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A systems perspective on ribavirin-induced hemolytic anemia: From network toxicology to atomic-level simulations

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摘要

Objective: The molecular mechanisms underlying ribavirin (RBV)-induced hemolytic anemia remain incompletely understood. Current research predominantly addresses individual targets or pathways, lacking a comprehensive integration of multi-target interaction networks. This study employs a combination of network toxicology (NT), multi-scale molecular dynamics (MD) simulation techniques, and in vitro experiments to conduct an in-depth analysis of the multi-target mechanisms responsible for RBV-induced hemolytic anemia. Methods: A multi-scale biological molecular network model was developed utilizing NT. Multi-scale molecular simulations were employed to integrate calculations across various temporal and spatial scales. This approach was complemented by red blood cell hemolysis assays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantitative analysis, and circular dichroism (CD) spectroscopy experiments, thereby elucidating the hierarchical patterns of molecular behaviors that contribute to RBV-induced hemolytic anemia across multiple scales. Results: The findings demonstrated that RBV/triphenyl ribavirin (RTP) disrupts the function of the Band 3 protein, inhibits glucose-6-phosphate dehydrogenase (G6PD) activity, induces oxidative stress, and triggers apoptotic pathways, ultimately resulting in the rupture of red blood cells. The integration of multi-scale data corroborated the "drug accumulation-oxidative stress-cell death" three-stage damage model, offering a quantitative characterization framework from atomic to systemic levels for elucidating the toxicity mechanism of nucleoside analogues. This approach advances the application of analytical toxicology in the evaluation of drug safety.

关键词

作者关键词: Network toxicology; Multi-scale molecular simulation; Ribavirin; Hemolytic anemia; Liquid chromatography-tandem mass spectrometry; Circular dichroism spectroscopy
Keywords Plus: MECHANISMS; PROLIFERATION; EXPRESSION; PREDICTION; PLATFORM; UPDATE; CANCER; GENES; CELLS

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A systems perspective on ribavirin-induced hemolytic anemia: From network toxicology to atomic-level simulations

Xumeng Shi^{a,b,1}, Shijia Lu^{a,1}, Guangman Zhu^a, Tianjiao Cui^a, Shunhang Wu^a, Mengdan Sang^a, Yiran Zhen^a, Xue Yang^a, Huaying Du^a, Miaoyan Han^a, Mengjia Yan^a, Jinle Wang^a, Yutong Zhang^a, Jiying Du^{a,*}

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ABSTRACT

Objective: The molecular mechanisms underlying ribavirin (RBV)-induced hemolytic anemia remain incompletely understood. Current research predominantly addresses individual targets or pathways, lacking a comprehensive integration of multi-target interaction networks. This study employs a combination of network toxicology (NT), multi-scale molecular dynamics (MD) simulation techniques, and *in vitro* experiments to conduct an in-depth analysis of the multi-target mechanisms responsible for RBV-induced hemolytic anemia. **Methods:** A multi-scale biological molecular network model was developed utilizing NT. Multi-scale molecular simulations were employed to integrate calculations across various temporal and spatial scales. This approach was complemented by red blood cell hemolysis assays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantitative analysis, and circular dichroism (CD) spectroscopy experiments, thereby elucidating the hierarchical patterns of molecular behaviors that contribute to RBV-induced hemolytic anemia across multiple scales. **Results:** The findings demonstrated that RBV/triphenyl ribavirin (RTP) disrupts the function of the Band 3 protein, inhibits glucose-6-phosphate dehydrogenase (G6PD) activity, induces oxidative stress, and triggers apoptotic pathways, ultimately resulting in the rupture of red blood cells. The integration of multi-scale data corroborated the “drug accumulation-oxidative stress-cell death” three-stage damage model, offering a quantitative characterization framework from atomic to systemic levels for elucidating the toxicity mechanism of nucleoside analogues. This approach advances the application of analytical toxicology in the evaluation of drug safety.

1. Introduction

First chemically synthesized in the 1970s, ribavirin (RBV) is a broad-spectrum nucleoside antiviral agent widely used to treat infections caused by RNA viruses, such as Hepatitis C virus and respiratory syncytial virus [1]. Current evidence suggests that RBV undergoes intracellular metabolism to form its active triphosphate metabolite, ribavirin triphosphate (RTP). The catabolic pathway of RTP typically mirrors that of endogenous nucleotides, primarily relying on enzymes involved in nucleotide metabolism [2]. The principal antiviral mechanism of RBV is attributed to the inhibition of inosine-5'-monophosphate dehydrogenase (IMPDH), the first rate-limiting enzyme in the *de novo* biosynthesis of guanine nucleotides. By blocking the conversion of inosine monophosphate to xanthosine monophosphate (a precursor to guanosine monophosphate), RBV depletes intracellular guanosine triphosphate (GTP) pools, thereby inhibiting the synthesis of viral nucleic acids

and demonstrating efficacy against a range of DNA and RNA viruses (Fig. 1) [3].

However, the clinical utility of RBV is significantly challenged by its dose-dependent side effect, hemolytic anemia. According to data from the China National Center for Adverse Drug Reaction Monitoring up to 2023, RBV administration has been associated with hundreds of reported cases of hemolytic anemia, 20 cases of aplastic anemia, hundreds of cases of leukopenia, 10 cases of agranulocytosis, and 5 fatal cases of systemic hemorrhage, in addition to numerous co-occurrences of leukopenia and anemia [4]. These findings underscore the urgent need to elucidate the underlying molecular mechanisms. Existing research has largely focused on phenotypic descriptions or isolated signaling pathways, lacking a systematic and quantitative characterization of drug-target interactions—a critical gap in the field of analytical toxicology [5]. Recently, analytical toxicology has provided a new paradigm for the systematic, quantitative dissection of drug-induced toxicity by

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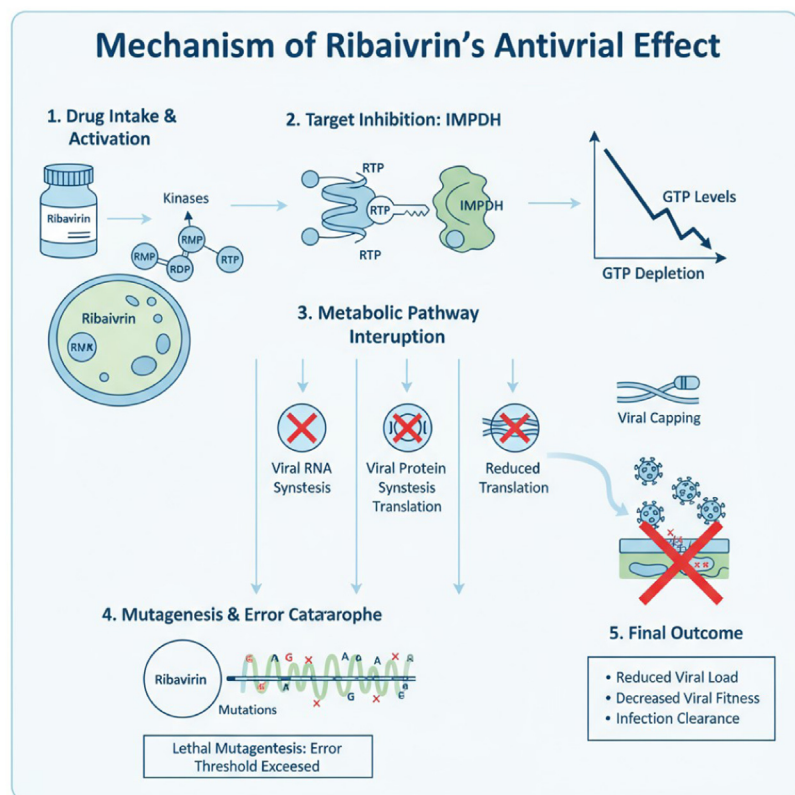


Fig. 1. The main antiviral mechanisms of RBV.

integrating network toxicology (NT) with multi-scale molecular simulations. NT decodes the synergistic perturbation mechanisms involving multiple targets and pathways by constructing biomolecular interaction networks [6], while multi-scale simulations—ranging from molecular docking to molecular dynamics (MD) simulations—enable the quantification of interactions at atomic resolution [7].

This study aims to systematically decipher the molecular mechanism of RBV-induced hemolytic anemia by integrating NT, MD simulations, and experimental validation within a multi-scale framework that spans from atomic-scale drug-target interactions to macroscopic pathway networks. Our work seeks to fill the gap in the quantitative characterization of nucleoside analog toxicity and advance the application of analytical toxicology in drug safety evaluation.

2. Materials and methods

2.1. Data sources and tools

2.1.1. Database

During the analytical phase of this study, a diverse array of databases (Table 1) was extensively utilized to enhance the richness of data sources, thereby increasing the reliability of the analytical outcomes.

2.1.2. Software and platform

AutoDock Vina [33], GROMACS [34], Cytoscape3.7.1 [35], Kdeep [36], fastDRH [37], CDPro [38]

2.1.3. Materials and instruments

The study utilized healthy adult peripheral anticoagulant whole blood sourced from Henan Huanqiu Biotechnology Co., LTD., characterized by the inclusion of 3.8% sodium citrate as an anticoagulant (License number: Henan Medical Device Registration No. 202312345), phosphate-buffered saline (PBS), trichloroacetic acid, ammonium formate, acetonitrile, ribavirin (RBV) standard (Sigma-Aldrich, Cat. No. R9644, purity $\geq 98\%$ as analyzed by reversed-phase high-performance

liquid chromatography), refrigerated high-speed centrifuge (Thermo Scientific Sorvall ST 16R), constant temperature incubator (Memmert INCOmed, temperature control accuracy $\pm 0.5\text{ }^{\circ}\text{C}$), full-wavelength microplate reader (BioTek Synergy H1, used for hemolysis rate and oxidative stress indicator detection), malondialdehyde (MDA) assay kit (Nanjing Jiancheng Bioengineering Institute, Cat. No. A003-1), reduced glutathione (GSH) assay kit (Beyotime Biotechnology, Shanghai, Cat. No. S0053), catalase (CAT) activity assay kit (Solarbio Science & Technology, Beijing, Cat. No. BC0200), Agilent ZORBAX Eclipse Plus C18 column, AB Sciex Triple Quad 5500 mass spectrometer, J-1500 circular dichroism (CD) spectropolarimeter (JASCO, Japan).

2.2. Research methods

2.2.1. Acquisition of ribavirin action targets

The chemical structure of RBV was retrieved using the Chemical Structure function in the DrugBank database. Detailed drug structural data and physicochemical information were downloaded. Additional searches were conducted using the DrugBank database, SWISS Target Prediction, and PubChem to obtain and save RBV molecular information, followed by the download and compilation of target-related data.

2.2.2. Identification of RBV targets associated with hemolytic anemia

Targets related to hemolytic anemia were queried using the keyword “Hemolytic Anemia” in databases including Disgenet, MalaCards, GeneCards, and CTD. The retrieved targets were subsequently collated. All RBV-related targets and hemolytic anemia-associated targets were imported into the Venny 2.1.0 online tool. A Venn diagram was generated to identify the intersection, which represented the common targets through which RBV may mediate hemolytic anemia.

2.2.3. Construction of a protein–protein interaction network for key protein targets

To further elucidate the mechanism of RBV-induced hemolytic anemia, a protein interaction network was built using the STITCH database

Table 1
List of databases used.

PubChem [8]	https://pubchem.ncbi.nlm.nih.gov
DrugBank [9]	https://go.drugbank.com/
CTD [10]	https://ctdbase.org
RCSB PDB [11]	https://www.rcsb.org/
GEO [12]	https://www.ncbi.nlm.nih.gov/geo/
ADMET Lab [13]	https://admetlab3.scbdd.com/server/screening
ProTox-II [14]	https://tox.charite.de/protox3/index.php?site=home
VenomPred [15]	https://www.mmvsl.it/wp/venompred2/
Disgenet [16]	https://disgenet.cn/
OMIM [17]	https://www.omim.org/
GeneCards [18]	https://www.genecards.org/
MalaCards [19]	https://www.malacards.org/
PharmMapper [20]	http://lilab-ecust.cn/pharmmapper/index.html
SuperPred [21]	https://prediction.charite.de/
SwissTargetPrediction [22]	http://swisstargetprediction.ch/
TargetNet [23]	http://targetnet.scbdd.com/home/index/
Therapeutic Target Database [24]	http://db.idrblab.net/ttd/
Similarity ensemble approach [25]	https://sea.bkslab.org/
STITCH [26]	http://stitch.embl.de/
DAVID [27]	https://david.ncifcrf.gov/
KEGG [28]	https://www.genome.jp/kegg/
Uniprot [29]	https://www.uniprot.org/
ESMFold [30]	https://bio-web1.nsc-gz.cn/app/esmfold
CB-Dock2 [31]	https://cadd.labshare.cn/cb-dock2/index.php
HydraScreen [32]	https://hydrascreen.ro5.ai/paper

for predicting gene and chemical interactions. The common key targets identified above were input into the STITCH platform. Targets that could not form a PPI network or had too few nodes were excluded. The interaction score was set to high confidence, and line thickness was used to represent the strength of data support. The resulting PPI topological network was analyzed and optimized using Cytoscape 3.7.1, with node color indicating the number of interaction relationships.

2.2.4. Gene ontology (GO) biological process analysis

GO enrichment analysis was performed using the DAVID database. The core target proteins identified above were imported into DAVID, with Homo sapiens (taxonomy ID: 9606) selected as the species. The enrichment results were categorized into molecular function (MF), biological process (BP), and cellular component (CC). Terms with a P-value greater than 0.01 were excluded; only those with $P \leq 0.01$ were retained, indicating high statistical significance. The BP, CC, and MF terms were ranked by gene count and visualized using bar charts for further analysis. GO biological process signaling analysis provides deeper insight into the functional roles of different genes and helps reveal correlations and mechanisms among targets.

2.2.5. Gene expression differential analysis and KEGG pathway analysis

This study utilized the GEO database to examine changes in gene expression levels following RBV treatment, as well as differences between hemolytic anemia and normal cells, in order to validate RBV's mechanism of action at a macro-network level. THP-1 cells [39] were selected as the model system, with the control group consisting of untreated cells and the experimental group comprising RBV-treated cells. Differential gene expression analysis was conducted between the two groups. In addition, KEGG pathway enrichment analysis of key targets was carried out using the DAVID database. The resulting pathways were further investigated by incorporating gene expression differential data.

2.2.6. Drug molecule toxicity prediction

To systematically quantify the toxicity profiles of RBV and its active metabolite RTP, the ProTox-II database and VenomPred 2.0 platform were employed. These tools integrate molecular similarity, pharmacophore mapping, fragment propensity, and machine learning models to predict multiple toxicity endpoints, including acute toxicity, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity, Tox21 adverse outcome pathways, and toxicological targets [40].

2.2.7. Molecular docking and dynamics simulations

This study employed an integrated computational approach combining molecular docking and molecular dynamics simulations to systematically investigate the interaction mechanisms between RBV and key target proteins in red blood cells. First, molecular docking software such as AutoDock was used to perform rigid/flexible docking of RBV with potential target proteins, predicting the binding mode, key binding sites, and types of interaction forces, and screening for the optimal binding conformation. Subsequently, based on the docking results, all-atom MD simulations (100–200 ns) were conducted using GROMACS under physiological conditions (310 K, 1 atm, aqueous environment) to evaluate the dynamic stability, binding free energy, and conformational changes of key residues in the complex system. By analyzing parameters such as RMSD, RMSF, and hydrogen bond occupancy, the long-term binding characteristics of RBV with the target proteins and its potential impact on protein conformation were elucidated, providing an atomic-level theoretical basis for explaining the molecular mechanism by which RBV interferes with red blood cell structure and function.

2.2.8. Experimental validation of RBV-Induced hemolysis

Fresh anticoagulated whole blood from healthy adult donors (Henan Huanqiu Biotechnology Co., Ltd., containing 3.8% trisodium citrate anticoagulant) was used. A total of 4 mL of anticoagulated whole blood was mixed with 36 mL of normal saline, inverted and shaken gently, then centrifuged at 1000 r/min for 15 min. The supernatant was discarded, and the precipitated red blood cells were washed 2–3 times with 36 mL of normal saline using the same procedure until the supernatant was no longer red. The resulting red blood cells were suspended in normal saline to prepare a 5% suspension for experimental use. An RBV working solution with a concentration of 16 mg/mL was prepared and stored temporarily at 4 °C; for long-term storage, it was transferred to –20 °C.

(1) To verify the dose- and time-dependent characteristics of RBV-induced hemolytic anemia and its molecular mechanism, the experiment included six concentration gradients: 0, 2, 4, 6, 8, and 10 mg/mL. The RBV solution was added proportionally to the red blood cell suspension, followed by incubation at 37 °C for 1 h. After centrifugation, the supernatant was collected, and its absorbance was measured at 540 nm using a full-wavelength microplate reader (BioTek Synergy H1) to calculate the hemolysis rate. To analyze the time kinetics, a concentration of 8 mg/mL RBV was selected, and samples were taken at 0, 2, 4, 6, and 8 h to determine the hemolysis rate, revealing the nonlinear

characteristics of the hemolysis process. Hemolysis rate (%) = (Sample absorbance – Blank absorbance) / (Full hemolysis absorbance – Blank absorbance) $\times$ 100 %.

(2) To further clarify the oxidative stress mechanism, the levels of malondialdehyde, reduced glutathione, and catalase activity in red blood cells were measured. MDA content was determined by the thio-barbituric acid method (Kit A003–1, Nanjing Jiancheng Bioengineering Institute), GSH level was quantified based on the DTNB chromogenic reaction (Kit S0053, Beyotime Biotechnology, Shanghai), and CAT activity was assessed by monitoring the decomposition rate of H_2O_2 (Kit BC0200, Solarbio, Beijing). All assays were performed in strict accordance with the kit instructions. Data were expressed per milligram of protein or per gram of hemoglobin.

All experiments were performed in triplicate, and data are presented as mean $\pm$ standard deviation. Statistical analysis was carried out using two-way ANOVA or *t*-test, with a *P*-value < 0.05 considered statistically significant. Through multi-dimensional experimental correlation, this study comprehensively elucidates the hemolytic toxicity mechanism of RBV, from macroscopic hemolytic phenotypes to microscopic molecular functions.

2.2.9. LC-MS/MS quantitative analysis

To directly verify the core hypothesis that RBV undergoes irreversible phosphorylation and accumulates as its active metabolite RTP within red blood cells, LC-MS/MS quantitative analysis was performed. First, erythrocyte suspensions incubated with different concentrations of RBV (2, 4, 6, and 8 mg/mL) at 37 °C for varying durations (1, 2, 4, and 6 h) were centrifuged, and the supernatant was discarded. The red blood cell pellet was washed three times with pre-cooled PBS to thoroughly remove surface-adsorbed drug. Subsequently, the cells were lysed by repeated freeze-thaw cycles in pure water, and proteins were precipitated using 10 % trichloroacetic acid. After centrifugation, the supernatant was collected for analysis. Chromatographic separation was carried out using an Agilent ZORBAX Eclipse Plus C18 column (2.1 $\times$ 100 mm, 1.7 μm) maintained at 40 °C. Mobile phase A consisted of ultrapure water containing 5 mM ammonium formate, and mobile phase B was mass spectrometry-grade acetonitrile. A gradient elution program was applied as follows: 0–2.0 min, 5 % B; 2.0–5.0 min, a linear increase to 90 % B, held for 1 min; 6.0–6.1 min, B reduced to 5 % and equilibrated for 2.9 min. The total run time was 9 min at a flow rate of 0.3 mL/min. Mass spectrometric detection was performed on an AB Sciex Triple Quad 5500 system equipped with an electrospray ionization (ESI) source operating in positive ion mode. The ion transitions (precursor $\rightarrow$ product ion) optimized for multiple reaction monitoring (MRM) were: RBV, *m/z* 245.1 $\rightarrow$ 113.0; RTP, *m/z* 585.0 $\rightarrow$ 245.1.

2.2.10. Experimental validation of protein conformation by circular dichroism spectroscopy

To validate the effect of RBV and its active metabolite on the conformation of Band 3 protein, circular dichroism (CD) spectroscopy was employed to quantitatively analyze changes in secondary structure. Band 3 protein was diluted with PBS to a final concentration of 0.2 mg/mL and divided into three experimental groups: native Band 3 protein (control group), protein mixed with 100 μM RBV, and protein mixed with 100 μM RTP. All samples were incubated in the dark at 25 °C for 30 min before being transferred to a quartz cuvette for measurement. Spectra were acquired in the far-UV region (190–260 nm) with a scan speed of 50 nm/min, bandwidth of 1 nm, and response time of 4 s. Each sample was scanned three times and the average spectrum was used. To eliminate background interference, all CD spectra were subtracted from the PBS blank signal and smoothed using the Savitzky–Golay algorithm. The resulting ellipticity data (in millidegrees) were deconvoluted using the CONTIN algorithm within the CDPPro software package to quantitatively assess the relative content of secondary structure elements, including α -helix, β -sheet, β -turn, and random coil.

Disease Targets

Drug Targets

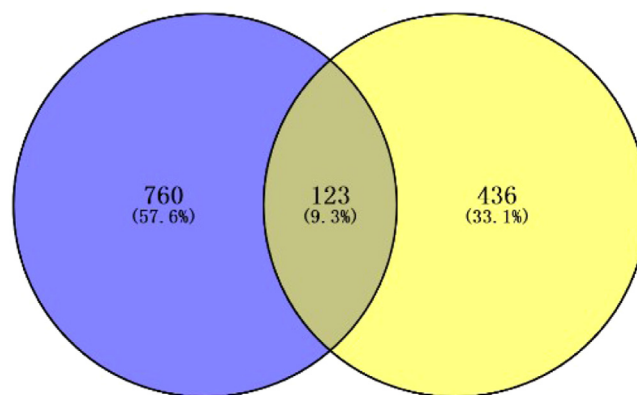


Fig. 2. Venn diagram of disease targets and drug targets.

3. Results and analysis

3.1. Drug–disease target mining results

Target data for RBV were retrieved from databases including PubChem and DrugBank, yielding a total of 559 potential targets. Related targets for hemolytic anemia were collected from Disgenet, MalaCards, GeneCards, and CTD, resulting in 883 disease-associated targets. The intersection between RBV-related targets and hemolytic anemia-related targets was identified using a Venn diagram, revealing 123 common targets potentially involved in RBV-induced hemolytic anemia, as shown in Fig. 2.

3.2. Construction and analysis of the key protein target interaction network

To further explore the mechanism of hemolytic anemia, a protein–protein interaction (PPI) network was constructed using the STITCH database for predicting gene and chemical interactions. The common targets obtained above were imported into the STITCH platform. Targets that could not form a PPI network or had too few connecting nodes were removed. The interaction score threshold was set to high confidence, and edge thickness was used to represent the strength of supporting data. The resulting PPI network was analyzed and optimized using Cytoscape 3.7.1, with node color indicating the number of interaction partners. The final network is shown in Fig. 3.

The network, constructed based on the STITCH database and topologically optimized with Cytoscape, incorporated 31 core targets and 218 interaction edges. Notable network properties (average node degree = 14.2, clustering coefficient = 0.78) indicated a high degree of functional coupling and cooperative interaction among the targets. Hub node analysis revealed that TNF (tumor necrosis factor; betweenness centrality = 0.42) [41] occupied a central role in network coordination, directly linking the apoptosis module [42], inflammatory cascade (IL6) [43], and oxidative stress hub (SOD2) [44], together forming a regulatory axis for the “inflammation–oxidation–apoptosis” triad of damage. Although not among the most highly central nodes, targets such as G6PD (degree = 9) and SLC4A1 (Band 3 protein; degree = 7) formed a tightly connected functional cluster through key oxidative effectors including SOD2 (superoxide dismutase 2; degree = 21) and CAT (catalase; degree = 18).

3.3. GO biological process analysis

The 31 core target proteins identified above were imported into the DAVID database for GO enrichment analysis, with Homo sapiens (taxonomy ID: 9606) selected as the species. The enrichment results

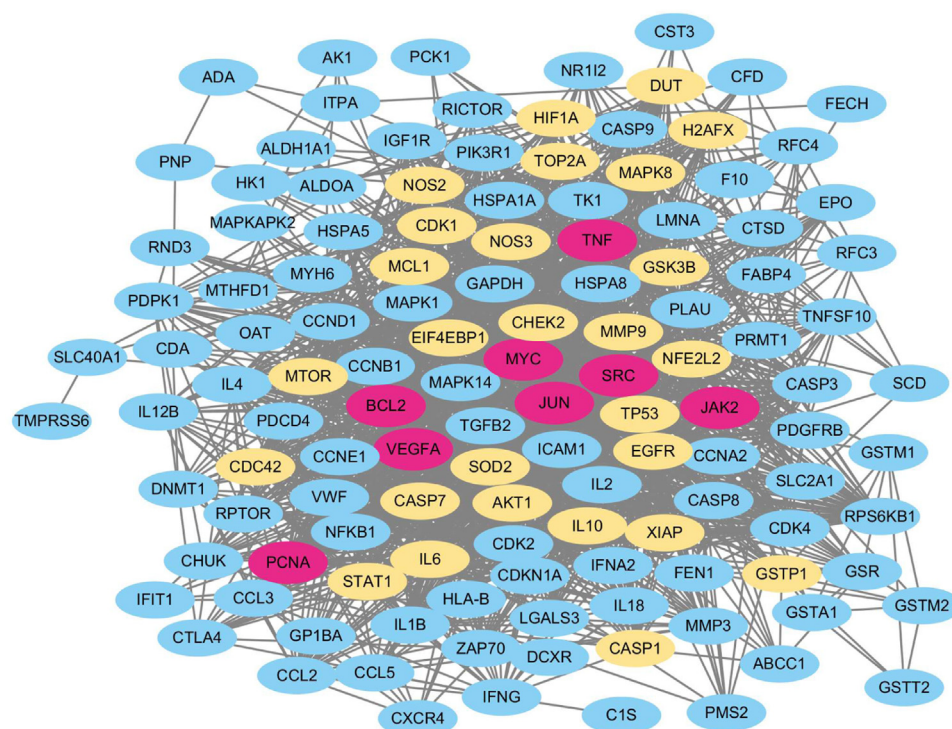


Fig. 3. PPI Network Diagram.

were categorized into MF, BP, and CC. Terms with a P -value > 0.01 were excluded; only those with $P \leq 0.01$ were retained, indicating high statistical significance. The BP, CC, and MF terms were ranked by gene count and visualized using bar charts for analysis, as shown in Fig. 4.

Based on the PPI analysis (Fig. 3) and GO enrichment results (Fig. 4), the molecular mechanism of RBV-induced hemolytic anemia may involve synergistic actions of multiple pathways and targets. Dysfunction of glutathione metabolism-related genes (GSTM1, GSTP1, GSR) [45] can reduce intracellular levels of reduced glutathione (GSH), impairing the antioxidant capacity of red blood cells. Insufficient SOD2 activity leads to superoxide anion accumulation, increased reactive oxygen species (ROS), and subsequent lipid peroxidation and erythrocyte membrane damage. The significant GO terms “cellular response to oxidative stress” and “negative regulation of ROS metabolic process” [46] further support this mechanism. Additionally, RBV may activate the CASP8/CASP3 cascade via death receptor signaling [47], as well as promote mitochondrial cytochrome c release to activate CASP9/CASP3. Dysregulation of the BCL2 family may enhance mitochondrial membrane permeability, which is closely associated with RBV’s impact on hematopoietic lineages [48]. Aberrant expression of pro-apoptotic TP53 and the apoptosis inhibitor XIAP may further exacerbate erythrocyte programmed death [49]. Overexpression of pro-inflammatory cytokines (TNF, IL6, IFNG) can activate the NF- κ B signaling pathway [50], upregulate adhesion molecule ICAM1 on the erythrocyte surface, and promote erythrophagocytosis by immune cells. TNF may also directly trigger apoptosis through death receptor pathways such as TNFRSF1A.

Abnormal expression of cell cycle regulators CDK1 and CCNB1 may interfere with the differentiation and maturation of erythroid precursors [51]. Dysregulation of hypoxia-inducible factors could affect erythrocyte energy metabolism. Impaired function of SCD, which is involved in membrane lipid composition, may increase erythrocyte membrane fragility. Decreased activity of FECH, a key enzyme in heme synthesis, may lead to heme deficiency and impaired erythrocyte maturation [52]. Impaired SLC2A1 function can cause energy metabolism disorders and accelerate erythrocyte lysis [53].

3.4. Gene expression differential analysis and KEGG pathway analysis

This study utilized THP-1 cells from the GEO database as the research model, with the control group consisting of untreated cells and the experimental group comprising RBV-treated cells. The differential gene expression results are shown in Fig. 5.

Based on the transcriptomic data of THP-1 cells from the GEO database (Fig. 5), systematic clustering of differentially expressed genes (DEGs) after RBV exposure revealed the multi-pathway regulatory nature of hemolytic toxicity. Heatmap analysis showed that, compared with the control group, the RBV-treated group (10 μ M, 24 h) exhibited a total of 1286 significantly differentially expressed genes ($|\log_2FC| > 1$, FDR < 0.05). Among these, genes associated with key toxicity-related pathways showed consistent alterations: (1) Oxidative stress pathway: SOD2 (superoxide dismutase 2) expression was significantly downregulated ($\log_2FC = -1.8$, $p = 0.003$); meanwhile, GPX4 (glutathione peroxidase 4) expression was suppressed ($\log_2FC = -1.2$, $p = 0.01$), providing a molecular basis for GSH depletion. (2) Apoptosis pathway: Caspase-3 (CASP3) was activated ($\log_2FC = +1.8$, $p = 0.007$), while BCL2 expression was suppressed ($\log_2FC = -2.1$, $p = 0.001$), indicating synergistic pro-apoptotic effects. (3) Inflammatory cascade pathway: TNF (tumor necrosis factor) expression was upregulated ($\log_2FC = +2.1$, $p = 0.004$), driving activation of NFKB1 (nuclear factor kappa B subunit 1; $\log_2FC = +1.6$, $p = 0.005$).

KEGG pathway enrichment analysis was performed on the key targets from the PPI network, and the results are shown in the corresponding figure. The enriched pathways were further investigated using the differential expression data, as illustrated in Fig. 6.

Integrated analysis of PPI, GO, KEGG, and gene expression data following RBV treatment indicates that RBV influences oxidative stress increase and antioxidant system imbalance in erythrocytes through key pathways including the FoxO signaling pathway [54] and AMPK signaling pathway [55]. FoxO transcription factors participate in regulating the expression of antioxidant enzymes (SOD2, GSR). Downregulation of glutathione metabolism genes such as GSTM1 and GSTP1 is consistent with the significant enrichment of the “glutathione metabolism” pathway in KEGG analysis.

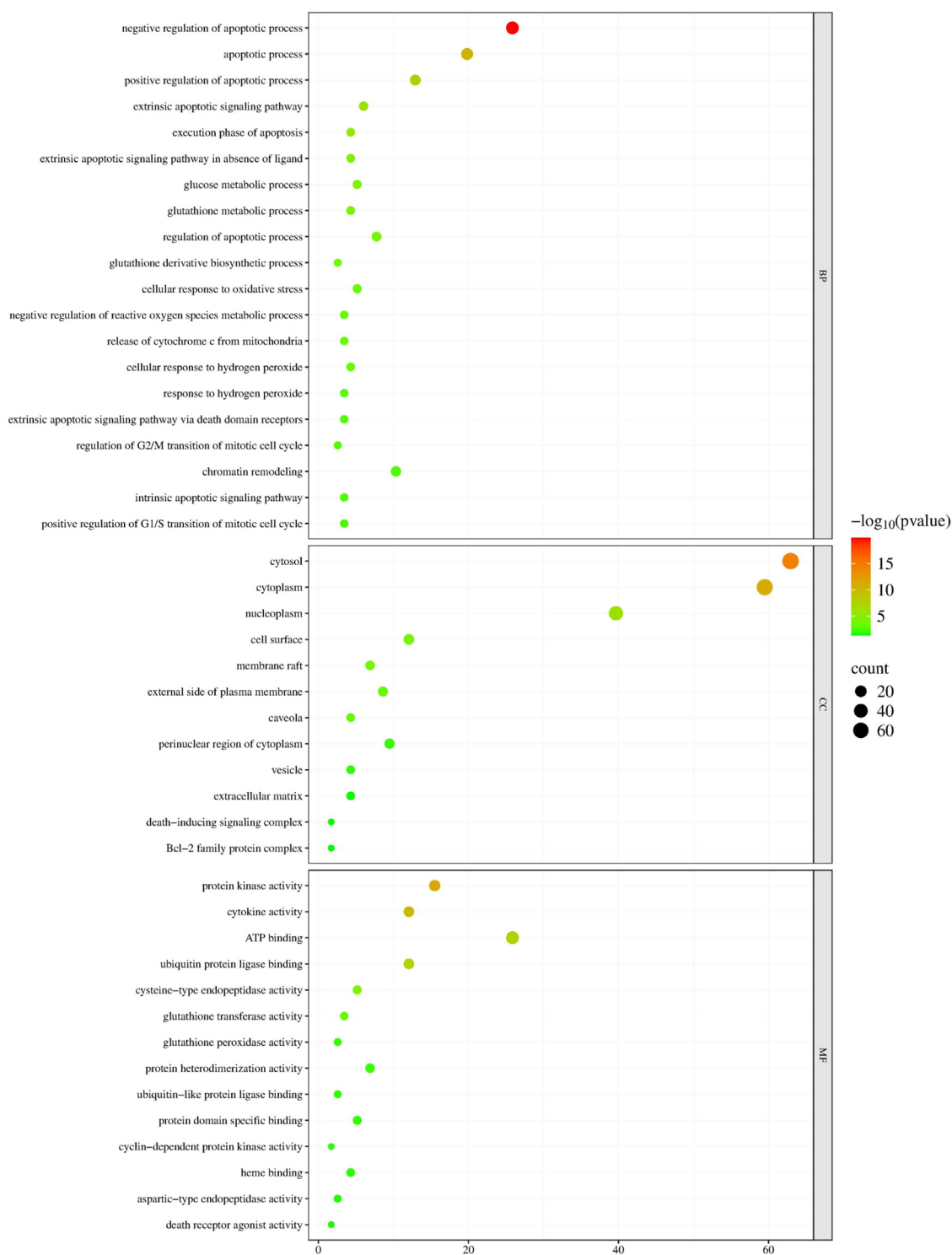


Fig. 4. GO enrichment analysis of the core target protein.

Key pathways including Apoptosis [56], p53 signaling pathway [57], and MAPK signaling pathway [58] are associated with abnormal activation of erythrocyte apoptosis. The TNF signaling pathway, mediated by TNF- α binding to its receptor, activates the CASP8/CASP3 cascade, directly leading to erythrocyte membrane rupture [59]. The p53 pathway upregulates pro-apoptotic genes via TP53, while dysregulation of the BCL2 family promotes mitochondrial cytochrome c release and activates CASP9/CASP3 [60]. Enhanced JNK phosphorylation further amplifies apoptotic signaling, resulting in programmed death of erythrocytes.

Key pathways such as the NF- κ B signaling pathway [61] and IL-17 signaling pathway [62] are involved in inflammation and immune-mediated damage to erythrocytes. RBV may induce pro-inflammatory factors such as TNF and IL6, activate NF- κ B, and upregulate adhesion molecules including ICAM1, enhancing macrophage-mediated phagocytosis of erythrocytes. Upregulation of IL17A synergistically amplifies inflammatory responses via MAPK and NF- κ B, leading to erythrocyte membrane damage and vascular endothelial activation, thereby exacerbating hemolysis. Key pathways including the HIF-1 signaling pathway

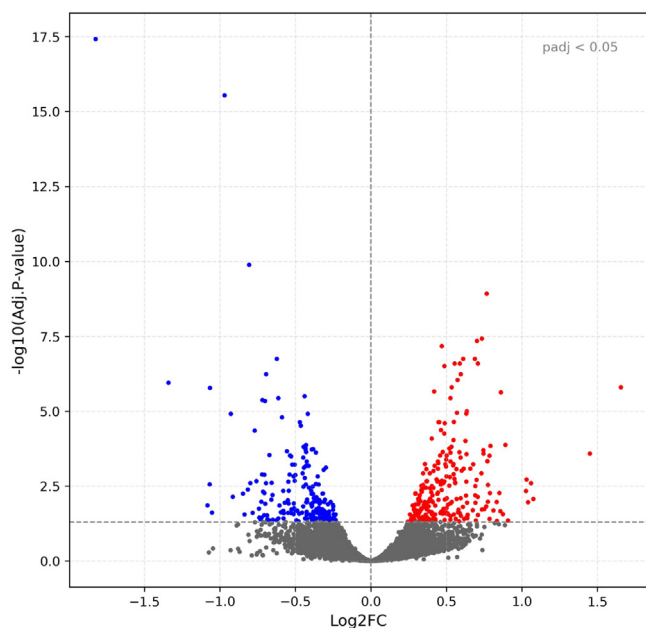


Fig. 5. Gene expression differences in THP-1 cells.

and PI3K-Akt signaling pathway are associated with erythrocyte energy metabolism and hypoxic stress [63]. Under hypoxic conditions, HIF1A expression is upregulated, but inhibition of the PI3K-Akt pathway attenuates the response to erythropoietin (EPO), impairing erythrocyte maturation. Downregulation of SLC2A1 reduces glucose uptake, leading to insufficient ATP production and further compromising membrane stability.

3.5. Drug molecule toxicity prediction

To systematically quantify the toxicity profiles of RBV and its active metabolite RTP, this study utilized the ProTox-II database and VenomPred 2.0 platform, integrating molecular similarity, pharmacophore matching, fragment propensity, and machine learning models to predict multiple toxicity endpoints, including acute toxicity, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity, Tox21 adverse outcome pathways, and toxicological targets.

Computational toxicology models were employed to quantitatively reveal the systemic toxicity risks of RBV and its active metabolite RTP. As shown in the left panel of Fig. 7, toxicity prediction using the ProTox-II platform indicated that the core risks of RBV were concentrated in hepatotoxicity (probability score 0.82) and mutagenicity (probability score 0.78), while carcinogenicity and immunotoxicity risks were relatively low (probability scores < 0.3). These results are mechanistically consistent with the clinical pharmacokinetic behavior of RBV: with approximately 50 % oral bioavailability, RBV undergoes phosphorylation to RBV monophosphate in the liver by adenosine kinase. Its prolonged retention (half-life 12 days) disrupts hepatocellular energy metabolism and induces mitochondrial dysfunction. In contrast, the toxicity profile of RTP (right panel of Fig. 7) showed significant differences—with substantially increased risks of carcinogenicity (0.91) and genotoxicity (0.85).

The consensus prediction strategy of the VenomPred 2.0 platform, which integrates multiple machine learning models, systematically quantified the toxicity characteristics of RBV and RTP (Fig. 8). As shown in the left panel of Fig. 8, the toxicity profile of RBV was dominated by acute oral toxicity (LD_{50} = 310 mg/kg, confidence 0.85) and carcinogenicity (probability 0.72).

The right panel of Fig. 8 revealed that the toxicity risk of RTP increased by orders of magnitude: acute oral toxicity (LD_{50} = 190 mg/kg,

confidence 0.91) and carcinogenicity (probability 0.89) were significantly higher than those of RBV. This difference is primarily attributed to the triphosphate group of RTP—its strong negative charge exacerbates toxicity through two mechanisms: (1) Membrane transport escape mechanism: RTP cannot be effluxed via equilibrative nucleoside transporters (ENTs), leading to intracellular accumulation in red blood cells at concentrations up to 30 times that in plasma; (2) DNA damage cascade: The phosphate groups mimic endogenous ATP, competitively inhibiting the DNA repair enzyme PARP-1 and reducing the efficiency of single-strand break repair.

3.6. Microscopic mechanism of RBV-Induced hemolytic anemia

3.6.1. RBV speciation

In this study, the Marvin software was used to compute the possible species of RBV under different pH conditions. A total of 15 distinct forms (A–O) were identified, primarily differing in the protonation/deprotonation states of various ionizable functional groups. The results are shown in Fig. 9.

As illustrated in Fig. 9, the RBV molecule exhibits up to 15 distinct protonation/deprotonation species (A–O) across a range of pH conditions. These variations arise mainly from differences in the ionization states of multiple functional groups within the molecule, including nitrogen atoms in the triazole ring and hydroxyl groups on the ribose moiety. Notably, under physiological pH conditions (pH 7.4), the predominant form of RBV is its neutral molecule, as indicated by the red marker in Fig. 9. Detailed analysis of the structural characteristics of these 15 species confirms that at physiological pH, the RBV molecule overall exhibits an electronegative character. This key physicochemical property—clearly illustrated in Fig. 10—plays a decisive role in its interaction with the erythrocyte membrane and its subsequent accumulation and metabolic behavior within red blood cells, providing important structural and chemical insights into the molecular basis of RBV-induced hemolytic anemia.

3.6.2. Pharmacokinetic analysis of RBV

While defective metabolism of RBV in red blood cells is an important factor contributing to RBV-induced hemolysis, the process of RBV uptake into erythrocytes constitutes the molecular basis of hemolytic anemia. Therefore, we used ADMETLab 3.0 to predict the pharmacokinetic properties of RBV. The results indicated that RBV is rapidly absorbed after administration, with Caco-2 permeability and MDCK permeability values of -5.877 and -4.88 , respectively. The PAMPA assay also showed favorable permeability. The plasma protein binding (PPB) rate was only 6.7 %, suggesting that RBV exists predominantly in an unbound monomeric form in plasma. The steady-state volume of distribution (VDss) was 1.345 L/kg, the half-life in vivo was 2.134 h, and the plasma clearance rate was 4.78 mL/min/kg [64].

3.6.3. Molecular basis of RBV uptake in erythrocytes

The primary organs responsible for RBV metabolism are the liver and the kidneys, which mediate phosphorylation, dephosphorylation, degradation, reabsorption, and excretion of RBV. Among these processes, hENT1 (SLC29A1) [65] plays a critical role in the absorption, transport, and metabolism of RBV. We investigated the interaction between RBV and hENT1; molecular docking results and key interacting residues are shown in Fig. 11.

According to the molecular docking results, the transport channel of hENT1 can accommodate the molecular diameter of RBV. RBV interacts with specific residues of hENT1 with a binding energy of -6.7 kJ/mol. To further evaluate the binding, we used the Kdeep neural network model to calculate the Gibbs free energy of the RBV-hENT1 interaction as -3.5983 kcal/mol, ligand efficiency as -0.2117 kcal/mol, binding intensity as 2.6654, and pIC50 as 2.5275. The corresponding IC50 value was calculated as 2.98 mM, indicating weak affinity between RBV

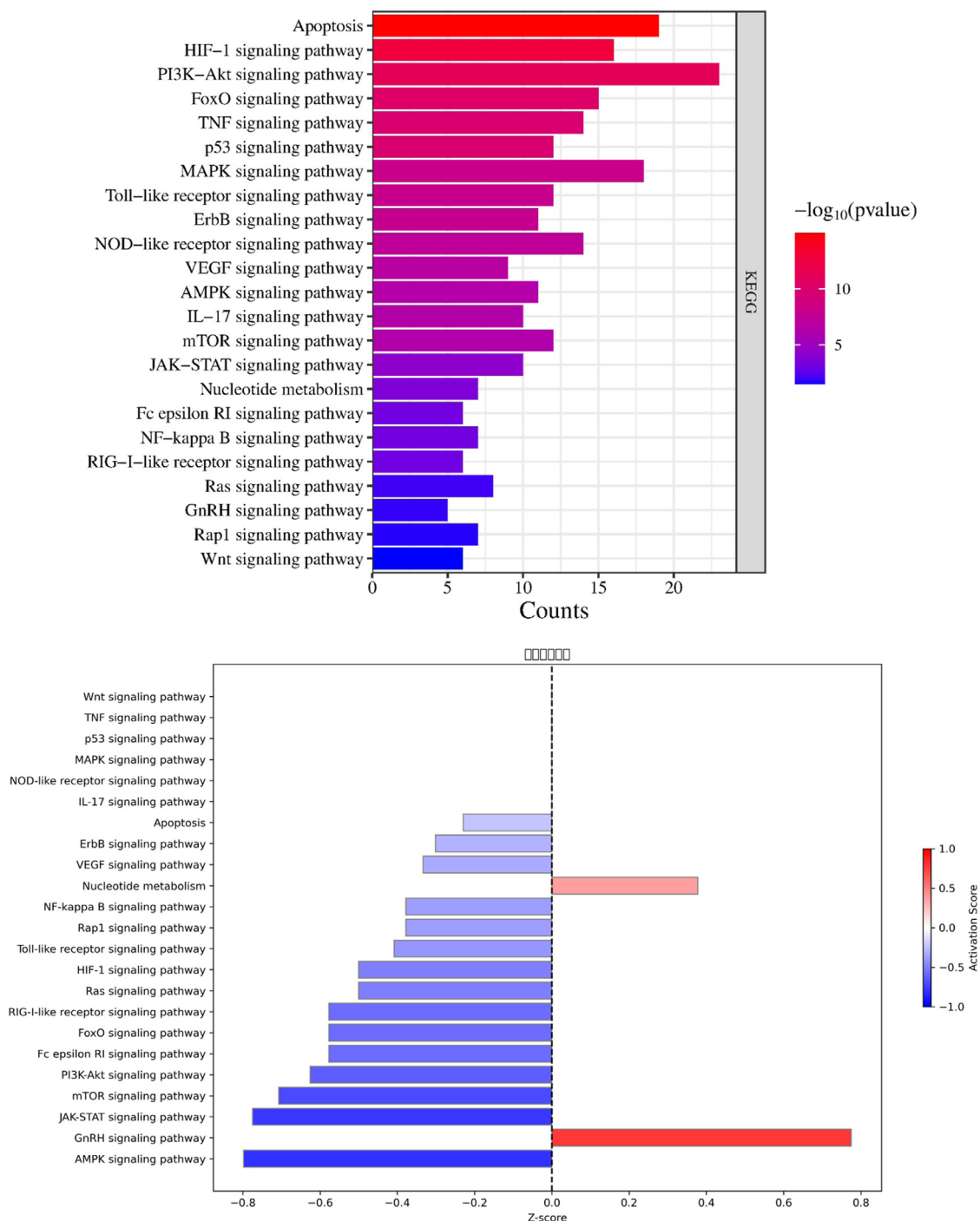


Fig. 6. KEGG enrichment analysis.

and hENT1 and inefficient binding. These results suggest that hENT1-mediated transport alone is insufficient to account for the significant accumulation of RBV in erythrocytes and its consequential severe effects.

Previous studies have proposed that Band 3 protein may be involved in RBV uptake [66]. We performed molecular docking to elucidate the interaction modes of RBV and RTP with Band 3 protein; the results are presented in Fig. 12.

As shown in Fig. 12, both RBV and RTP bind to the same pocket of Band 3 protein (pocket volume: 2338 Å³), which is distinct from the transport channel of hENT1. The binding energy of Band 3 protein with

RBV is -6.8 kJ/mol, while that with RTP is -8.1 kJ/mol. Based on these results, we speculate that RBV is phosphorylated by adenosine kinase into RTP within erythrocytes and becomes anchored to the erythrocyte membrane, thereby interfering with the normal function of Band 3 protein.

3.6.4. Molecular dynamics simulation analysis of RBV with band 3 protein

To further investigate the functional impact of RBV binding to Band 3 protein, semi-flexible molecular docking was first performed. A suitable conformation was then selected for all-atom MD simulations. The simulations were based on Newton's equations of motion, which were



Fig. 7. Toxicity prediction graph of RBV (left) and toxicity prediction graph of RTP (right).

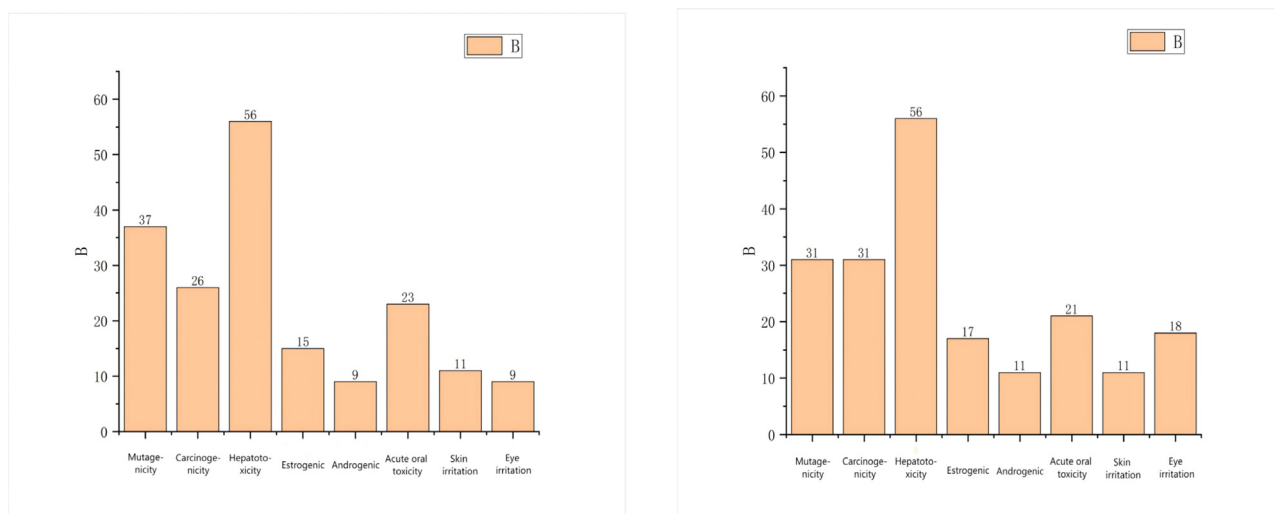


Fig. 8. Toxicity prediction diagram of RBV (left) and toxicity prediction diagram of RTP (right).

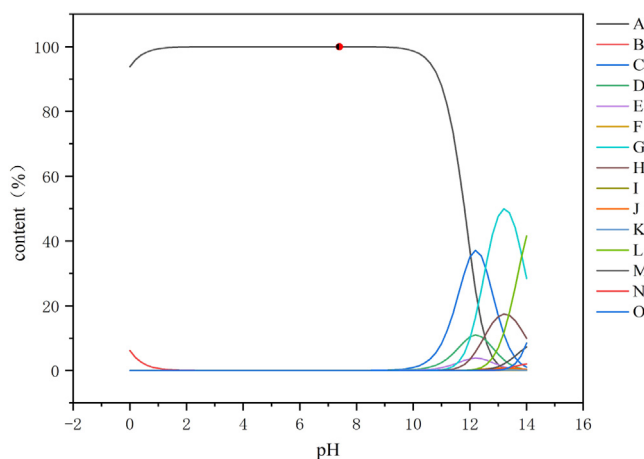


Fig. 9. The existence forms of ribavirin at different pH values.

numerically integrated to sample the phase space defined by atomic coordinates and velocities. The future positions and velocities of atoms were computed from their current states. Statistical analysis of particle motions allowed the prediction of possible conformations, thermody-

amic properties, dynamic behavior of molecules, solvation effects, and various equilibrium properties of the system. The results of the MD simulations for the RBV-Band 3 protein complex are presented in Fig. 13.

As shown in Fig. 13, the RMSD values of the RBV-Band 3 protein complex fluctuated within the range of 0.0–0.5 nm, with no sustained increasing trend. The low RMSD values indicate that the overall conformation of the Band 3 protein–RBV complex remained stable during the simulation, without significant structural drift. An initial adjustment phase (0–20000 ps) was observed, possibly reflecting minor conformational relaxation, after which the system reached equilibrium. The radius of gyration (R_g) values (Y1–Y4) showed no notable fluctuations over the 100 ns time scale, suggesting that the protein maintained a compact structure without noticeable unfolding or collapse, further supporting the structural stability of the complex. Combined with the RMSD results, it can be inferred that RBV binding did not induce global conformational disorder in the protein.

The number of hydrogen bonds remained relatively stable over time, with no consistent decrease. This stability suggests that persistent polar interactions were formed at the binding interface between RBV and Band 3 protein. Analysis of the RMSF revealed that regions with low fluctuations ($\text{RMSF} < 0.2 \text{ nm}$) likely correspond to trans-membrane helices of Band 3 protein or residues near the RBV-binding pocket, indicating increased rigidity due to ligand binding. Regions with high fluctuations ($\text{RMSF} > 0.5 \text{ nm}$) are likely located in flexible

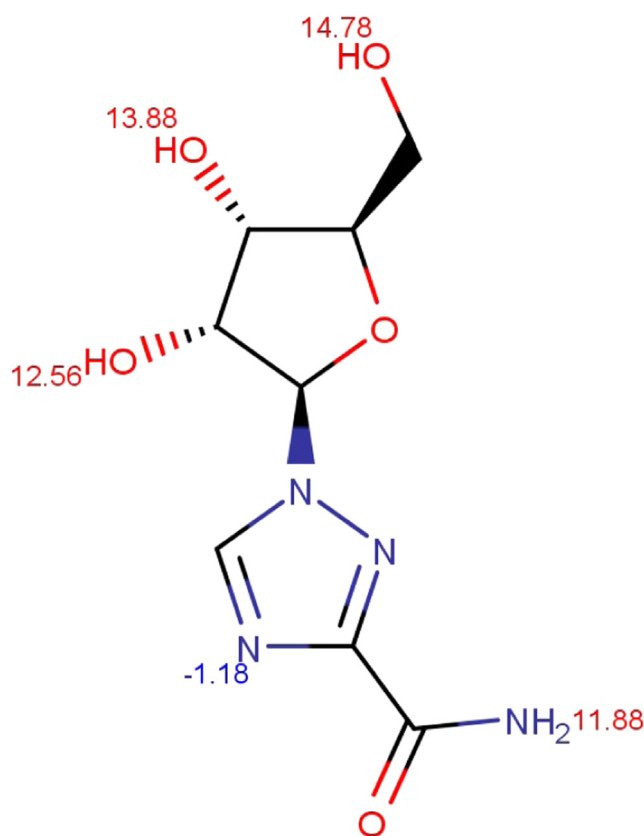


Fig. 10. The charged state of ribavirin in the physiological condition.

loops or domains not involved in binding, reflecting natural dynamic behavior.

From a functional perspective, if the low-fluctuation regions overlap with the anion transport active site of Band 3 protein, RBV may inhibit its physiological activity by occupying functional domains. Moreover, the SASA values of the RBV–Band 3 protein complex fluctuated between 155 and 180 nm², with no sharp changes within the 10 ns timeframe, indicating that the complex surface did not undergo major conformational rearrangements and that RBV binding did not lead to large-scale exposure of the hydrophobic core.

In summary, the MD simulations collectively demonstrate that the RBV–Band 3 protein complex reached equilibrium during the extended

Table 2
Results of molecular docking between RBV and RTP and G6PD.

Ligand	Pocket	Binding energy	Cavity volume	Center coordinate
RBV	C2	−8.0	5383	37, −7, −3
RBV	C5	−6.2	2403	−4, 26, 31
RTP	C5	−7.9	2403	−4, 26, 31
RTP	C4	−7.7	2607	12, 10, 32

Table 3
Predictive results of kdeep for RBV and RTP in relation to G6PD.

Ligand	ΔG (kcal/mol)	Lig. Efficiency (kcal/mol)	pIC50	pK _d
RBV	−3.5983	−0.2117	2.5275	2.6654
(std)	(−0.8596)	(−0.0506)	(0.7527)	(0.6368)
RTP	−3.3035	−0.1139	2.7838	2.447
(std)	(−0.5457)	(−0.0188)	(0.5198)	(0.4042)

simulation time, and that RBV forms a stable complex with Band 3 protein.

3.7. Mesoscopic mechanism of erythrocyte damage

Glucose-6-phosphate dehydrogenase (G6PD) is a key enzyme in the pentose phosphate pathway of erythrocytes, responsible for converting glucose-6-phosphate to 6-phosphogluconate [67]. This reaction simultaneously generates reduced nicotinamide adenine dinucleotide phosphate (NADPH), a potent antioxidant that helps maintain the reduced state of glutathione (GSH) within red blood cells, neutralizing reactive oxygen species and protecting erythrocyte membranes and hemoglobin from oxidative damage [68]. Some studies suggest that RBV may interfere with G6PD enzyme activity, reducing NADPH production, which in turn depletes GSH and disrupts the cellular oxidative stress balance. This leads to the accumulation of reactive oxygen species (ROS), resulting in lipid peroxidation and damage to the cell membrane [69]. In this study, molecular docking and MD simulations were employed to investigate the binding mode and interaction stability of RBV and RTP with G6PD. The molecular docking results are summarized in Table 2.

Through kdeep, calculations and simulations were conducted on RBV and RTP in combination with G6PD. The results are shown in Table 3.

Based on the molecular docking results, RBV exhibited the strongest binding energy at the C2 pocket, which also features a relatively large cavity volume, indicating more stable binding to G6PD. The strong binding of RBV at this site may directly interfere with the enzyme's catalytic activity, thereby inhibiting NADPH production. Although the binding energies of RTP at the C5 and C4 pockets were slightly lower than that of

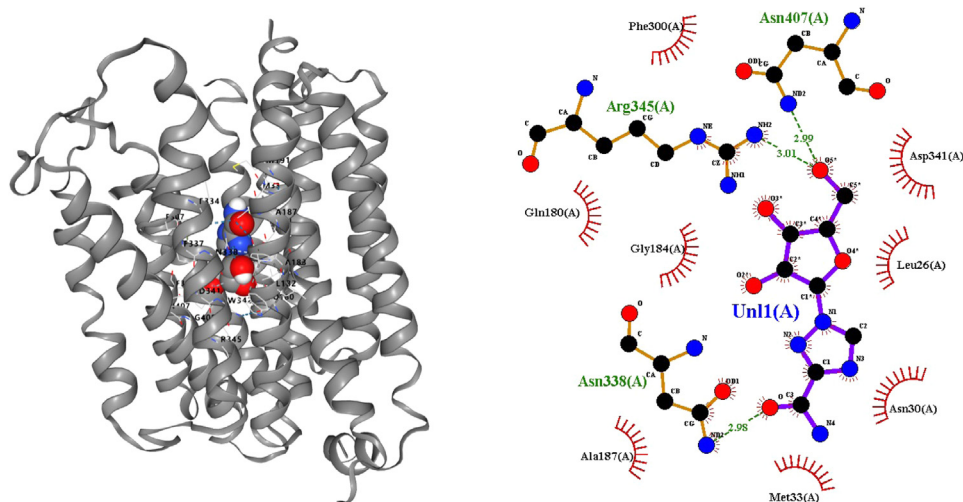


Fig. 11. The HENT1 transport mode of RBV (left) and the interacting residues (right). Unit: Å, 1 Å = 10^{−10} m.

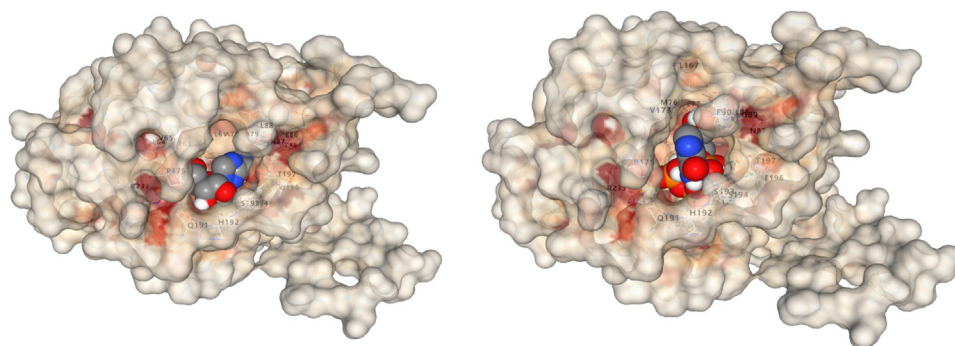


Fig. 12. Binding patterns of Band 3 protein with RBV (left) and RTP (right).

RBV at C2, the C5 and C4 sites are potentially located near the allosteric regulatory region or the substrate-binding channel of the enzyme. The more favorable ΔG of RBV suggests greater overall binding stability. The higher pIC_{50} value of RBV implies a lower half-maximal inhibitory concentration (IC_{50}), reflecting stronger inhibitory potency under physiological conditions. This may be attributed to the phosphate groups of RTP enhancing interactions within the enzyme's active site. RBV also showed higher ligand efficiency, indicating effective binding with a smaller molecular weight. As the phosphorylated metabolite of RBV, RTP binding at C5/C4 may indirectly inhibit G6PD through allosteric effects or by obstructing the substrate channel. Despite its slightly lower binding energy, the higher pIC_{50} of RTP suggests more efficient enzyme inhibition, which could significantly reduce NADPH levels and exacerbate oxidative stress. Therefore, all-atom molecular dynamics simulations were performed for the RBV-G6PD complex, with the results shown in Fig. 14.

As shown in Fig. 14, the RMSD values of the RBV-G6PD complex fluctuated between 0.00–0.40 nm, eventually stabilizing within the range of 0.20–0.30 nm. The low RMSD values indicate that the overall conformation of the G6PD-RBV complex remained stable during the simulation without significant structural drift. The radius of gyration (R_g) provided supplementary validation, showing no significant changes and being consistent with the RMSD results, further supporting the global structural compactness of the protein. In the RMSF analysis, high fluctuation regions ($RMSF > 0.5$ nm) were concentrated at residue numbers 50–100 and 250–300, possibly corresponding to flexible loop regions or terminal regions of G6PD, representing normal dynamic behavior. Low fluctuation regions ($RMSF < 0.2$ nm) were located at residues 150–200 and 320–350, likely representing the catalytic domain or RBV-binding pocket of G6PD, indicating that these regions gained rigidity due to ligand binding. If the low fluctuation regions overlap with G6PD's active center, RBV may directly inhibit enzyme activity by occupying key sites. Hydrogen bond analysis showed stable hydrogen bond numbers, supporting the formation of persistent interactions between RBV and active site residues. Meanwhile, the SASA values fluctuated between 170–195 nm² with no significant trend observed during the 0–60 ns timeframe. The stable SASA values indicate that no large-scale conformational adjustments occurred at the complex surface, and RBV binding did not lead to exposure of the protein's hydrophobic core or structural loosening.

3.8. Erythrocyte hemolysis assay

Hemolysis assays were performed using anticoagulated whole blood from healthy adult donors purchased from Henan Huanqiu Biotechnology Co., Ltd. The results are shown in Fig. 15.

Based on the results shown in Fig. 15, the dose- and time-dependent characteristics of RBV-induced hemolytic anemia were systematically validated using an in vitro erythrocyte suspension model (Fig. 15A). As the RBV concentration gradient increased (0–10 mg/mL), the erythrocyte hemolysis rate exhibited a significant positive correlation

($p < 0.01$). When the RBV concentration reached 8 mg/mL, the hemolysis rate surged to $68.5 \pm 3.2\%$, and in the 10 mg/mL group, it reached $82.1 \pm 4.7\%$, clearly confirming a dose-dependent membrane damage effect. Further time-course analysis (Fig. 15B) revealed the kinetic characteristics of hemolysis development. Under 8 mg/mL RBV exposure, the hemolysis rate increased gradually during the initial stage (0–2 h, $< 15\%$), suggesting the presence of compensatory repair mechanisms in erythrocyte membranes; however, during the 4–6 h period, the hemolysis rate rose sharply to $54.3 \pm 2.8\%$, reaching a plateau of $76.5 \pm 3.5\%$ at 8 h.

Concurrently, the detection of MDA, GSH, and CAT elucidated the molecular mechanism of RBV-induced erythrocyte hemolysis from the perspective of oxidative damage, providing critical experimental evidence for evaluating drug toxicity, optimizing therapeutic regimens, and developing protective strategies. As shown in Fig. 15C, the MDA content in erythrocytes of the RBV-treated group (8 mg/mL, 6 h) increased sharply to (12.7 ± 0.8) nmol/mg prot, a six-fold increase compared to the control group ((2.1 ± 0.3) nmol/mg prot, $p < 0.001$), indicating pathological levels of membrane lipid peroxidation damage. A collapse of the key antioxidant defense system was fully demonstrated in Fig. 15D. After RBV exposure, the intracellular GSH level decreased drastically from a baseline of (25.3 ± 1.5) μ mol/g Hb to 6.2 ± 0.9 μ mol/g Hb ($p < 0.001$), a reduction of 75.5%. Fig. 15E systematically revealed the cascade amplification mechanism of oxidative stress in erythrocytes under RBV exposure by quantitatively monitoring the dynamic changes in CAT activity. After 6 h of treatment with 8 mg/mL RBV, erythrocyte CAT activity increased significantly to (48.7 ± 3.5) U/mg prot, an increase of 118.4% compared to the control group ((22.3 ± 2.1) U/mg prot, $p < 0.001$). This abnormal upregulation in activity does not represent an enhancement of antioxidant defense but is rather a direct biomarker of intracellular hydrogen peroxide (H_2O_2) accumulation.

3.9. LC-MS/MS quantitative analysis

To directly verify the key hypothesis that RBV is metabolically converted to RTP and accumulates intracellularly in erythrocytes, this study employed high-resolution LC-MS/MS to accurately quantify RBV and its active metabolite RTP in erythrocyte lysates.

Mass spectrometry identification results are shown in Fig. 16. In high-resolution mass spectrometry, the protonated molecular ion ($[M+H]^+$) of RBV exhibited an accurate mass-to-charge ratio (m/z) of 245.0915 (Fig. 16A), whereas the $[M+H]^+$ ion of RTP showed an m/z of 585.0418 (Fig. 16B). The measured accurate mass values deviated from the theoretical values by < 1.0 ppm, providing decisive evidence for compound identification. The $[M+H]^+$ ion of RBV further fragmented to yield major characteristic fragment ions at m/z 113.0 (glycosidic bond cleavage product) and 96.0 (base fragment), which provided secondary mass spectrometric evidence for the qualitative confirmation of RBV. In the mass spectrum of RTP, in addition to the $[M+H]^+$ base peak, its

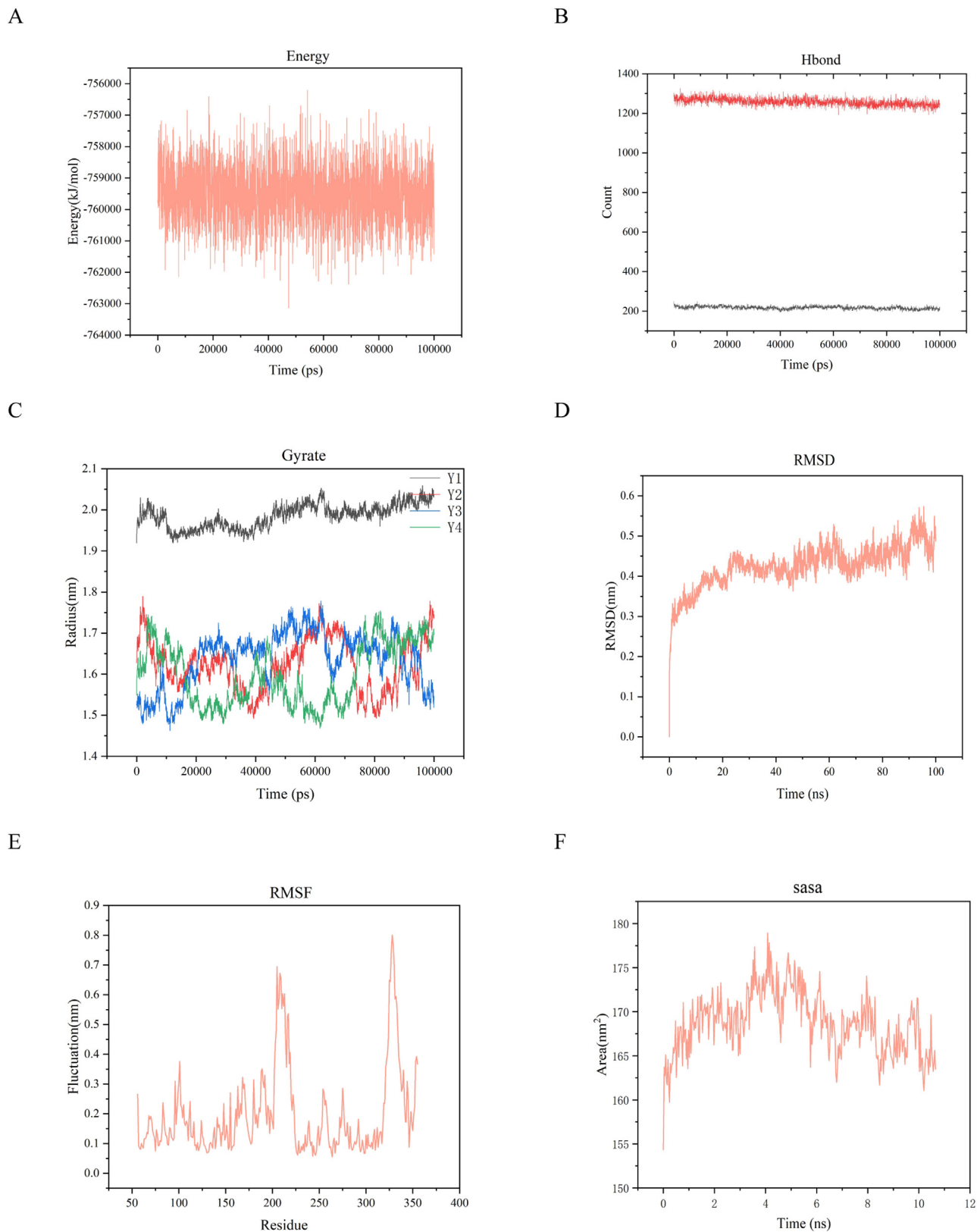
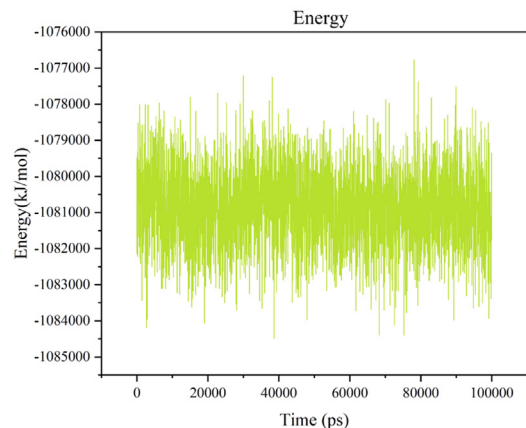
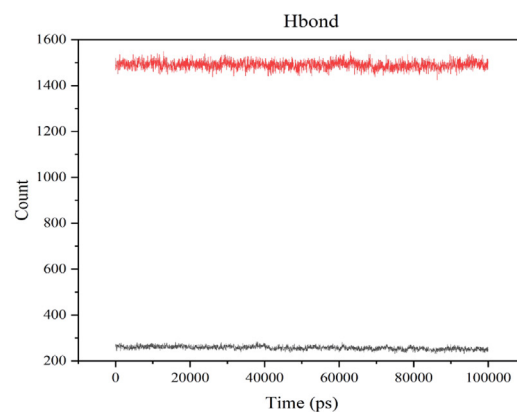


Fig. 13. MD simulation results of RBV and Band 3 protein. (A) Energy analysis of RBV and Band 3 protein; (B) Analysis of hydrogen bond dynamic changes of RBV and Band 3 protein; (C) Analysis of the evolution behavior of the radius of gyration of RBV and Band 3 protein; (D) RMSD analysis of RBV and Band 3 protein; (E) RMSF analysis of RBV and Band 3 protein; (F) Evolution behavior analysis of solvent accessible surface area (SASA) of RBV and Band 3 protein.

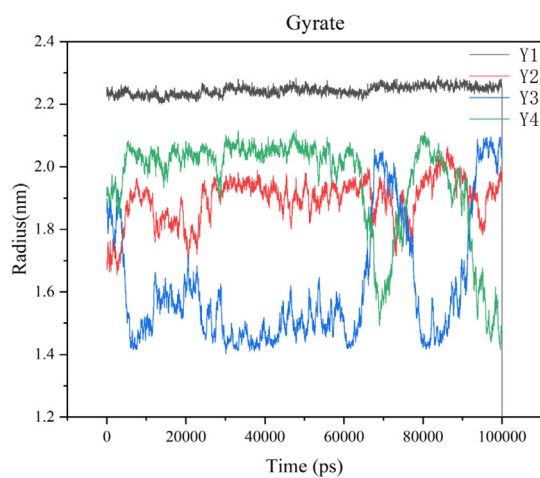
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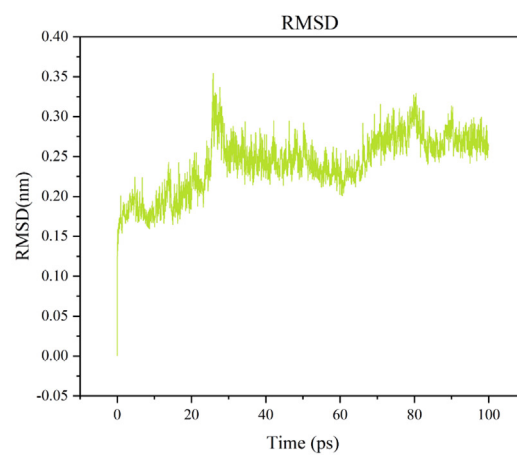
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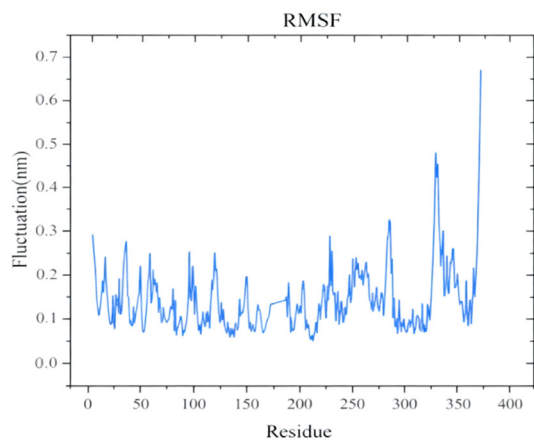
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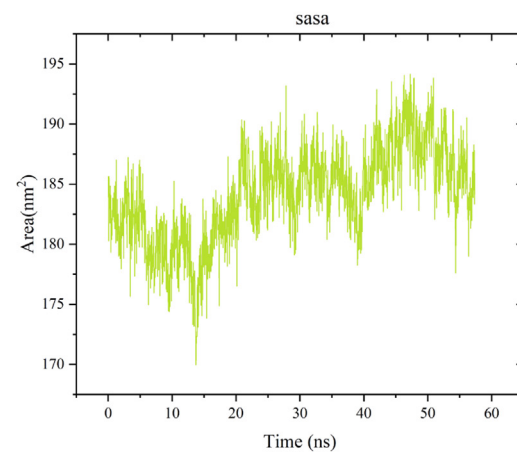


Fig. 14. MD simulation results of RBV and G6PD. (A) Energy analysis of RBV and G6PD; (B) Analysis of hydrogen bond dynamic changes of RBV and G6PD; (C) Analysis of the evolution behavior of the cyclization radius of RBV and G6PD; (D) RMSD analysis of RBV and G6PD; (E) RMSF analysis of RBV and G6PD; (F) Evolution behavior analysis of solvent accessible surface area (SASA) of RBV and G6PD.

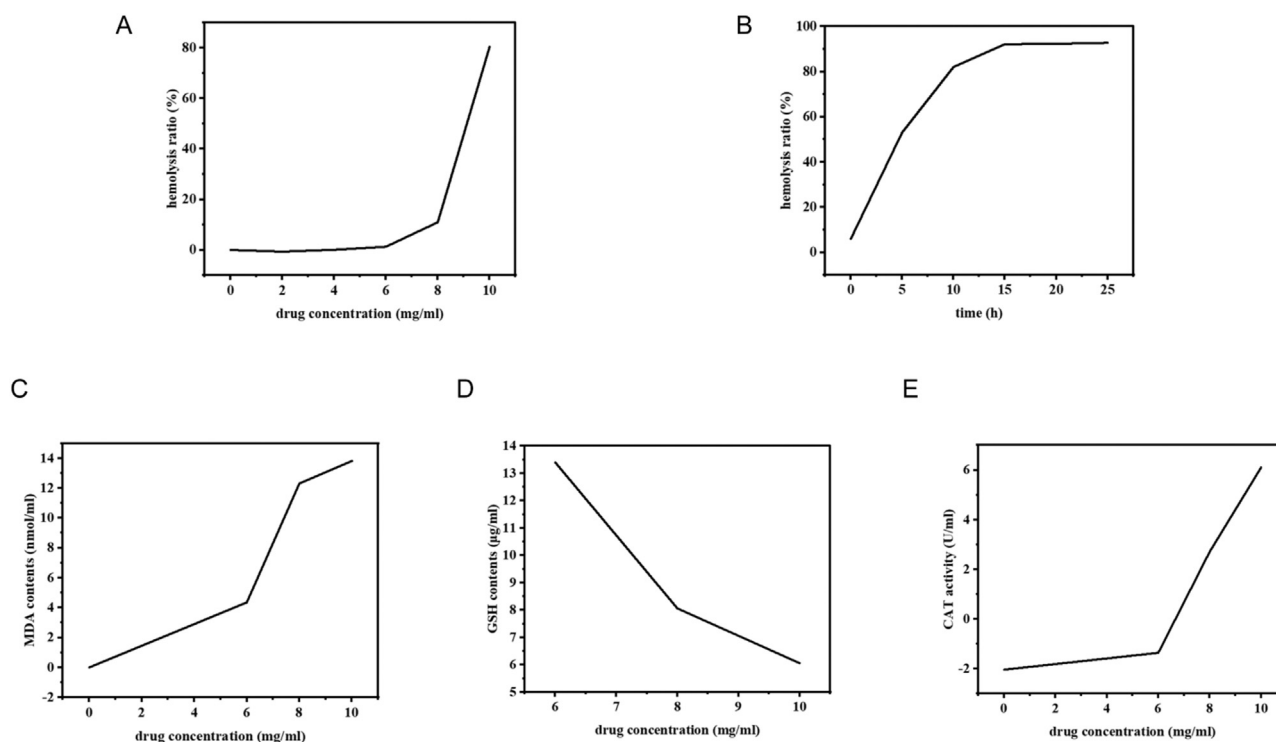


Fig. 15. Results of RBV hemolysis test. (A) Results of RBV concentration-dependent hemolysis; (B) Results of RBV time-dependent hemolytic anemia; (C) Effect of RBV on the lipid peroxidation level of red blood cells; (D) Effect of RBV on the antioxidant capacity (GSH) of red blood cells; (E) Effect of RBV on the CAT activity of red blood cells.

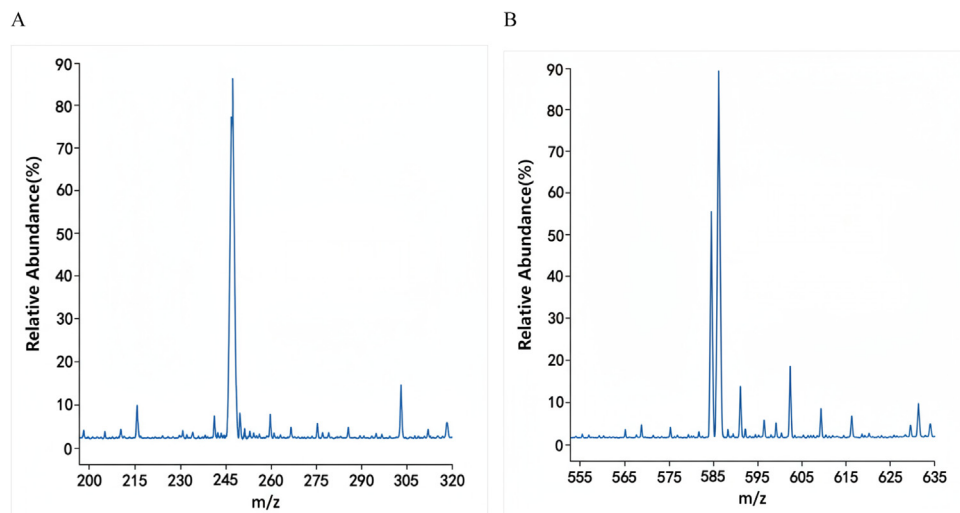


Fig. 16. LC-MS/MS quantitative analysis of high-resolution mass spectra. A: high-resolution mass spectrum of RBV. The figure shows the protonated molecular ion peak $[M+H]^+$ of RBV at m/z 245.0915. The data were directly injected through the injection pump and collected using the Q-TOF high-resolution mass spectrometer in the electrospray positive ion (ESI^+) mode. B: High-resolution mass spectrum of RTP. The figure clearly displays its protonated molecular ion peak $[M+H]^+$ at m/z 585.0418. The data were collected using the Q-TOF high-resolution mass spectrometer in the electrospray positive ion (ESI^+) mode.

characteristic isotopic distribution pattern was also observed, further confirming the elemental composition of the molecule.

Validation of the quantitative analytical methodology demonstrated that the established LC-MS/MS method exhibited excellent specificity, linear range, and sensitivity. Both RBV and RTP showed good linearity over the concentration range of 1–500 ng/mL ($R^2 > 0.999$). The method displayed high sensitivity, with the limit of detection (LOD) and limit of quantification (LOQ) for RBV reaching 0.3 ng/mL and 1.0 ng/mL, respectively, while the LOD and LOQ for RTP were 0.8 ng/mL and 2.5 ng/mL, respectively. Intra-day and inter-day precision (RSD) were better than 10%, and accuracy (RE) was within $\pm 8\%$, fully meeting the requirements for trace-level quantification in biological samples.

Analysis of intracellular drug concentrations revealed that after incubation with different concentrations of RBV (2–8 mg/mL), significant

levels of both the parent drug RBV and its metabolite RTP were detected within erythrocytes. Notably, after 6 h of incubation with 8 mg/mL RBV, the intracellular concentration of RTP was approximately 2.8 times that of the parent RBV. This result directly confirms that RTP is the primary form of drug accumulation within red blood cells. Drug concentrations showed clear dose- and time-dependent increases, and the accumulated intracellular concentration of RTP exhibited a highly significant positive correlation ($p < 0.001$) with the observed hemolysis rate and oxidative stress indicators (MDA, GSH).

In summary, LC-MS/MS quantitative analysis provided the most direct and conclusive experimental evidence for the core hypothesis that “RBV is phosphorylated to RTP and undergoes irreversible accumulation within erythrocytes”. This firmly links the pharmacokinetic profile of the drug to its cytotoxic phenotype, thereby completing the molecular mechanistic pathway of RBV-induced hemolytic anemia.

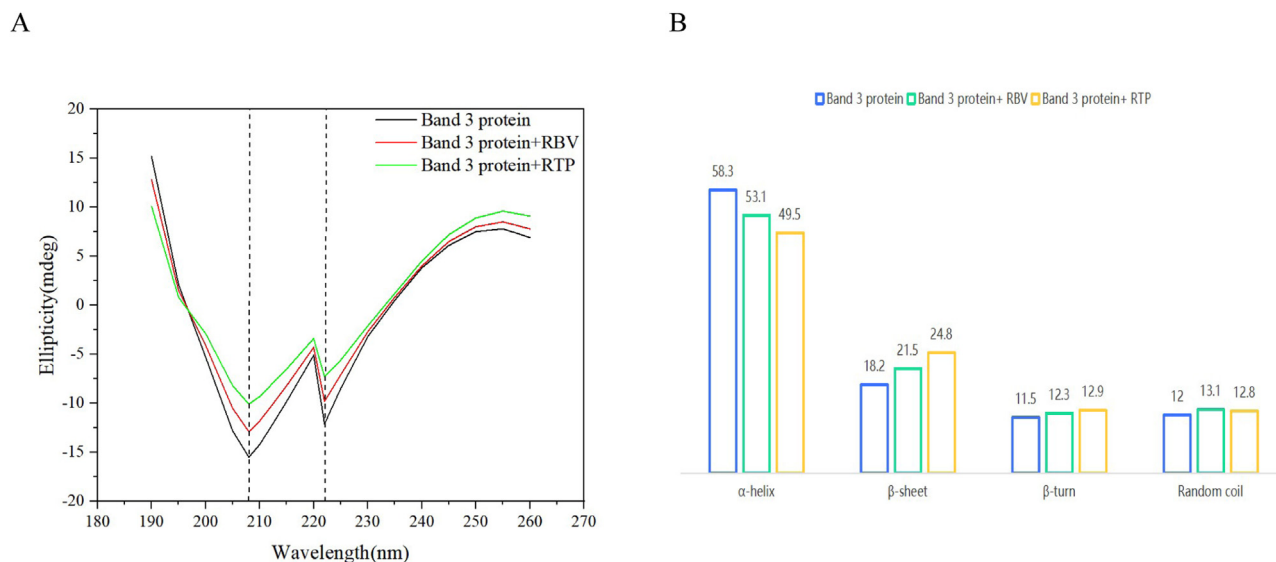


Fig. 17. Experimental results of protein conformation analysis using CD spectroscopy. (A) Overlay of the original CD spectra (qualitatively showing the occurrence of conformational changes, vertical dotted lines indicate the characteristic wavelengths of 208 nm and 222 nm, which are the indicators of the α -helix structure); (B) Effects of RBV and RTP on the secondary structure of Band 3 protein (unit: %).

3.10. Experimental validation of protein conformation by circular dichroism spectroscopy

To validate the effects of RBV and its active metabolite on the conformation of Band 3 protein, circular dichroism (CD) spectroscopy was employed to quantitatively analyze changes in protein secondary structure. The results are shown in Fig. 17.

As depicted in Fig. 17A, the far-UV CD spectrum of native Band 3 protein exhibited characteristic double negative peaks at 208 nm and 222 nm, indicative of a high α -helical content. In comparison with the native protein, the CD spectra of the protein treated with RBV and RTP showed noticeable shifts. Specifically, the absolute values of negative ellipticity at both 208 nm and 222 nm displayed a systematic decrease. However, the reduction in ellipticity was significantly greater in the RTP-treated group than in the RBV-treated group, indicating a more pronounced impact of RTP on the protein's secondary structure.

Deconvolution of the CD spectra allowed quantitative analysis of the changes in secondary structure components (Fig. 17B). The results revealed that, compared to the native protein, RBV binding led to a significant decrease in α -helical content, accompanied by a corresponding increase in β -sheet and random coil structures. RTP binding induced even more dramatic structural alterations, with a further reduction in α -helical content. These results clearly demonstrate that the binding of RBV/RTP to Band 3 protein indeed induces unfolding of its secondary structure, promoting a transition from ordered α -helices to more disordered β -sheet and random coil conformations.

These experimental findings are highly consistent with the MD simulation results, forming a robust computational–experimental evidence chain. MD simulations showed a significant decrease in the root mean square fluctuation (RMSF) of the transmembrane domain of Band 3 protein after RBV/RTP binding. The observed reduction in α -helical content in the CD experiments provides a structural basis for this conformational rigidification: to accommodate ligand binding, local helical structures may undergo unwinding or distortion, resulting in an overall loss of helicity. This “rigidified” conformation may underlie the impaired anion-exchange function of the protein. The more substantial conformational change induced by RTP is also consistent with its stronger binding free energy and more notable RMSF alterations observed in MD simulations. Together, these data confirm at both atomic and molecular levels that RTP is the key toxic molecule responsible for Band 3 protein dysfunction.

4. Results and discussion

This study systematically deciphered the molecular mechanism of ribavirin (RBV)-induced hemolytic anemia by integrating network toxicology (NT), multi-scale molecular simulations, and experimental validation. We innovatively adopted a cross-scale modeling and verification strategy, seamlessly connecting computational predictions with experimental data. The results not only confirm the hypothesis that RBV and its active metabolite RTP cause erythrocyte damage through synergistic perturbations of multiple targets and pathways but also, more importantly, provide a quantitative characterization framework—from atomic-scale interactions to macroscopic phenotypes—from the perspective of analytical toxicology. This work fills a critical gap in the integration of multi-scale data and quantitative verification within the toxicity mechanism of nucleoside analogs. MD simulations accurately quantified the binding free energy and conformational dynamics parameters of RBV/RTP with target proteins (Band 3 protein, G6PD). These computational results provide a solid foundation for mechanistic interpretation at the atomic level. Meanwhile, the prediction of binding modes using molecular docking is widely recognized for its reliability in nucleoside analog research. The multi-target interaction network constructed by NT revealed a synergistic perturbation pattern involving “oxidative stress–apoptosis–inflammatory response” at the systems level. This “atom-to-system” multi-scale research paradigm represents the core approach of modern analytical toxicology for resolving complex toxicity mechanisms.

The LC-MS/MS method used in this study provided conclusive quantitative evidence supporting the hypothesis of “RTP accumulation within erythrocytes.” The method demonstrated high sensitivity, with limits of detection (LOD) for RBV and RTP reaching 0.3 ng/mL and 0.8 ng/mL, respectively, and limits of quantification (LOQ) of 1.0 ng/mL and 2.5 ng/mL, fully meeting the requirements for trace-level quantification in biological samples. MD simulations predicted that RBV/RTP binding induces conformational rigidification of Band 3 protein, a prediction experimentally verified by circular dichroism (CD) spectroscopy. CD analysis showed that compared to the native protein, RBV treatment significantly decreased the α -helical content of Band 3 protein, while RTP treatment induced even more pronounced changes. The weakening of the characteristic negative peaks at 208 nm and 222 nm visually reflected this alteration. The CD experimental results highly align with the reduced RMSF values observed in the transmembrane helical regions in

MD simulations: the conformational rigidification indicated by computational simulations manifested experimentally as a transition from ordered α -helical structures to looser β -sheet and random coil formations. This conformational change is likely the direct structural cause of the impaired anion exchange function of Band 3 protein, thereby mediating the decrease in membrane stability.

The results support a “three-stage injury model”: RBV enters erythrocytes via hENT1, is phosphorylated to RTP and irreversibly accumulates, binds to Band 3 protein interfering with its function, while simultaneously inhibiting G6PD activity, depleting NADPH and GSH, leading to ROS accumulation and triggering lipid peroxidation; oxidative damage combined with death receptor pathway activation (e.g., by TNF) activates CASP3, ultimately causing erythrocyte apoptosis or rupture. This mechanism elucidates the dual dose- and time-dependence of RBV hemolytic toxicity and provides precise targets for clinical monitoring and intervention. For example, in patients with G6PD deficiency (favism), whose inherent NADPH production capacity is already insufficient, RBV administration would exacerbate oxidative stress, posing a very high risk of hemolysis. This study suggests that monitoring intracellular RTP concentration or oxidative stress indicators (e.g., GSH/MDA ratio) in patient erythrocytes could become an effective strategy for predicting and preventing hemolytic anemia.

5. Study limitations and future directions

This study has certain limitations. First, although the timescale of the MD simulations was sufficient to capture key conformational changes, longer-scale simulations may reveal rarer conformational events. Second, while the study primarily focused on the direct damage mechanisms in red blood cells, the inhibitory effect of RBV on the differentiation of hematopoietic progenitor cells in the bone marrow also warrants further investigation, which would require integration with techniques such as single-cell sequencing.

In summary, by innovatively integrating multi-scale computational and experimental approaches, this study not only systematically elucidates the molecular mechanism of RBV-induced hemolytic anemia but, more importantly, provides a reusable research paradigm for the quantitative characterization and validation of drug toxicity mechanisms within the field of analytical toxicology. In the future, this paradigm can be extended to the safety evaluation of other nucleoside analogs or drugs with complex toxicity mechanisms.

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The authors declare that they have no competing interests.

CRedit authorship contribution statement

Xumeng Shi: Data curation, Conceptualization. **Shijia Lu:** Formal analysis. **Guangman Zhu:** Investigation, Funding acquisition. **Tianjiao Cui:** Software, Resources. **Shunhang Wu:** Validation. **Mengdan Sang:** Writing – original draft. **Yiran Zhen:** Funding acquisition. **Xue Yang:** Writing – original draft. **Huaying Du:** Writing – original draft. **Miaoyan Han:** Writing – original draft. **Mengjia Yan:** Visualization. **Jinle Wang:** Writing – original draft. **Yutong Zhang:** Writing – original draft. **Jiying Du:** Writing – review & editing, Conceptualization.

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