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ORIGINAL RESEARCH

Potential Mechanisms of Tetramethylpyrazine in the Treatment of Traumatic Brain Injury Based on Network Pharmacology, Molecular Docking, Molecular Dynamics Simulations, and in vivo Experiments

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Background: Traumatic brain injury (TBI) is a leading cause of global disability and mortality. Tetramethylpyrazine (TMP), an active compound from Chuanxiong, holds promise for treating cerebrovascular diseases, but its precise mechanism of action against TBI remains incompletely understood. This study aimed to elucidate the therapeutic effects and underlying mechanisms of TMP in TBI.

Methods: Potential targets of TMP against TBI were identified using Swiss Target Prediction, PharmMapper, and GeneCards databases. Core targets and mechanisms were predicted through network pharmacology, molecular docking, and molecular dynamics (MD) simulations. These computational predictions were then experimentally validated in a rat TBI model, employing behavioral tests, ELISA, RT-qPCR, and Western blot analysis.

Results: Through network pharmacology analysis, 39 potential targets associated with TMP were identified. Molecular docking and MD simulations manifested that key genes like MMP3, MMP2, MMP13, and GSK3B, showed a strong binding affinity to TMP. GO analysis and KEGG analysis corroborated that such targets strongly related to the IL-17 signaling pathway and the relaxin signaling pathway. In vivo tests proved that TMP could improve the modified Neurological Severity Score (mNSS) and foot defect test scores among rats. ELISA confirmed that TMP could decrease the expression of inflammatory factors, encompassing interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 17A (IL-17A), and tumor necrosis factor-alpha (TNF-α). Furthermore, RT-qPCR analysis exhibited that the levels of MMP3, MMP2, MMP13, and GSK3B were increased within the rat cortex after TBI. Significantly, TMP treatment alleviated such upregulation. Western blot analysis validated that TMP down-regulated the expression of p-GSK3β (Ser9), active MMP13, active MMP3, and P65 NF-κB proteins after TBI, while TMP increased the expression of occludin protein.

Conclusion: This study demonstrates that TMP exerts therapeutic effects on TBI by targeting the IL-17 and relaxin signalling pathways, providing evidence for its potential as a clinical therapy.

Keywords: tetramethylpyrazine, traumatic brain injury, molecular docking, molecular dynamic simulation, network pharmacology

Introduction

Traumatic Brain Injury (TBI) is among the main reasons for disability and mortality on a global scale.[1,2] Its pathological mechanisms involve two primary factors: primary mechanical injury and secondary cascade reactions.[3] The core mechanisms of secondary injury encompass multiple pathological processes, including neuroinflammation, oxidative stress, calcium overload, mitochondrial disorder, disruption of the blood-brain barrier (BBB), among which, the

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excitotoxicity of glutamate, the peroxidation of free radical-mediated lipid, and the activation of apoptotic signaling pathways have been regarded as pivotal factors in neuron death.[4–7] In spite of progress in contemporary medicine used for sustaining vital signs and decreasing intracranial pressure, there is still a scarcity of efficacious pharmacological therapies targeting these intricate multi-target pathological networks. Hence, survivors frequently undergo sequelae, like cognitive disorder and dyskinesia.[8,9] As a result, investigating multipath intervention strategies has been an important method of dealing with the present therapy limitations.

Traditional Chinese medicine is key to treating TBI, since its theoretical bases are comprehensive and distinctive.[10] Its therapeutic means is multifaceted, due to various constituents, pathways, and targets. Chuanxiong (Ligusticum chuanxiong), a traditional TCM herb known for its blood-activating and stasis-eliminating properties, primarily exerts pharmacological effects by enhancing blood circulation, preventing thrombosis, repairing microcirculation disorders, protecting nerve function, reducing inflammation, and modulating the immune response. Tetramethylpyrazine (TMP) is the primary active alkaloid of chuanxiong, exhibiting multi-target and multi-pathway pharmacological effects.[11] Research has demonstrated that TMP enhances cardiovascular health by suppressing oxidative stress and decreasing the oxidative modification of low-density lipoprotein.[12,13] In the nervous system, it activates the Nrf2 pathway to augment antioxidant capacity while concurrently inhibiting NLRP3 inflammatory vesicles to mitigate nerve damage.[14,15] In terms of anti-inflammatory effects, TMP targets NF-κB signaling, decrease the secretion of pro-inflammatory factors, and enhances anti-inflammatory factors.[16,17] The effects endow TMP with considerable potential for treating numerous diseases. Research has revealed that TMP might ameliorated neurological function in TBI patients via diverse mechanisms, like modulating the inflammatory response and decreasing cerebral edema.[18–20] Despite the promising therapeutic potential of TMP in decreasing the inflammatory reaction to brain lesion, there exists a lack of research on whether other signaling pathways regulated by the drug can be considered key targets for future pharmacological development.

Our research employed molecular docking, molecular dynamics (MD) simulations, network pharmacology, and in vivo tests to explore genes, proteins, and pathways modulated by TMP that are relevant to TBI pathology. The main target of this study is to integrate and clarify the complicated mechanisms of the influence of TMP upon TBI.

Materials and Methods

Data Acquisition and Target Prediction

The chemical structure and canonical SMILES notation of TMP were searched from the PubChem database[21] (https://pubchem.ncbi.nlm.nih.gov/). The SMILES data of the compound were subsequently imported into the PharmMapper server[22] (https://www.lilab-ecust.cn/pharmmapper/) and Swiss Target Prediction[23] (http://www.swisstargetprediction.ch/) to forecast potential drug targets. Disease-associated targets were filtered from the GeneCards database[24] (https://www.genecards.org/) using the keyword "Traumatic Brain Injury", with relevance scores serving as the filtering criteria. This study utilized exclusively pre-existing, anonymized data from public databases. The ethical review of this specific analysis was exempted as per the national legislation of China ("Measures for the Ethical Review of Life Science and Medical Research Involving Human Subjects", Article 32, Items 1 and 2). This exemption was formally confirmed by the Ethics Committee of the Second People's Hospital of Wuhu, and the requirement for informed consent was accordingly waived.

Intersection Analysis and Construction of Protein-Protein Interaction (PPI) Networks

Shared targets between compound-predicted and disease-associated targets were identified through intersection analysis using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/), before the overlapping targets were observed. Subsequently, the common targets were recorded into the STRING database[25] (https://cn.string-db.org/) to establish one PPI network, and interaction confidence scores was set to medium stringency (≥0.4). Network topology analysis and visualization were performed using Cytoscape 3.9.1.[26] Through the CytoHubba plugin, hub genes were recognized by evaluating node centrality metrics.[27]

Pathway Enrichment and Functional Annotation

Including Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, functional enrichment analyses were executed through the online bioinformatics platform GENEDENOVO (https://www.genedenovo.com/)[28]. The core targets underwent GO enrichment analysis, which encompassed three ontologies: biological activities, cell components, and molecular functions. Further, KEGG pathway enrichment was implemented to recognize associated signaling pathways. The visualization outputs included KEGG bubble plots and GO bar charts.

Molecular Docking and Binding Affinity Assessment

As a drug design strategy, Molecular docking employs the property of receptors and the interplay between these receptors and drug molecules. Network pharmacological analyses were conducted to identify key targets for TMP intervention based on the main active ingredients. These predictions were made using the molecular docking software Discovery Studio 2021 and AutoDock Vina1.5.6.[29] The structures of the key gene active ingredients and potential therapeutic targets were acquired from the RCSB PDB database (https://www.rcsb.org/) and the PubChem database, separately. Subsequently, molecular docking was adopted to delve into the interplay between the compounds and the key gene targets, and the binding energies were calculated. The binding energy value indicates the strength of the interaction between the active component and the therapeutic target. Commonly, binding energies below −5.0 kcal/mol have a powerful binding activity, with lower values and larger binding strength.

Molecular Dynamics Simulation

To substantiate the stability of conjugates after molecular docking, 100 ns MD simulations of the complexes were executed through Gromacs 2022.[30,31] The CHARMM36 force field parameters were used for the protein, whereas the ligand topology was formulated according to the GAFF2 force field parameters.[32] Following that, the protein-ligand complex was put into a cubic box under cyclic boundary conditions. The box was full of water molecules through the TIP3P water model. Hence, one water box emerged with a cyclic boundary of 1.2 nm.[33] After that, the mechanism was subjected to MD simulations via Gromacs 2022 at the temperature of 310 K under the pressure of 1 bar for 100 ns. As a credible measurement of the stability of proteins and ligands, root mean square deviation (RMSD) was used for figuring out the extent of atomic location deviation from their initial configurations. Reportedly, a smaller deviation signifies a greater conformational stability of proteins. In addition, root mean square fluctuation (RMSF) was utilized for determining the resilience of amino acid residues. Actually, lower RMSF values illustrate higher stability and better resilience. In order to appraise the stability of molecular docking results, our study employed RMSD and RMSF.

TBI Model Preparation and Drug Administration

Our research selected adult male Sprague-Dawley rats weighing from 250 to 300 grams. A weight-drop model was employed to induce TBI, as this method is a well-established and reproducible model of closed-head impact injury that effectively mimics key clinical features of human TBI, including cortical contusion, blood-brain barrier disruption, and neuroinflammation.[34,35] This model is particularly suitable for the evaluation of neuroprotective agents and neurological functional outcomes, which aligns with the primary objectives of our study. Induced through a 10% chloral hydrate solution, anesthesia was injected intraperitoneally at a dose of 3 mL/kg. A medial incision was executed along the sagittal suture to expose the skull, after disinfection with iodophor. The rat's head was secured to maintain a horizontal position of the skull. A hole was drilled 3 mm behind to the coronal suture and 2–3 mm lateral to the midline. A 5 mm diameter round bone window was created, ensuring that the dura mater remained intact. A 4.0 mm diameter craniotomy was performed between the amniotic membrane of the right hemisphere and the fontanelle, positioned 3.0 mm from the sagittal suture. Weights of 40 grams were dropped freely from a height of 10 cm. After the injury, the scalp incision was sutured. The sham group were subjected to craniotomy with no cortex lesion. The rats were segregated stochastically into five groups (n=12): a sham group, a TBI model group, a low-dose TMP group (L-TMP, 20 mg/kg), a medium-dose TMP group (M-TMP, 60 mg/kg), and a high-dose TMP group (H-TMP, 100 mg/kg). The dosage selection was based on previously established effective ranges of TMP in rodent models of neurological disorders. The low dose (20 mg/kg)

aligns with the minimal effective dose reported for neuroprotection in TBI and stroke models.[18,19] The medium (60 mg/kg) and high (100 mg/kg) doses were selected to evaluate a dose-dependent therapeutic effect, covering the upper range of commonly used and effective doses in cerebral ischemia and neurodegenerative studies.[20,36,37] TMP was digested in saline and administered once via intravenous injection 6 hours after the TBI surgery. From postoperative days 2 to 14, TMP was administered to the rats every day. This treatment regimen was designed to specifically target the subacute to chronic phases of TBI pathogenesis. The initiation of treatment from day 2 allows for the intervention during the peak of secondary injury processes, including robust neuroinflammation, oxidative stress, and blood-brain barrier disruption, while the continuation through day 14 covers critical periods of repair and plasticity, such as neurogenesis, angiogenesis, and synaptic remodeling, as demonstrated in multiple experimental models of neural injury.[18,38–40] This extended treatment regimen is supported by previous studies demonstrating that TMP exerts potent pro-regenerative effects during this critical time window, including the promotion of neurovascular restoration, neural stem cell migration, and functional recovery.[38,40,41] The sham and TBI model groups received phosphate-buffered saline (PBS) as a control treatment. All animal experiments were approved by the Ethics Committee of the Second People's Hospital of Wuhu (IRB number:2024-KY-003). We strictly followed the ethical and welfare requirements for the protection of experimental animals (GB/T 35892–2018) and adhered to the "3R" principles (Replacement, Reduction, Refinement) to use experimental animals in a scientific, reasonable, and humane manner.

Evaluation of Neurological Outcomes

The modified neurological severity score (mNSS) of each rat was assessed preoperatively and at 1, 3, 7, and 14 days after TBI. The mNSS incorporates sensory, reflex, and motor experiments and has been widely utilized in prior research. One point is awarded for each aberrant behavior or a scarcity of a experimented reflex. Neurological scores range from 0 (normal) to 18 (maximum deficit). Significantly, if the scores are higher, it will indicate poorer performance. The foot fault test was conducted concurrently with the mNSS at the identical time points. Such rats were allowed to walk on the grid for one minute, after which the misalignment rate was calculated as (contralateral misalignment - ipsilateral misalignment) / 100% of total forepaw steps. Neurobehavioral parameters were independently evaluated by two researchers.

Western Blot Analysis

The cortical tissue at the site of injury was lysed in RIPA buffer for 30 minutes and then subjected to centrifugation to harvest the total protein. The resulting proteins were separated on a 10% SDS-PAGE gel, following which they were electrophoretically delivered to a PVDF membrane. The primary antibodies used included p-GSK3β (Ser9) (ab107166, 1:1000, Abcam), active MMP13 (ab84594, 2 μg/mL, Abcam), active MMP3 (ab96555, 150 ng/mL, Abcam), occludin (ab216327, 1:1000, Abcam), and p65 NF-κB (ab16502, 0.5 μg/mL, Abcam). To ensure consistent protein loading across all samples, the membranes were reprobed with a monoclonal antibody against GAPDH (ab8245, 1:1000, Abcam) as an internal control. Visualization of the membranes was achieved through an improved chemiluminescence Western blotting system (Thermo Fisher Scientific). Densitometric quantitative analyses were performed through ImageJ.

Real-Time Quantitative Polymerase Chain Reaction (PCR)

Total RNA was obtained from the cortical tissues of TBI rats with TRIzol reagent. Subsequently, the RNA specimens were reversely transcribed to produce complementary DNA (cDNA). PCR amplification was implemented through GAPDH as an internal reference gene. The selection of GAPDH is supported by its demonstrated stable expression under cerebral ischemic and traumatic conditions in prior studies.[42,43] The relative mRNA expression was evaluated using the $2^{-\Delta\Delta Ct}$ method. Table 1 displays the primer sequences.

The Enzyme-Linked Immunosorbent Assay

The rats were euthanized three days post-surgery. This time point was selected because the expression of key pro-inflammatory cytokines peaks during the acute phase following TBI, allowing for a robust assessment of the early anti-inflammatory effects of TMP intervention.[18,19] After euthanasia, the cortical tissue at the injury site was collected and

Table 1 List of Primers Used for mRNA qPCR

Gene	Forward	Reverse
MMP2	CAAGTTCCCCGGCGATGTC	TTCTGGTCAAGGTCACCTGTC
MMP3	ACATGGAGACTTTGTCCCTTTTG	TTGGCTGAGTGGTAGAGTCCC
MMP13	TCCATCCCGAGACCTCATGT	CTCAAAGTGAACCGCAGCAC
GSK3B	TCGCCACTCGAGTAGAAGAAA	ACTTTGTGACTCAGGAGA ACT
GAPDH	TGGTGAAGCAGGCATCTGAG	TGCTGTTGAAGTCGCAGGAG

lysed. Following homogenization in phosphate-buffered saline (PBS) using a high-throughput homogenizer, the tissue was centrifuged at 12,000 rpm for 15 min at 4°C. Then, the resultant pellet was gathered for further analysis. The levels of interleukin-17A (IL-17A), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (IL-17A, catalog no. ZC-36387; IL-1β, catalog no. ZC-36391; IL-6, catalog no. ZC-36404; TNF-α, catalog no. ZC-37624; ZCIBIO Technology Co., Ltd, Shanghai, China), strictly following the manufacturer's protocols. The optical density (OD) was measured at a wavelength of 450 nm using a microplate reader. Then, the measured OD values were converted to corresponding concentration values.

Statistical Analysis

Data were denoted as the mean ± standard deviation and were statistically construed through GraphPad Prism software (v 8.0). The comparison was implemented between two groups through an independent, unpaired, two-tailed *t*-test, whereas the comparison in multiple groups was executed through one-way analysis of variance before Dunnett's test. A p-value below 0.05 held statistical significance.

Results

Network Pharmacology Predicts the Targets of TMP's Effects on TBI

Figure 1A displays the molecular formula. In total, 144 potential targets for TMP were predicted through the Swiss Target Prediction and PharmMapper databases, as shown in Figure 1B. Subsequently, we utilized the keyword "traumatic brain injury" in GeneCards to filter for disease-associated genes, resulting in 5,461 genes. Through the intersection of diseases and drugs, our study identified 39 common genes between the disease-associated and drug-target genes. In the Venn diagram, the central intersection stands for these 39 common targets for diseases and drugs (Figure 1B). The 39 potential gene targets for treatment were documented in the STRING database, where we obtained the PPI network diagram of intersection targets (Figure 1C). After that, the data were documented in Cytoscape to discern the top 20 hub genes; our research adopted the degree algorithm in the CytoHubba plugin for this analysis. As depicted in Figure 1D, the color intensity signifies the importance of each gene target. Redder colors and more edges indicate that gene targets are more significant. In light of the Maximal Clique Centrality analysis, 20 core targets were identified, which include CASP3, ESR1, EGFR, PPARG, MMP2, GSK3B, MDM2, IL2, ESR2, JAK2, BMP2, MMP3, CASP1, CCNA2, FGFR1, PPARD, TGFB2, KIT, MMP13, and CASP7.

Enrichment Analysis of Target Functions and Pathways

GO enrichment analysis was used for assessing the biological functions of TMP targets, which include cellular components, molecular functions, and biological processes (Figure 2A). As for BP classification, the results for the target genes mainly contained reactions to stimuli, metabolic processes, cellular processes, cellular anatomical entities, and immune system processes. As to CC categorization, enrichment was visualized among cellular anatomical entities and protein-containing complex. For MF categorization, enrichment was noted among catalytic activities, molecular function regulation, molecular transducer activity, and transcription regulation activity. The KEGG enrichment analysis, as illustrated in Figure 2B, identified 172 pathways. The top 25 enriched pathways suggest that TMP may influence TBI

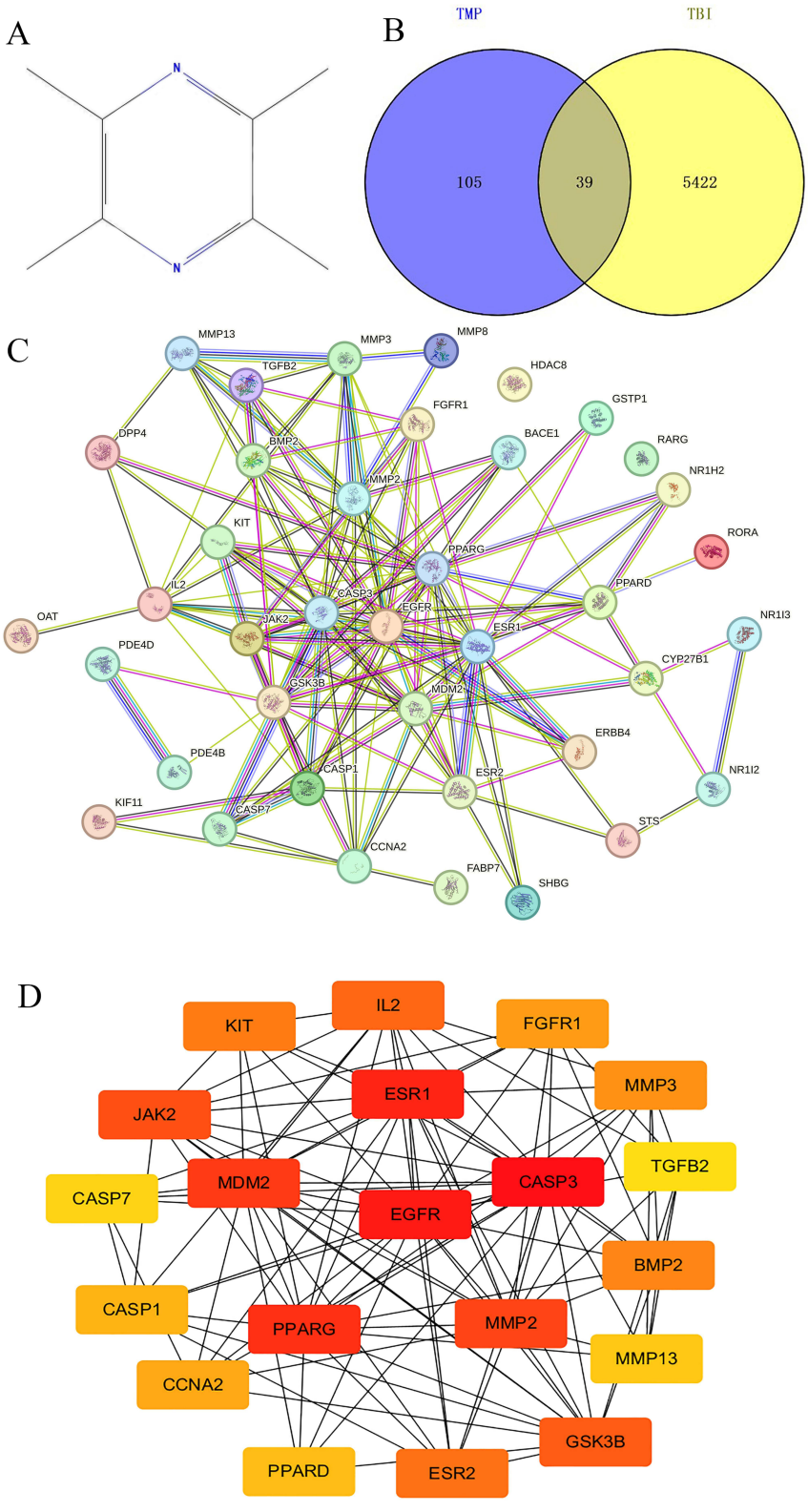

Figure 1 Network pharmacology analysis of TMP on TBI. (**A**) The chemical structure of TMP; (**B**) Venn diagram illustrating the potential targets of TMP and the targets associated with TBI; (**C**) PPI network of the intersecting targets of TMP and TBI; (**D**) Among the intersecting targets, the top 20 key target genes are identified using Cytoscape, with a deeper red color indicating a higher ranking.

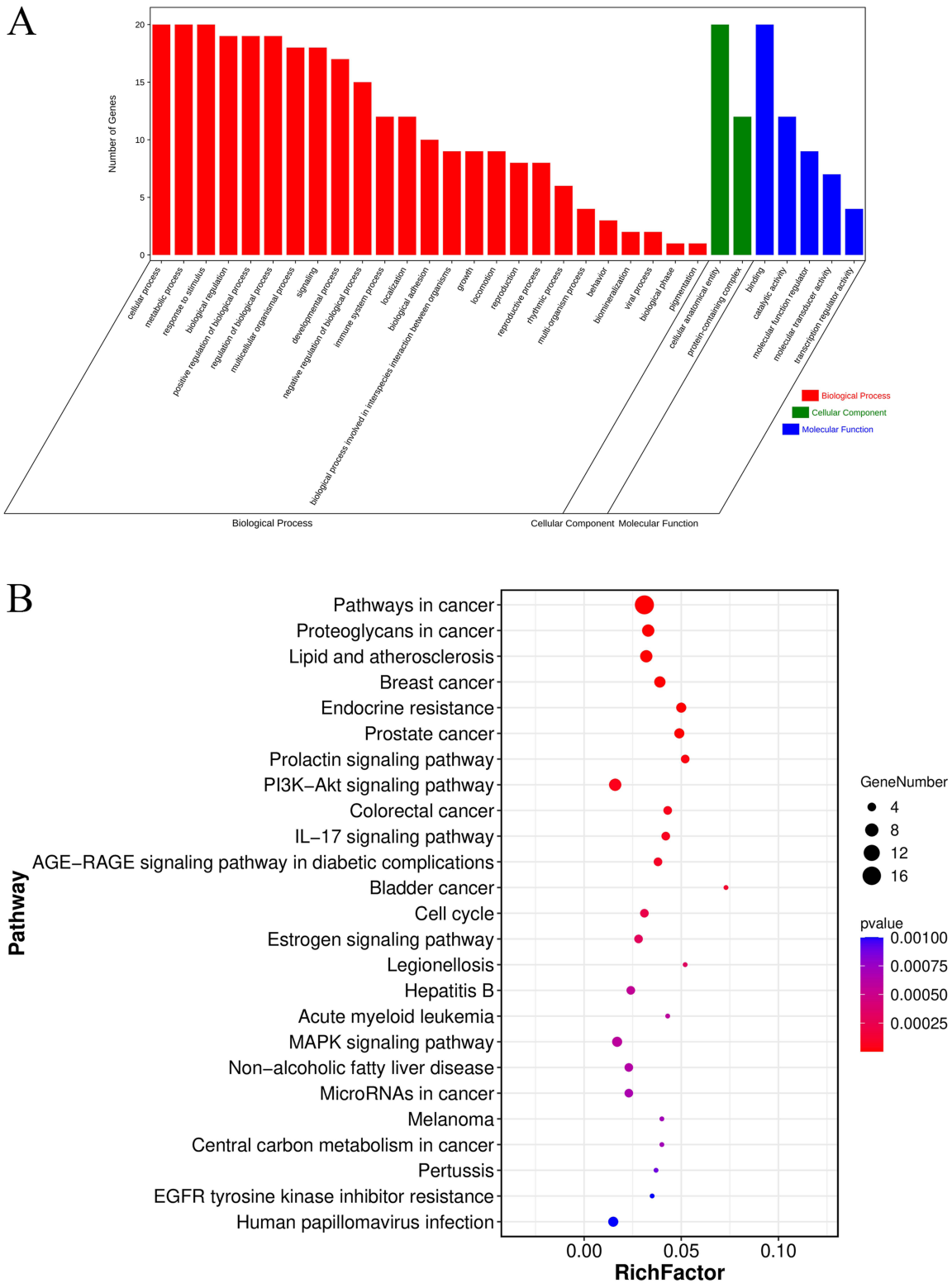

Figure 2 Functional enrichment analysis of the top 20 intersecting target genes of TMP for the treatment of TBI. (**A**) GO enrichment analysis results, showing the significantly enriched biological processes, cellular components, and molecular functions; (**B**) KEGG pathway enrichment analysis showing the top 25 significantly enriched pathways.

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via multiple mechanisms. Signaling pathways, including the lipid and atherosclerosis pathways, prolactin signaling pathway, PI3K-Akt signaling pathway, IL-17 signaling pathway, etc.

Verification of Molecular Docking

To deeply investigate the interplay between TMP and associated genes in the core pathway and elucidate a novel drug progress strategy, additional molecular docking simulations were conducted. The research findings are presented in Table 2. The top five gene targets showing the highest binding energies were chosen for in-depth analysis: MMP13, MMP3, MMP2, GSK3B, and CCNA2. The interactions between these molecules and the target proteins were subjected to visual analysis (Figure 3A–E). Moreover, the top five genes underwent GO and KEGG analyses. Such analyses revealed that the primary involvement of these genes was in the relaxin and IL-17 signaling pathways (Figure 3F).

Molecular Dynamic Simulation

After completing the molecular docking analysis and KEGG pathway enrichment analysis of the targets, four key genes showing significant associations with IL-17 signalling pathway and relaxin signalling pathway were screened for the study. Subsequently, the stability of the binding of these targets to the corresponding compounds was evaluated using MD simulation. The equilibrium of the simulated systems was appraised using RMSD. As demonstrated in Figure 4A, the MMP3 complex system attained equilibrium following 60 ns, the MMP2 complex system satisfied the balance following 85 ns, the GSK3B complex system satisfied the balance following 95 ns, and the MMP13 complex system reached equilibrium after 85 ns. Consequently, TMP exhibited elevated stability when bound to MMP3, MMP2, GSK3B and MMP13, respectively. RMSF is a measure of the resilience of amino acid residues among proteins. According to Figure 4B–E, the MMP3, MMP2, GSK3B and MMP13 complexes exhibited comparatively low RMSF values and high stability. Consequently, the TMP fraction demonstrated effective binding to MMP3, MMP2, GSK3B and MMP13.

TMP Treatment Alleviates Neurological Deficits Following TBI

mNSS scores and foot fault tests are generally utilized as credible indicators of neurological function among rats. In the TMP group, rats showed a swift and remarkable reduction in mNSS scores relative to controls (Figure 5A). Furthermore,

Table 2 Analysis of the Binding Energy of TMP with 20 Target Proteins

Target Protein	PDB ID	The Lowest Binding Energy (kcal/mol)
MMP3	1CIZ	−6.4
MMP13	3TVC	−6.3
MMP2	7XJO	−6.0
GSK3B	7B6F	−5.9
CCNA2	6GVA	−5.7
ESR2	4ZI1	−5.6
PPARG	6K0T	−5.6
FGFR1	3DPK	−5.6
KIT	4HVS	−5.5
JAK2	3UGC	−5.5
PPARD	7WGN	−5.4
BMP2	2H64	−5.2
CASP7	4JR2	−5.2
ESR1	7QVJ	−5.1
MDM2	5J7G	−5.1
CASP1	8WRA	−4.8
EGFR	8SC7	−4.7
CASP3	4JJE	−4.3
IL2	1M47	−3.9
TGFB2	6I9J	−3.8

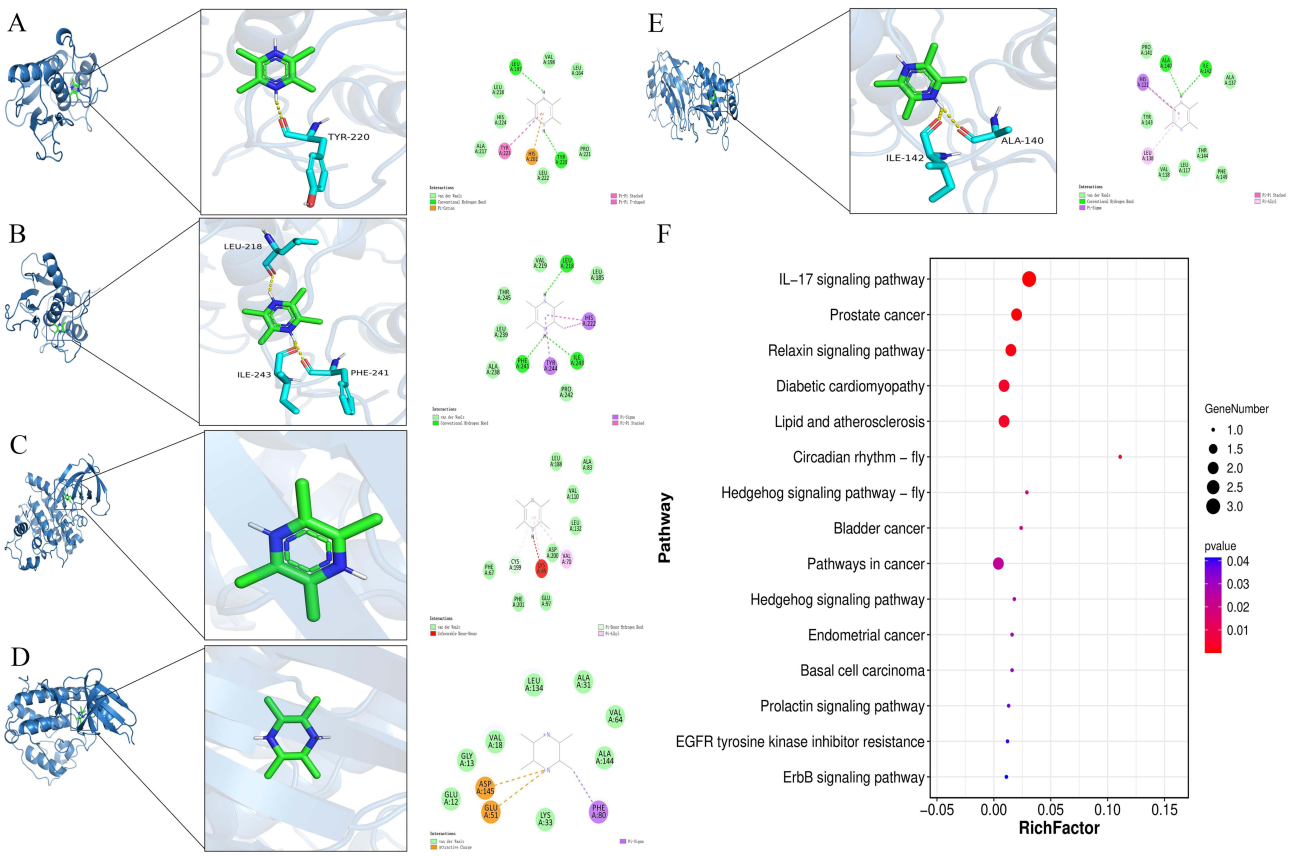

Figure 3 Molecular docking and KEGG enrichment analysis of key gene targets. (**A**) Molecular docking of MMP3 with TMP; (**B**) Molecular docking of MMP13 with TMP; (**C**) Molecular docking of GSK3B with TMP; (**D**) Molecular docking of CCNA2 with TMP; (**E**) Molecular docking of MMP2 with TMP; (**F**) KEGG enrichment analysis of the top five gene targets based on molecular docking scores, showing the top 15 significantly enriched pathways.

the TMP group also exhibited a significant decrease in the frequency of forelimb foot faults relative to the TBI model group (Figure 5B).

Effects of TMP on Serum Levels of TNF-α, IL-6, IL-1β, and IL-17A in TBI Rats

In Figure 5C–F, the levels of IL-6, IL-1β, IL-17A, and TNF-α were remarkably elevated in the TBI model group relative to the sham group ($p < 0.05$). Nevertheless, the L-TMP group showed remarkably disparities in serum levels of IL-6, IL-1β, and IL-17A, and TNF-α relative to the TBI model group. Furthermore, both the M-TMP and H-TMP groups demonstrated remarkably decreased levels of IL-1β, IL-6, IL-17A, and TNF-α relative to the TBI model group ($p < 0.01$).

TMP Suppresses IL-17 and Relaxin Pathway Activation in TBI

q-PCR was employed to assess the levels of MMP3, MMP2, MMP13, and GSK3B mRNA in the rat`s cortical tissue in the TBI model group. These levels were exceedingly elevated relative to those in the sham operation group ($P < 0.05$). Furthermore, the levels of MMP3, MMP2, MMP13, and GSK3B in the rat`s cortical tissue within the high-dose TMP group remarkably came short of those in the TBI model group ($P < 0.05$) (Figure 6A).

Western blot analysis was utilized for detecting the expressions of p-GSK3β (Ser9), active MMP13, active MMP3, occludin, and P65 NF-κB proteins in the rat`s cortical tissue in the TBI model group (Figure 6B–F). Evidently, relative to the sham operation group, the levels of p-GSK3β (Ser9), active MMP13, active MMP3, and P65 NF-κB proteins in the rat`s cortical tissue in the TBI model group were prominently elevated ($P < 0.05$). In the high-dose TMP treatment group, the expressions of p-GSK3β (Ser9), active MMP13, active MMP3, and P65 NF-κB proteins in the rat`s cortical tissue were obviously reduced relative to those in the TBI model group ($P < 0.01$). Notably, the protein expression of occludin

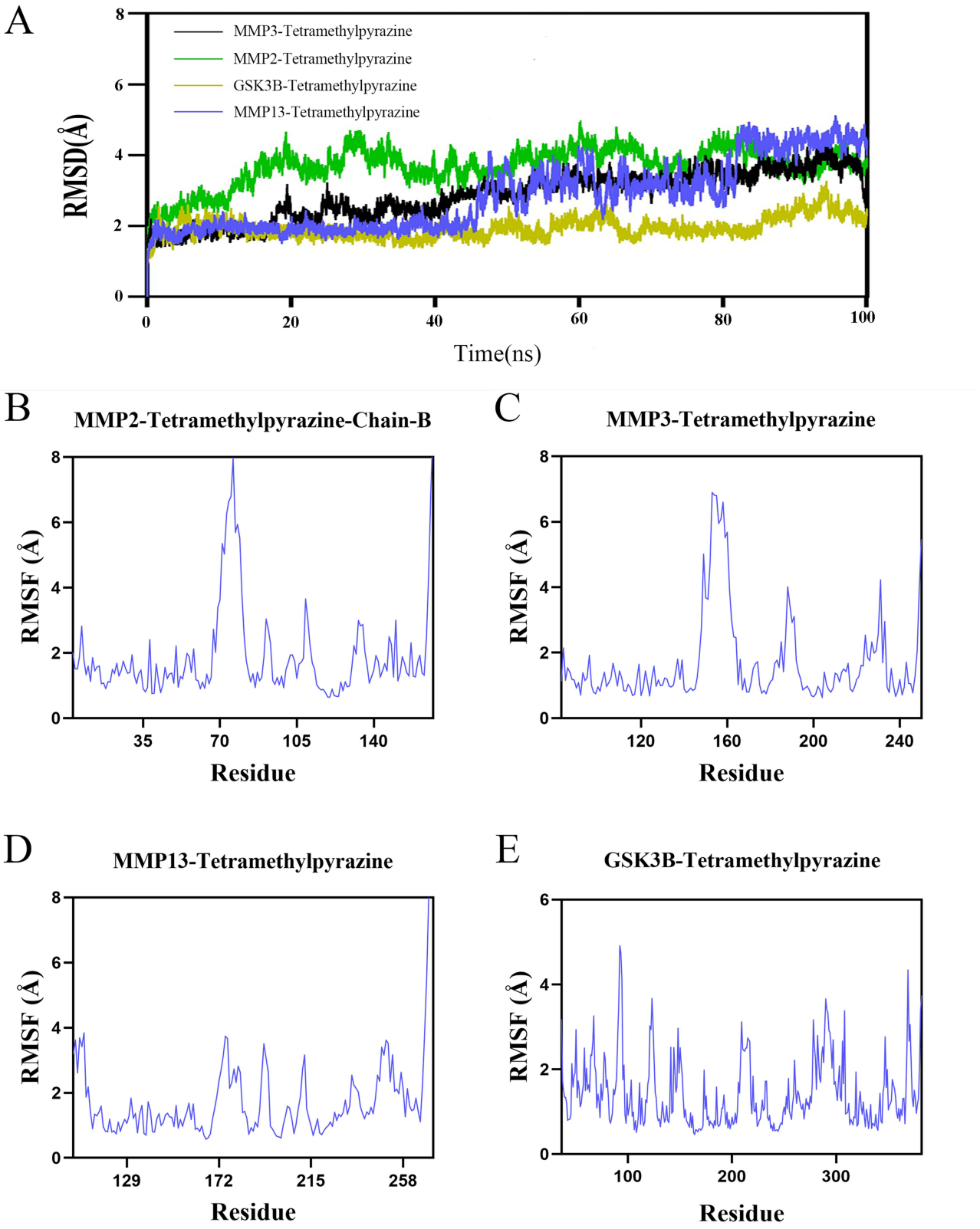

Figure 4 Molecular dynamics simulation of key gene targets interacting with TMP. (**A**) RMSD values for four protein-ligand complexes; (**B**) RMSF of MMP2 in complex with TMP; (**C**) RMSF of MMP3 in complex with TMP; (**D**) RMSF of MMP13 in complex with TMP; (**E**) RMSF of GSK3B in complex with TMP.

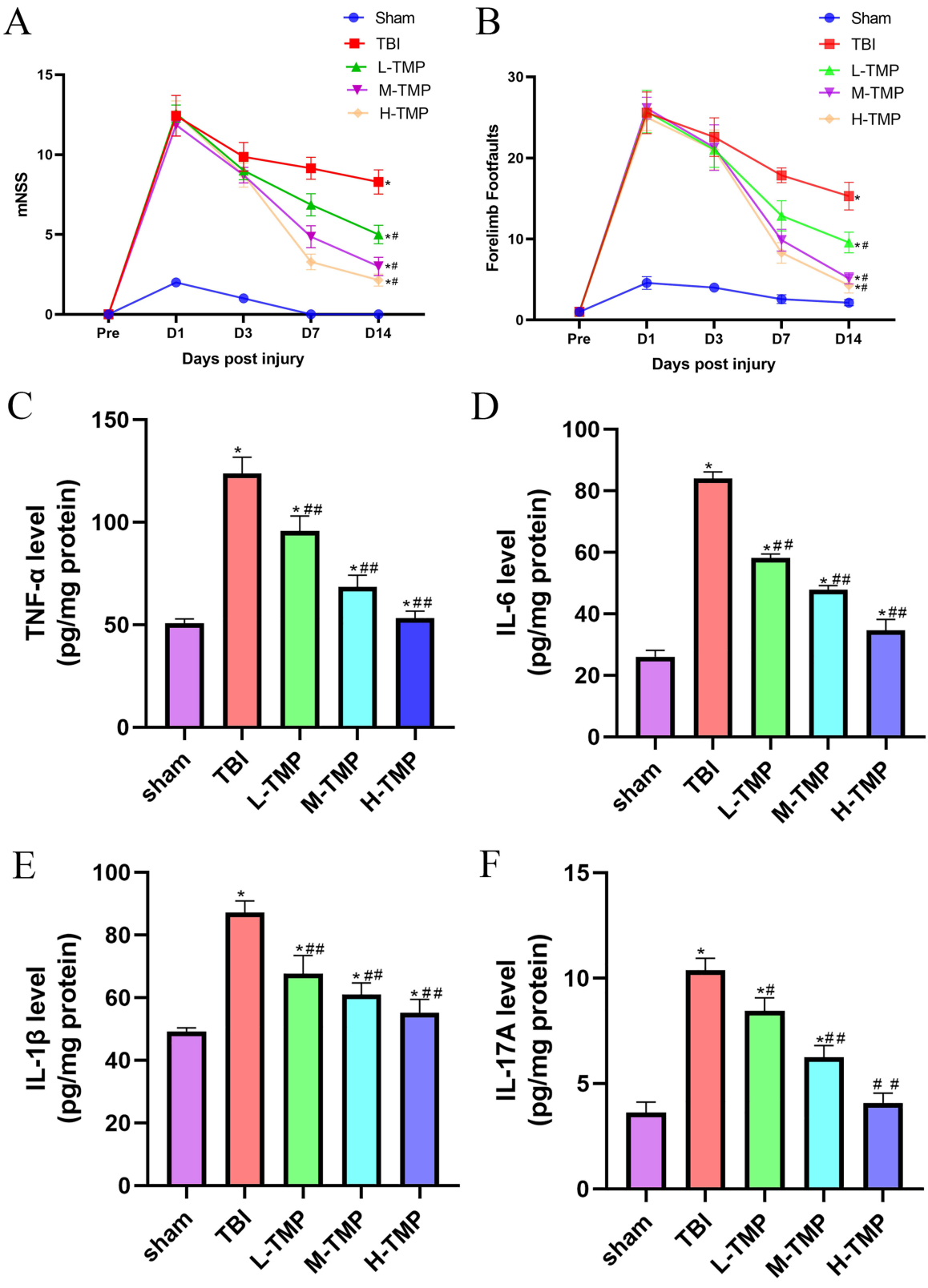

Figure 5 Neurological function scores and levels of various inflammatory factors in cortical tissue. (**A**) Neurological deficits were assessed by mNSS evaluation, n = 7; (**B**) Sensorimotor function was measured with the foot fault test, n = 7; ELISA results of TNF-α (**C**), IL-6 (**D**), IL-1β (**E**), and IL-17A (**F**), n =7. *P < 0.05 vs the sham group; #P < 0.05 vs the TBI group; ##P < 0.05 vs the TBI group.

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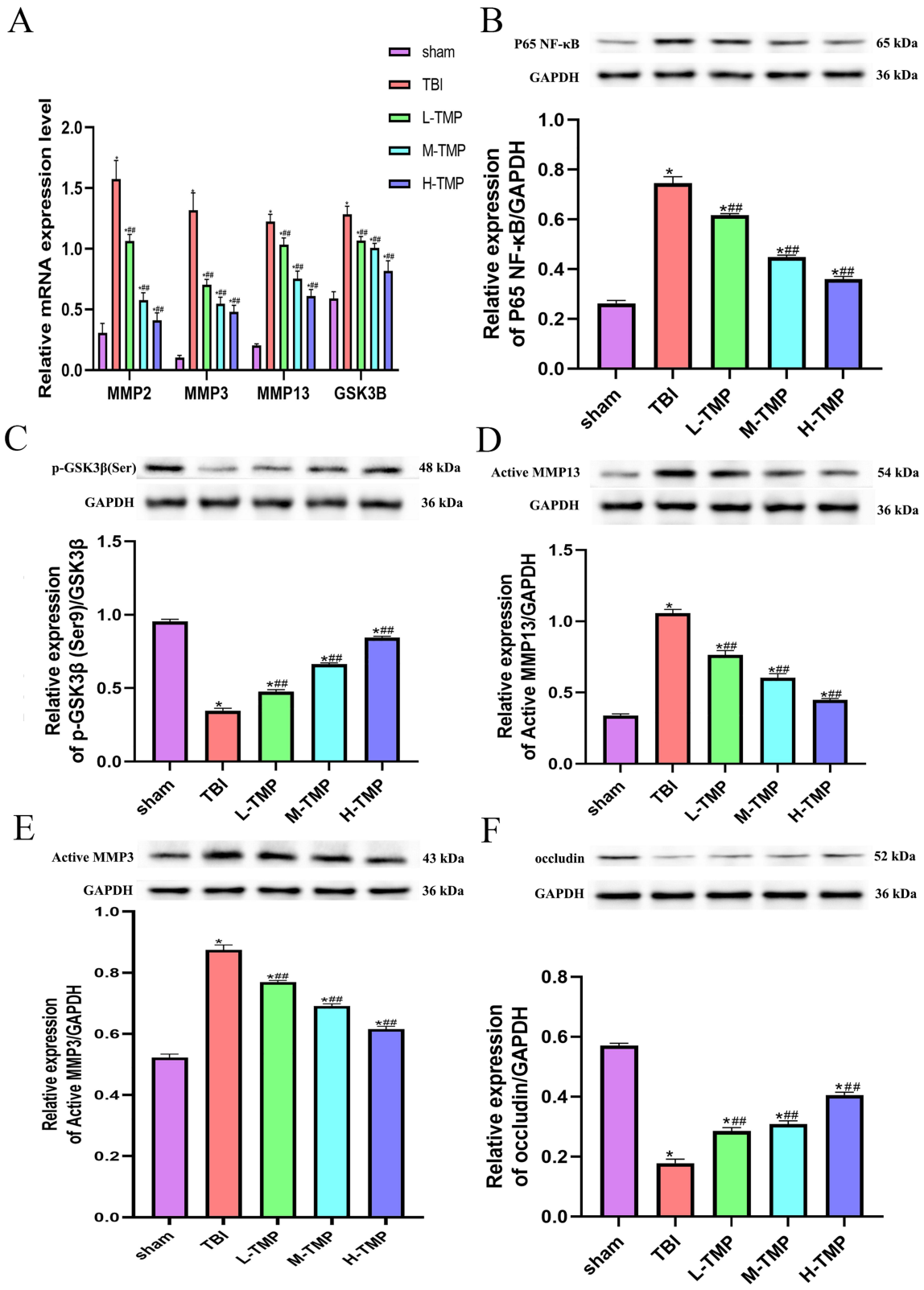

Figure 6 TMP inhibits the TBI-induced upregulation of key gene targets and protein expression. (**A**) The relative mRNA expressions of MMP3, MMP2, MMP13, and GSK3B were measured using RT-qPCR. The relative protein expressions of P65 NF-κB (**B**), p-GSK3β (Ser9) (**C**), active MMP13 (**D**), active MMP3 (**E**), and occludin (**F**) were assessed by Western blot analysis. *P < 0.05 vs the sham group; ##P < 0.05 vs the TBI group.

was much higher in the rat`s cortical tissues in the high-dose TMP-treated group than in the TBI model group ($P < 0.01$), approaching levels visualized in the sham group (Figure 6F).

Discussion

In this study, a comprehensive database was analyzed to identify core proteins that serve as common targets for drugs and diseases. The database included 20 proteins, like CASP3, ESR1, EGFR, PPARG, MMP2, GSK3B, MDM2, IL2, ESR2, and JAK2. Then, such proteins were detected through network pharmacology. In terms of MMP13, MMP3, MMP2, and GSK3B, key gene targets were recognized via molecular docking and MD simulations. The pivotal mechanism of TMP therapy for TBI was construed by GO and KEGG enrichment. Our results were deeply authenticated via animal tests.

Through network pharmacology and MD techniques, our research excellently recognized core gene targets according to the docking scores. Subsequently, the targets were verified by means of MD simulations and in vivo tests. MMP13, MMP3, MMP2, and GSK3B were regarded as the main gene targets of TMP to treat TBI. MMP13 belongs to the matrix metalloproteinase family, and is famous for degrading diverse collagen proteins.[44] Indeed, it can degrade various collagen proteins, and especially has effects on soluble type II collagen, which is important to embryonic bone growth, wound healing, tumor invasion, and so on.[45,46] Evidence suggested that MMP13 expression was obviously upregulated one day following spinal cord injury model, which indicated its engagement in extracellular matrix (EM) remodeling in the early phases of nerve regeneration.[47] Simultaneously, MMP13 might aggravate the permeability of BBB by degrading type IV collagen in the basement membrane, therefore facilitating vasogenic cerebral edema. In addition, MMP13 knockdown caused aberrant expression of inflammatory factors and delayed wound healing, along with angiogenesis-associated genes, suggesting that it coordinated inflammatory reactions after central nervous system damage.[48] The MMP3 gene is implicated in tissue remodeling, atherosclerosis, and tumor metastasis by degrading diverse EM components, and it is aberrantly expressed in rheumatoid arthritis and coronary artery disease.[49,50] Peaking one day post-injury in the spinal cord injury model, MMP3 upregulates the permeability and hemorrhage of blood-spinal cord barrier by degrading tight junction proteins.[51] It also activates other MMPs, exacerbating inflammatory cell infiltration and neuronal death.[52,53] The MMP2 gene is widely involved in angiogenesis, myocardial repair, and tumor metastasis.[54] In TBI, MMP2 is secreted by immune cells and promotes vascular reconstruction by breaking down the extracellular matrix. Inhibition of MMP2 significantly reduces the quantity of repaired blood vessels.[55] Under the action of a cerebral ischemia model, MMP2 is observed to disrupt the BBB in the early stages, leading to edema, while promoting neurovascular regeneration in the later stages.[56] Moreover, it has been demonstrated that MMP2 knockdown decreases the volume of hemorrhage and infarction.[57] After cerebral hemorrhage, MMP2 relates to inflammatory cell infiltration, and its expression levels positively relate to the extent of BBB disruption.[58] The GSK3B gene, located on chromosome 3q13.3, encodes a serine/threonine kinase.[59,60] Under the influence of TBI, the suppression of GSK3B stimulates the mTORC1 pathway and facilitates synaptic protein synthesis, while enhancing axonal damage and cognitive ability after hypoxia-ischemia.[61] In addition, it is proven that GSK3B suppressors increase β-catenin and tight junction proteins, thus alleviating the BBB disruption following cerebral hemorrhage.[62,63] Further, the evidence suggests that GSK3B knockdown improves the activity of antioxidant enzyme via the Nrf2/ARE pathway while decreasing oxidative damage after ischemia-reperfusion damage.[64] Such discoveries indicate that MMP13, MMP3, MMP2, and GSK3B prominently affect the therapy of TBI.

GO and KEGG analyses indicate that TMP might have therapeutic effects upon TBI via the relaxin and IL-17 signaling pathways. The latter involves the IL-17 family members and their receptors, primarily yielded by Th17 cells.[65] These IL-17 family members and associated receptors stimulate downstream signaling pathways that trigger the expression of pro-inflammatory factors.[66] Such pro-inflammatory factors are implicated in both host defense and the development of autoimmune diseases.[67] The upregulation of IL-17A following cerebral hemorrhage has been shown to promote microglial autophagy and inflammatory responses. Furthermore, it is demonstrated to increase the permeability of BBB and exacerbate cerebral edema, while facilitating leukocyte infiltration and neurological damage through the activation of chemokine-releasing endothelial cells.[68] In cases of peripheral nerve injury, Schwann cells upregulate chemokines via the IL-17B/IL-17RB signaling pathway, recruiting macrophages to remove the myelin sheath.[69] This pathway is essential for nerve repair, while IL-17A exacerbates the pathological progression of multiple

sclerosis and Alzheimer's disease.[70,71] The relaxin signaling pathway primarily takes the responsibility for the actions of RXFP1 and RXFP3/4, which have been demonstrated to show anti-fibrotic, vasodilatory, and anti-inflammatory properties.[72] This pathway involves the engagement of multiple signaling mechanisms to promote the level of matrix metalloproteinases and to impede fibrosis.[73] Under the impact of TBI, serelaxin is demonstrated to reduce inflammation, decrease neuronal apoptosis, and improve cognitive function through the activation of the eNOS/NO-cGMP pathway.[74] Additionally, it inhibits the activity of TLR4 and NLRP3 inflammasomes, thereby reducing the levels of various pro-inflammatory factors.[75] In models of subarachnoid hemorrhage, relaxin is proven to suppress apoptosis via the PI3K/Akt pathway, upregulate the anti-apoptotic protein, and promote the level of MMP-2/9 to enhance brain tissue remodeling.[76] Furthermore, Relaxin-3/RXFP3 signaling regulates the hypothalamic-pituitary axis, attenuates the stress response, and promotes neural regeneration.[77] Relaxin protects hippocampal neurons by impeding epithelial-mesenchymal transition via the Wnt/β-catenin pathway.[78] The present study assessed protein and gene expression using ELISA, Western blot and q-PCR, respectively. Occludin is a crucial structural protein found in the tight junctions of the blood-brain barrier, playing a vital role in maintaining its integrity.[79,80] Expression levels of occludin are significantly reduced following traumatic brain injury, and our findings indicate that TMP can enhance its expression. The expression of target genes, like MMP13, MMP3, MMP2, and GSK3B, was found to increase following injury. However, TMP therapy prominently decreased their expression while suppressing the related IL-17 and relaxin signaling pathways.

Our study has several limitations. Firstly, while this research integrated multi-omics data from authoritative databases, the inherent biases present in public datasets may affect the generalizability of our conclusions. Secondly, although the network model identified several high-confidence targets and we validated them through molecular docking and in vivo experiments, the current validation lacks cellular resolution. Our mechanistic findings, based on tissue homogenates, do not provide spatial context or identify the specific cell types (eg, microglia, astrocytes, or endothelial cells) involved. Key mechanistic assumptions, therefore, still require validation at the cellular level through techniques such as immunofluorescence imaging or flow cytometry. Finally, existing approaches assume a linear superposition of drug-target interactions, which may oversimplify the nonlinear pharmacokinetic-pharmacodynamic relationships. The use of Boolean network models or stochastic simulations could provide a more comprehensive representation of multi-target regulatory mechanisms.

Conclusion

Through the framework of network pharmacology, we determined that the shared target genes exhibited a strong correlation with the efficacy of TMP in the management of TBI. The predicted core target genes were validated using molecular docking and MD simulation. GO and KEGG analyses demonstrated that the enriched pathways related to the IL-17 signaling pathway and the relaxin signaling pathway. In vivo experiments proved that TMP enhanced neurological function and inhibited the expression of inflammatory factors among TBI rats, while downregulating the level of the relaxin and IL-17 signaling pathways to varying extents. This study provides evidence that TMP has the potential for being a clinical therapy for TBI.

Data Sharing Statement

All data are available in the manuscript and they are showed in tables, figures.

Ethics Approval and Consent to Participate

The animal experiments were approved by the Ethics Committee of the Second People's Hospital of Wuhu (IRB number:2024-KY-003) and complied with the national standard GB/T 35892-2018 and the "3R" principles. This study utilized pre-existing, anonymized data from public databases. The Ethics Committee of the Second People's Hospital of Wuhu confirmed that this research is exempt from ethical review in accordance with Articles 32.1 and 32.2 of the "Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects" (China, 2023). Therefore, the requirement for informed consent was waived.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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