Integrated Network Pharmacology and Molecular Docking to understand the Mechanisms of Methotrexate-induced Intestinal Toxicity

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Abstract

Methotrexate, a versatile therapeutic agent, is associated with significant intestinal toxicity, posing clinical challenges. To comprehensively elucidate the underlying mechanisms, we employed an integrated approach combining network pharmacology and molecular docking. Our study identified 155 common targets associated with methotrexate and intestinal toxicity. Network analysis unveiled pivotal nodes, highlighting the TNF signaling pathway and PI3K-AKT signaling pathway. Gene ontology and KEGG pathway enrichment analysis revealed associations with inflammation, apoptosis and immune responses. The constructed D-C-T-P-D network illuminated complex interactions between methotrexate, target genes, pathways and diseases, emphasizing key targets including TNF, AKT1, CASP3 and others. Molecular docking simulations demonstrated robust binding affinities between methotrexate and these targets. AKT1 and CASP3 exhibited the strongest interactions, suggesting their pivotal roles in methotrexate-induced intestinal damage.

Our findings provide insights into the molecular basis of this adverse effect, offering potential therapeutic targets for intervention. However, further experimental validation and consideration of individual patient factors are warranted to translate these discoveries into clinical practice. This underscores the need for personalized approaches to mitigate methotrexate-induced intestinal toxicity.

Keywords: Methotrexate, Intestinal toxicity, Network pharmacology, Molecular docking, Molecular mechanism.

Introduction

Methotrexate, formerly known as amethopterin, is a folic acid analog employed in medical practice since 1950 for managing acute pediatric leukemia and malignancies[15]. In 1988, the Food and Drug Administration approved the limited use of this compound as a therapeutic intervention for adult rheumatoid arthritis. Subsequently, it has become the primary therapeutic approach for juvenile idiopathic arthritis[1]. Recently, methotrexate has gained recognition as a therapeutic option for various inflammatory conditions such as psoriasis, vasculitis and Crohn's Disease, owing to its multifaceted therapeutic mechanisms, which encompass the reduction of purine-pyrimidine synthesis, inhibition of polyamine accumulation and augmentation of adenosine levels[13]. This strategy proves particularly pertinent in cases where conventional treatment modalities have demonstrated ineffectiveness, especially in pediatric patients.

Additionally, in specific instances, methotrexate has been utilized as an alternative to surgical intervention to terminate ectopic pregnancies[28]. However, there exists comprehensive documentation establishing the adverse effects of low-dose methotrexate on various organs including the blood, lungs, liver, kidneys and intestines, primarily attributed to its cytotoxic properties[3,25]. The intestine is a pivotal organ in energy and nutrient absorption from ingested food and waste elimination and also serves as a protective barrier, facilitating drug and xenobiotic detoxification while modulating immune responses[22]. Concerns over drug-induced intestinal toxicity have intensified among physicians due to the critical functions of the intestines. Among adverse effects, methotrexate therapy predominantly manifests as intestinal damage, as supported by clinical trial NCT01535053[24].

Letertre et al reported that methotrexate can be metabolized by gut microbiota, yielding metabolites sensed by enterocytes, subsequently impairing enterocyte proliferation due to associated toxicity. This underscores the heightened susceptibility of the gastrointestinal tract to harmful agents[18]. Multiple clinical investigations indicate that approximately 70% of rheumatoid arthritis patients experience gastrointestinal tract damage during their two-year methotrexate therapy, resulting in nearly 50% discontinuation of treatment[10,12]. Moreover, our study noted that the intravenous administration of methotrexate at a dose of 0.4 mg/kg per day for a 5-day duration, with a repeat cycle every 14 days, had a deleterious impact on 92.31% of the subjects under investigation. The majority of patients (76.92%) within the study cohort presented with common mucositis.

In select instances, the manifestation of intestinal toxicity induced by low-dose methotrexate carries the potential for fatal outcomes[11]. Intestinal toxicity represents a prevalent adverse effect of methotrexate, exerting influence over the entire gastrointestinal system, thereby causing impaired nutrient absorption and subsequent weight loss. The

potential mechanism underlying this toxicity may be linked to the concurrent occurrence of methotrexate nonspecific cytotoxic effects on healthy intestinal cells, coupled with the disruption of cellular metabolic processes, though our comprehension of this remains incomplete[13].

It is noteworthy that there is currently no therapeutic approach available that effectively precludes or safeguards against intestinal toxicity arising from methotrexate treatment. The precise mechanism responsible for the observed toxicity remains elusive, with the cytotoxic properties of methotrexate and its potential interference with cellular metabolic processes posited as contributing factors[14]. The research conducted possesses a value that surpasses the scope of methotrexate, as it emphasizes the broader applicability of computational approaches in uncovering drug-induced toxicities. Moreover, it highlights the significance of comprehending pharmacological effects at a systems level.

The use of network pharmacology-based analysis has emerged as a reliable approach for elucidating the molecular mechanisms behind complex disorders[29]. The integration of information technology with systems biology is increasingly being considered as a potential alternative to conventional methodologies in the field of drug development within the pharmaceutical sector's next-generation approach[21]. This methodology is very appropriate for elucidating the underlying processes of any given chemical compound. To predict the underlying processes through which methotrexate induces intestinal damage, it is necessary to adopt an integrative approach.

Material and Methods

Data Retrieval: The methotrexate target proteins were sourced from multiple databases, including the Comparative Toxicogenomic Database (https://ctdbase.org/), Swiss Target Prediction Database (http://www.swisstargetprediction.ch/) and TargetNet (http://targetnet.scbdd.com/). These retrieved targets were subsequently transformed into the UniProt ID format (https://www.uniprot.org/). To forecast targets associated with conditions such as "mucositis", "intestinal toxicities", "gastroenteritis", "enteritis" and "ulceration", the GeneCards database (https://www.genecards.org/) was employed. The dataset of disease-related targets underwent curation to eliminate duplicated entries and convert the targets into UniProt ID format. All data retrieval took place in August 2023.

Network construction: The acquired target entities were graphically represented within a Venn diagram using Venny 2.1.0 accessible at (https://bioinfogp.cnb.csic.es/tools/venny/). Within this context, the pivotal molecular targets implicated in the onset of methotrexate-induced intestinal toxicity were designated as the focal points of interest. Subsequently, Cytoscape 3.7.2 was harnessed to construct a network that connects the overlapping targets of the various bioactive components with the underlying intestinal toxicity, denoted as the "component-target-intestine toxicity" network. The pivotal regulatory targets were then integrated into the String database (https://string-db.org/) to establish a comprehensive Protein-Protein Interaction (PPI) network.

Gene Ontology and Enrichment Analysis: The evaluation of protein interactions among the targets was conducted through STRING (https://string-db.org/) and subsequent Gene Ontology (GO) biological process analysis within the DAVID database (https://david.ncifcrf.gov/tools.jsp) was executed to enhance our understanding of potential therapeutic target-associated biological processes. Significance assessments were performed for the Biological Process (BP), Cell Component (CC) and Molecular Function (MF) categories, revealing statistically significant outcomes with a significance level of $P < 0.05$. Furthermore, KEGG pathway analysis was employed to comprehensively explore the essential biological relevance of therapeutic targets and to validate the robustness of the integrated findings.

Compound target pathway network construction: To gain insights into these mechanisms, a network analysis was conducted, wherein a network was constructed and visualized using Cytoscape version 3.9.1 software. This network consists of nodes representing both active components and target genes, with edges denoting interactions between these elements. The assembly of the PPI network involved the utilization of the STRING database (https://string-db.org/) in combination with the Network Analyzer plugin of Cytoscape, facilitating the identification and exclusion of hub genes.

Drug Compound Target Pathway Disease (D-C-T-P-D): The D-C-T-P-D network model was constructed employing Cytoscape software (version 3.9.1). This model integrated methotrexate-responsive genes and genetic disorders as input variables. The top-ranked central pathway identified in a prior KEGG pathway enrichment analysis was employed to investigate the interplay among these elements and to establish a comprehensive regulatory network, guided by the degree centrality metric for internal prioritization.

Molecular Docking Simulation: Six out of the top 10 proteins exhibited the highest degree of overlap, prompting their selection for molecular docking studies with methotrexate. The 3D structural data for these proteins were sourced from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) database, accessible at https://www.rcsb.org with the following identifiers: 7JRA (TNF), 1RHJ (CASP3), 3QKK (AKT), 1GKC (MMP9), 1VSC (ICAM1) and 3BQN (VCAM1).

Before docking simulations, we systematically removed water molecules from the protein structures and introduced polar hydrogen atoms, solvation effects and charges. The Auto Grid program was employed to generate predefined affinity maps with grid points representing the active binding sites of the proteins.

Subsequently, the protein-ligand docking process in AutoDock Vina was executed using a Lamarckian evolutionary algorithm. The resulting outcomes were analyzed based on ten distinct interactions for each docking complex, with a primary focus on identifying complexes exhibiting the most substantial binding energy. These selected complexes were then visualized using PyMOL and Discovery Studio software.

Results and Discussion

Network Pharmacology: The flow chart of this study is illustrated in fig. 1. A total of 2,657 putative methotrexate toxicity targets were compiled from the Comparative Toxicogenomic Database, while 580 targets were sourced from the TargetNet database. An intersection of 155 common targets was identified across both databases (Fig. 2A and Fig. 2B). For intestinal toxicity-related targets 1,386 candidates were retrieved using the GeneCards database. Employing a Venn diagram, 56 shared targets for both methotrexate and intestinal toxicity were identified and subsequently selected for subsequent analytical investigations.

PPI Network Analysis: Cytoscape version 3.9.1 was employed for the visualization of 56 intersectional targets concerning methotrexate-induced intestinal toxicity. This visualization is presented as an interactive network with a specific focus on elucidating the regulatory relationships associated with methotrexate-induced intestinal toxicity as illustrated in fig. 2C. This network diagram delineates the connections existing between chemical components and disease targets. In fig. 3A, a PPI network has been constructed utilizing data sourced from the String database. In the context of network analysis, the terminology 'network nodes' pertains to entities representing proteins, while 'edges' signify the connections or correlations between these proteins.

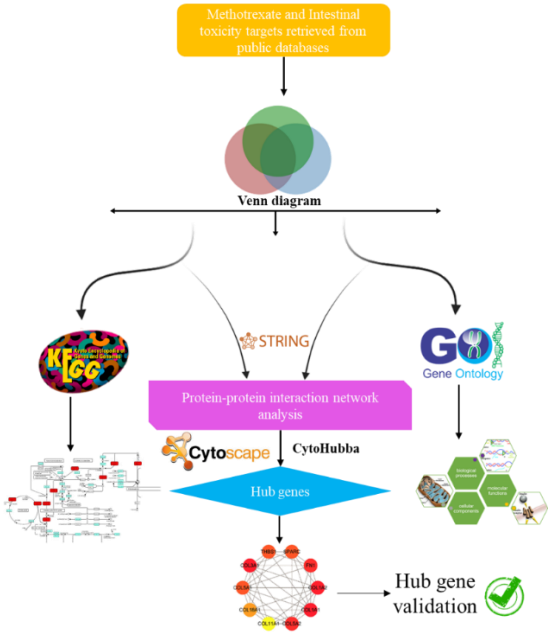

Fig. 1: Graphical representation of the work plan to establish the mechanism of methotrexate-induced intestinal toxicity.

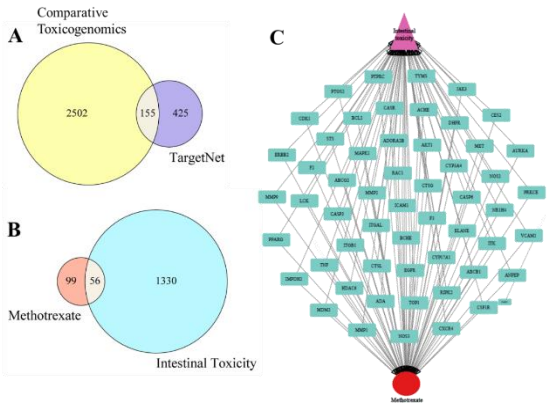

Fig. 2: Intersection of the methotrexate and intestinal injury-related target.
A) and B) Venn diagram of the methotrexate and intestinal toxicity targets;
C) List of the genes linked with the intersection of methotrexate and intestinal toxicity

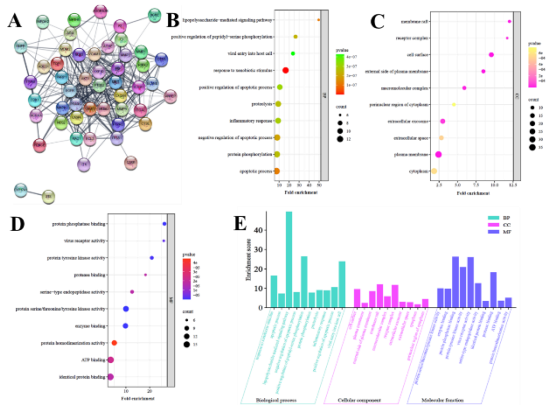

Fig. 3: PPI construction and GO biological function enrichment analysis. A) Establish Protein-Protein Interaction (PPI) by STRING where line and ellipse indicate the edges and nodes of the construct; B), C) and D) show the bubble plot of top 10 gene ontology enrichment function of biological process (BP), Cellular component (CC) and Molecular function (MF) which differentiated the count, p-value and enrichment score based on size, color and length; E) shows the bar chart of BP, CC and MF list identified for the cluster.

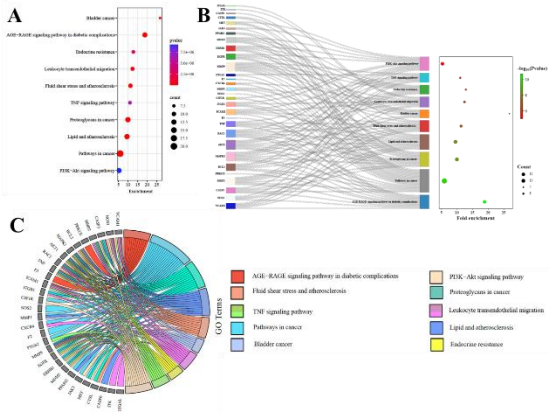

Fig. 4: KEGG enrichment analysis. A) Bubble plot of top 10 or significant (p<0.05) pathway list with enrichment with differentiated the count, p-value and enrichment score based on size, color and length;
B) and C) illustrate the Sankey diagram and Go chord plot of genes shared to the top 10 signaling pathways

GO Function and KEGG Pathway Enrichment Analysis: A total of 385 records containing GO items were collected. Among them, 48 records exhibited statistically significant association at a p-value threshold of <0.05. These GO items were categorized into 272 BP, 50 CC and 63 MF categories. To facilitate visualization, the top 10 items in each GO category were selected, as depicted in fig. 3. The results of the analysis of BP (Fig. 3B), MF (Fig. 3C) and CC (Fig. 3D) were visually represented using bubble plots and bar charts (Fig. 3E).

Further investigation through KEGG pathway enrichment analysis indicated a predominant association of methotrexate with 24 signaling pathways ($p < 0.05$), shared among 43 genes. Fig. 4A presents a bubble chart illustrating the top 10 enriched pathways where the X-axis represents gene enrichment, the bubble size correlates with the degree of gene enrichment and the color depth signifies the significance level (p-value). Key pathways highlighted in this study include the PI3K-AKT signaling pathway, TNF signaling pathway, endocrine resistance, leukocyte trans-endothelial migration, bladder cancer, fluid shear stress and atherosclerosis, lipid and atherosclerosis, proteoglycans in cancer, pathways in cancer and the AGE-RAGE signaling pathway in diabetic complications. Moreover, the top 10 enriched pathways were also explored using Shanky and dot plots, as well as Go Chort plots, which revealed associations with 30 genes (Fig. 4B and fig. 4C).

D-C-T-P-D Network Analysis: Fig. 5A provides a visual representation of the D-C-T-P-D network associated with methotrexate in the context of intestinal toxicity. This network elucidates the complex interplay between methotrexate and intestinal toxicity, comprising a total of 41 nodes. These nodes encompass one compound node, thirty target nodes and ten core pathway routes. Furthermore, the network exhibits interconnections through 297 edges as depicted in fig. 5A.

The observed high centrality value for a node within the network signifies its pronounced significance in network dynamics. Subsequently, utilizing MCODE and CytoHubba plugins, key targets were identified. The top ten core targets based on degree, namely TNF, AKT1, CASP3, EGFR, BCL2, CXCR4, MMP9, ICAM1, VCAM1 and MMP2, are illustrated in fig. 5B. Among these core targets, six proteins

specifically TNF, AKT1, CASP3, MMP9, VCAM1 and ICAM1, are predominantly shared within the TNF signaling pathway, as depicted in fig. 5C.

Molecular Docking Findings: The utilization of molecular docking was employed to get insights into the interaction between the six proposed hub targets and methotrexate. Methotrexate has the potential to form strong bonds with proteins by efficient docking, facilitated by both hydrophilic and hydrophobic interactions. The probable target proteins were examined as depicted in fig. 6A to fig. 6F. The proteins TNF, AKT1, CASP3, MMP9, VCAM1 and ICAM1 have relative binding affinities with methotrexate of -7.5, -8.5, -8.1, -7, -7.8 and -6.6 Kcal/mol as seen in fig. 6G. The targets with the highest binding affinity were AKT1 and CASP3, while ICAM1 had the lowest affinity. All of these targets had affinities below -5 Kcal/mol, indicating their strong interaction activity.

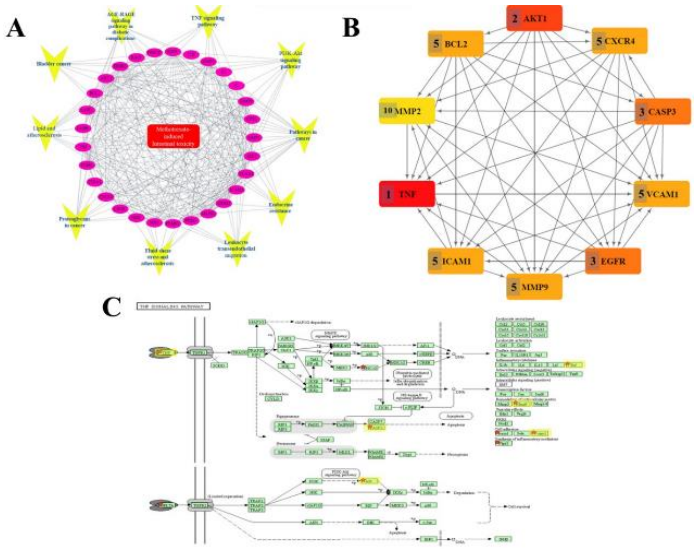

Fig. 5: Construction of D-C-T-P and screening for core genes. A)D-C-T-P constriction with enriched pathways where yellow shape indicates the pathways and pink shape indicates the core genes; B)Screened top 10 degree genes from D-C-T-P construct where color shades show the degree of the genes. C)The annotation map of the targets of methotrexate in intestinal toxicity in the AKT pathway was colored by a KEGG mapper, the green color represents the genes involved in the TNF signaling pathway, red star mapped are genes involved in methotrexate in intestinal toxicity targets and yellow highlighted are the core or hub and TNF common genes

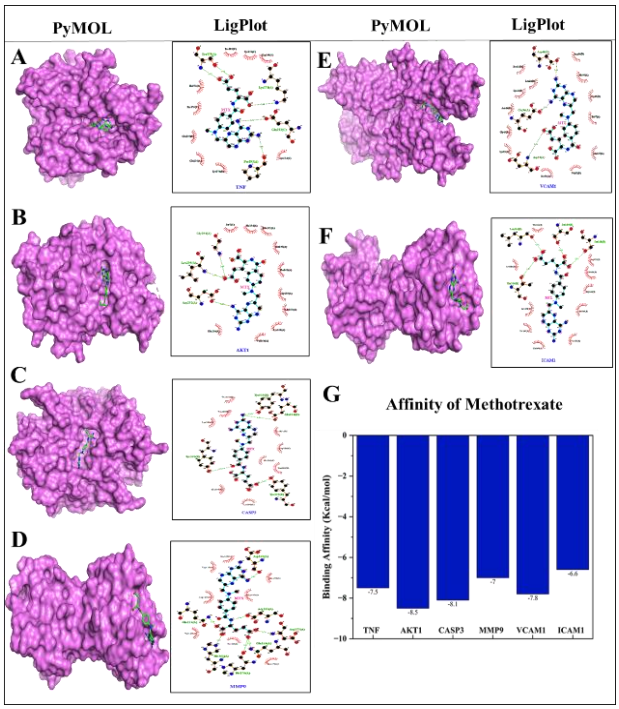

Fig. 6: Molecular docking of methotrexate with core genes. A) to F) show the 3D (PyMOL) and 2D interaction (LigPlot+) of the methotrexate with TNF (7JRA), AKT (3QKK), CASP3 (1RHJ), MMP9 (1GKC), VCAM1 (1VSC) and ICAM1 (3BQN); G Shows the binding affinity of the 6 genes with methotrexate

Methotrexate has demonstrated efficacy in the treatment of many malignancies and autoimmune disorders, either as a monotherapy or in conjunction with other treatments[15]. Regrettably, the administration of MTX is primarily limited by its intestinal toxicity, with MTX-induced gastrointestinal mucositis being a substantial challenge for patients. Several studies have indicated that the treatment of methotrexate leads to the occurrence of breakage of DNA in epithelial cells in the epithelium that undergoes fast proliferation[5,25,26]. Additionally, the regular use also results in the induction of substantial oxidative stress[2]. Moreover, it is significant to note that it has the potential to induce harmful consequences via dynamic series of intricate inflammatory processes that are triggered by direct cellular disruption in the intestinal epithelium and submucosal tissues[30] .

Multiple researches have indicated that the mononuclear phagocyte system (MPS) plays a crucial role in maintaining gut homeostasis and performs numerous tasks in responses from the immune system within the intestine. The major constituents of the MPS consist of dendritic cells and macrophages, which play a crucial role in initiating the adaptive immune response and serving as innate effector cells respectively. Nevertheless, there is a dearth of pertinent toxicological research conducted on animals[4,6,17]. Therefore, the comprehensive investigation conducted in this study sought to elucidate the intricate mechanisms underlying methotrexate-induced intestinal damage, a critical and often debilitating side effect of this widely utilized therapeutic agent.

The integration of network pharmacology and molecular docking techniques allowed us to explore the molecular interactions and pathways associated with this adverse effect, shedding light on potential avenues for therapeutic intervention. The integration of computer science and biology has garnered more interest in light of the advent of contemporary bioinformatics. The process of data mining plays a pivotal role in extracting significant insights through large-scale datasets, hence facilitating the pursuit of relevant research endeavors. Network pharmacology has emerged as a valuable approach for investigating the underlying mechanisms of disease therapy and prevention, supported by a substantial body of experimental data and clinical study findings[9,27].

Hence, this research endeavor represents the inaugural effort to comprehensively elucidate the underlying mechanism by which methotrexate induces intestinal damage. Furthermore, the molecular docking model of methotrexate and its target exhibited a favorable binding affinity, which was subsequently confirmed by the correlation between methotrexate and intestinal toxicity. GO and KEGG pathway enrichment analyses unveiled the biological processes and pathways associated with the identified targets. Our findings revealed a significant association between methotrexate and multiple pathways including the PI3K-AKT signaling pathway, TNF signaling pathway and endocrine resistance, among others. These pathways represent potential mechanistic links between methotrexate exposure and intestinal toxicity.

Sayed et al[23] proposed that the depletion of the anti-inflammatory peptide Angiotensin (1-7) may have been demonstrated to cause dysbiosis of the gut microbiota and deregulation of the PI3K/AKT signaling pathway in methotrexate intestinal injury rats. Katturajan et al[11], have proven that low dose methotrexate of 2.5 mg/kg once per week for 2 weeks has a significant increase of TNF and associated inflammatory molecules gene expression level in intestinal tissues. It is because TNF-α is promptly activated after an inflammatory stimulus and is believed to exert a pivotal function in many types of intestinal damage and mucositis caused by chemotherapeutic agents such as methotrexate, irinotecan and fluorouracil.

Moreover, it is important to note that TNF-α plays a crucial role in the development of mucous inflammation by enhancing the effects of certain inflammatory mediators[8,19]. In addition to that, compared to the placebo group, the fecal samples obtained from 36 pediatric leukemia patients undergoing methotrexate treatment, exhibited a significant reduction in the presence of *Bifidobacterium, Lactobacillus* etc. Radiotherapy leads to the disruption of the digestive tract and subsequent death of gut crypts. In a preliminary investigation, the combination of radiation and medicines demonstrated a decrease in *Firmicute* abundance and an increase in *Proteobacteria* abundance among three pediatric cancer patients diagnosed with pelvic rhabdomyosarcoma[16].

Consequently, this phenomenon leads to the suppression of cellular division, a reduction in the overall cell count and finally culminates in structural and functional alterations within the epithelium of the intestinal tract. The molecular process is dependent on the diminished TNF-α[7]. The PPI network analysis provided further insight into the functional relationships among the identified targets. This interactive network highlighted potential nodes of convergence between methotrexate and its impact on intestinal tissue. The identification of core targets, including TNF, AKT1, CASP3, EGFR, BCL2, CXCR4, MMP9, ICAM1, VCAM1 and MMP2, further emphasized the interconnectedness of these components in the context of intestinal toxicity.

The construction of the D-C-T-P-D network provided a holistic view of the interplay between methotrexate, its target genes, pathways and associated diseases. This network emphasized the complex nature of methotrexate-induced intestinal damage and highlighted the central role of specific targets and pathways in this process. Notably, the presence of predominant hub proteins within the TNF signaling pathway such as TNF, AKT1, CASP3, MMP9, VCAM1 and ICAM1 underscored their significance in the context of methotrexate-induced intestinal damage (Fig. 5C). Supportive to the previous findings, Natarajan et al[19],

proved that the administration of methotrexate led to an upregulation of TNF expression in the small intestine tissue, which subsequently induced the release of MMP9, ultimately resulting in damage to the intestinal tissue.

It could be accountable for the degradation of extracellular matrix components, leading to the subsequent decline in epithelial integrity. The breakdown of connective tissue and the transcription of several members of the MMP enzyme family are attributed to the MMP enzyme family members. The production of TNF in the initial phases following an inflammatory stimulus has the potential to trigger the infiltration of inflammatory cells into the colon. This occurs through the activation of VCAM and ICAM-1 along with other adhesion molecules. which proves that TNF is known to have a significant impact on many types of gastrointestinal (GI) injury, such as sub-acute damage caused by radiation[21].

A single dose (20 mg/kg i.p) of methotrexate exhibited an increased level of apoptosis in the organs including the liver, kidney and intestine where their caspase cascade is involved in the major contribution through intrinsic and extrinsic pathways by the activation of TNFR[20]. The same mechanism can be seen in our findings. Molecular docking studies with six selected hub targets provided valuable insights into the potential interactions between methotrexate and these proteins. The observed binding affinities, ranging from -6.6 to -8.5 Kcal/mol, indicated strong interactions between methotrexate and the selected proteins. Notably, AKT1 and CASP3 exhibited the highest binding affinities, suggesting their potential as key mediators of methotrexate-induced intestinal damage.

The findings of this study hold significant implications for both the understanding of methotrexate-induced intestinal damage and the broader application of computational approaches in drug-induced toxicity research. The identified targets and pathways offer potential avenues for therapeutic intervention and drug development. Specifically, the TNF signaling pathway, with its central role in our analysis, warrants further investigation as a potential target for mitigating intestinal toxicity associated with methotrexate. It is important to acknowledge the limitations of our study including the *in silico* nature of our approach. Further experimental validation of the identified targets and pathways is essential to confirm their relevance in the context of methotrexate-induced intestinal damage. Additionally, the individualized response of patients to methotrexate therapy may involve genetic and environmental factors that were not considered in our computational analysis.

Conclusion

In conclusion, our integrated network pharmacology and molecular docking study have provided valuable insights into the mechanisms underlying methotrexate-induced intestinal damage. This research represents a significant step toward a more comprehensive understanding of the adverse effects of methotrexate and highlights the potential for targeted interventions to improve the safety and efficacy of this widely used therapeutic agent.

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