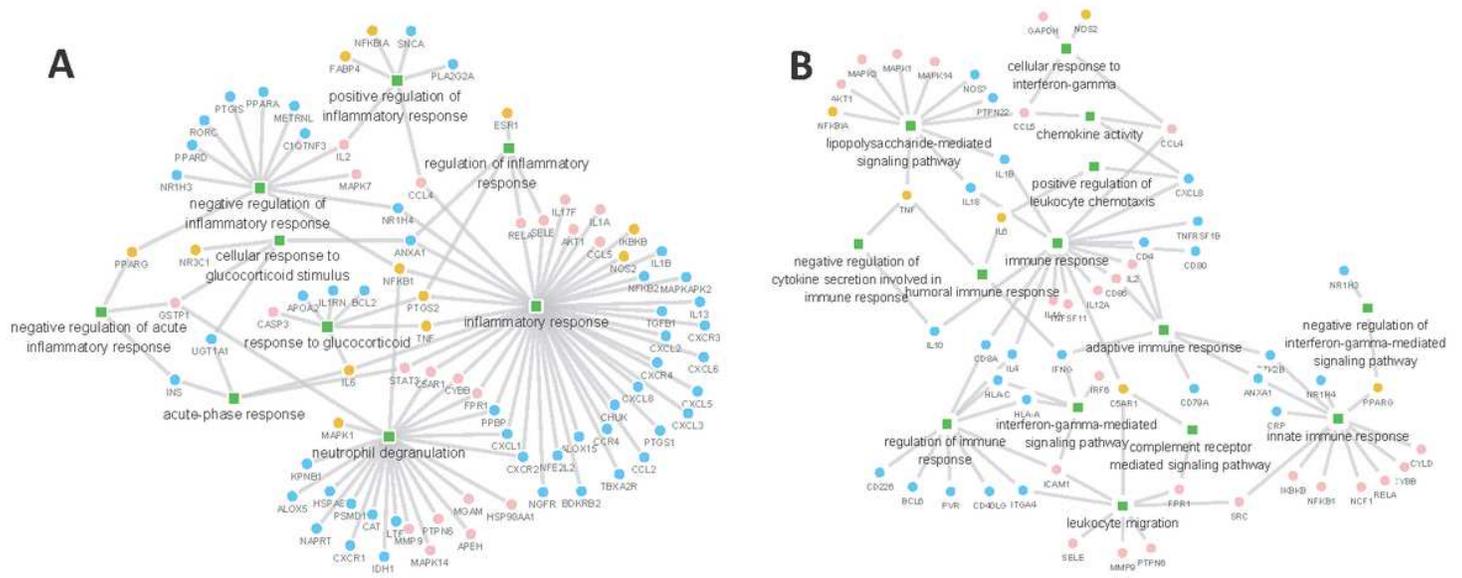


Figure 3

Comparison of XYPI and anti-inflammatory drugs in terms of inflammatory and immune pathways. (A) “drug-target-pathway” heterogeneous network of XYPI and ketoprofen s in terms of inflammatory response (B) “drug-target-pathway” heterogeneous network of XYPI and prednisolone in terms of immune response

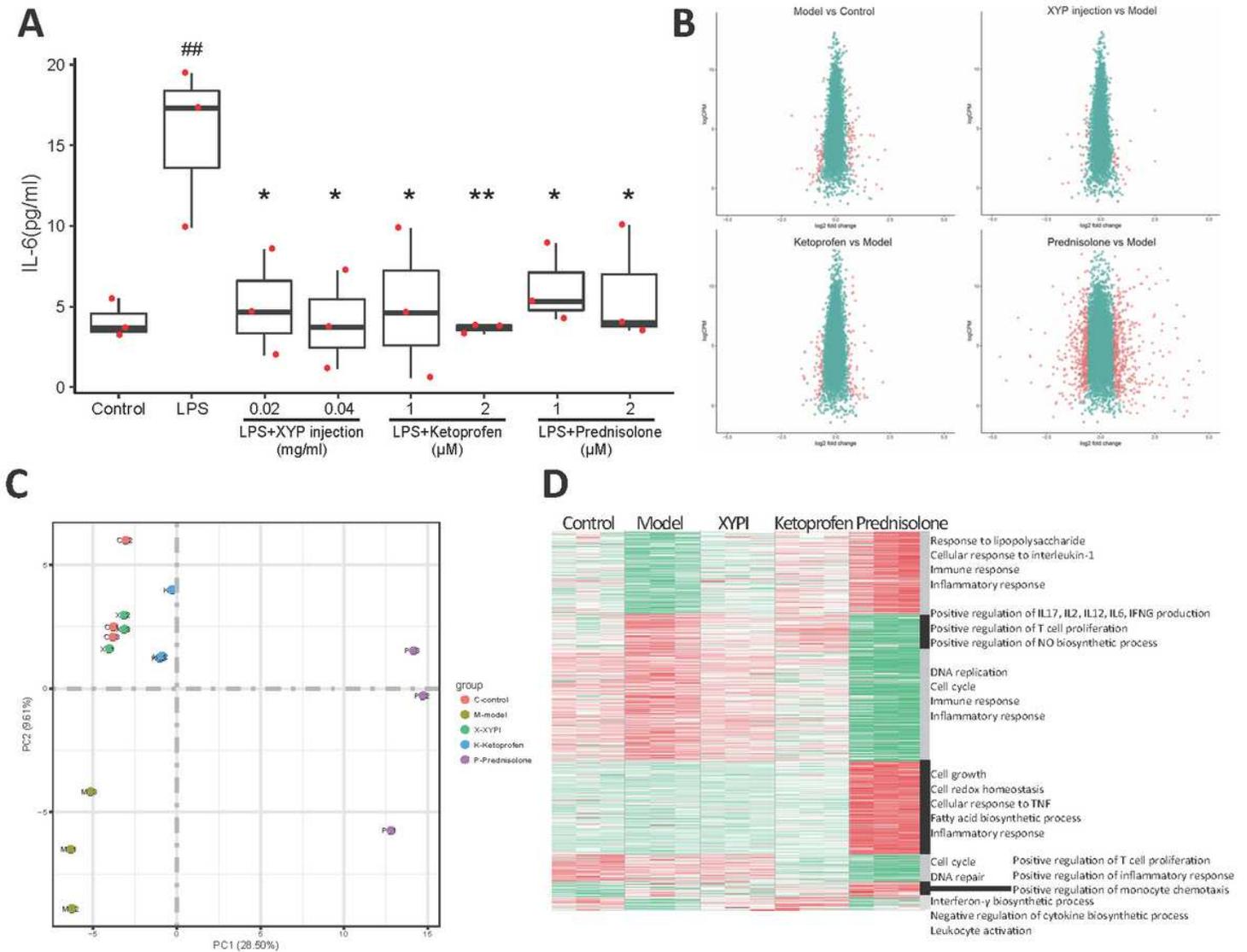


Figure 4

Transcriptome of XYPI pretreatment in an LPS-activated macrophage cell model. (A) Comparison of the anti-inflammatory effects of XYPI with those of ketoprofen and prednisolone. The effect of the three agents on expression of the inflammatory factor IL-6 induced by LPS in the supernatant of RAW264.7 cells after 24 hours of pre-administration. * indicates the administration group vs the model group $p < 0.05$, ** $p < 0.01$. # indicates the model group vs the blank control group $p < 0.05$, ## $p < 0.01$. (B-D) Volcano plot (B), PCA (C) and hierarchical clustering (D) of the transcriptome of the control group, model group (LPS-induced macrophage model), and drug group (pretreatment with high-dose XYPI, high-dose ketoprofen and high-dose prednisolone).

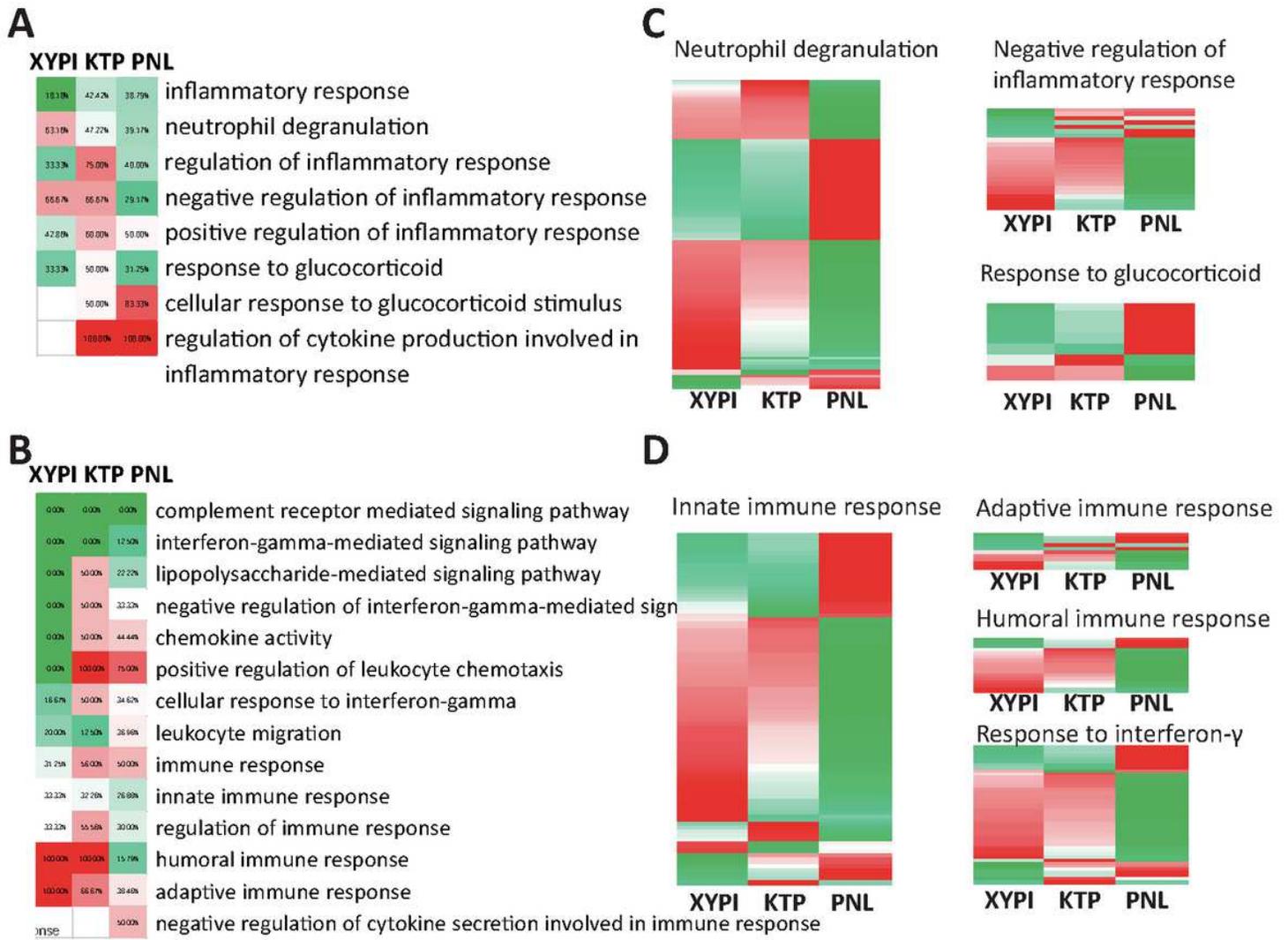


Figure 5

Expression of the shared pathways of XYPI, ketoprofen (KTP) and prednisolone (PNL) (A&B) Pathway activation and repression after drug treatment in terms of inflammatory (A) and immune pathways (B). (C&D) Heatmap of genes in the shared pathways after drug treatment in terms of the inflammatory (C) and immune pathways (D).