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The Synergistic Inhibition of IRF4/NF-κB/STAT3 Pathway by Lenalidomide Combined with BTK Inhibitor Enhances the Effect of Rituximab on Apoptosis in ABC Type Diffuse Large B-cell Lymphoma

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[#]Ting Wei and Xiaoting Liao are equal contribution to this study.

Abstract: AIM: Activated B-cell like diffuse large B-cell lymphoma (ABC-DLBCL) has poor response to standard rituximab combined chemotherapy regimen and poor clinical prognosis. The aim of this study is to investigate the in vitro experimental basis for the clinical application of the three-drug combination (Zanubrutinib, Lenalidomide and Rituximab), named as ZR2 regimen by enhancing the apoptotic effect and molecular mechanism of rituximab against ABC type diffuse large B-cell lymphoma. **Method:** The ABC-DLBCL cell line NU-DUL-1 was used as a cell model in vitro. (1)A culture system consisting of Zanubrutinib, Lenalidomide, and tumor cells was established. Cell proliferation was detected and subsequent experimental concentrations were determined using the CCK-8 method to determine the concentrations and coordinated effects of the two drugs; (2) The culture system of drugs including Zanubrutinib, Lenalidomide and Rituximab, peripheral blood mononuclear cells, serum, and tumor cells .This experiment was set up with blank control group, Zanubrutinib ,Lenalidomide or rituximab monotherapy group, Zanubrutinib combined with lenalidomide two drug group, and triple drug combination group. Flow cytometry was used to detect cell apoptosis, and Western blot was used to detect changes in the IRF4/NF-κB/STAT3 pathway and apoptosis related protein expression. **Results:** The combination of Zanubrutinib and Lenalidomide can synergistically enhance the anti-tumor effect of rituximab on ABC-DLBCL cells. The combination of three drugs can increase the level of apoptosis, and the overall effect showed a trend of three drug combination being better than two drug combination of Zanubrutinib and Lenalidomide ($p<0.05$) and rituximab monotherapy ($p<0.01$), respectively. Simultaneous combination therapy can downregulated the expression of IRF4 and inhibited the phosphorylation activation of NF-κB and STAT3, upregulated the expression of Cleaved caspase-3, and reduced the anti-apoptotic ability of tumor cells. **Conclusions:** Zanubrutinib combined with Lenalidomide can enhance the in vitro anti-tumor effect of rituximab (ZR2 regimen) on ABC-DLBCL cells, and its mechanism may be related to synergistic inhibition of IRF4/NF-κB/STAT3 resistance signaling axis activation.

Keywords: Diffuse Large B-cell Lymphoma; Rituximab; Zanubrutinib; Lenalidomide; IRF4/NF-κB/STAT3 Signaling Pathway.

1. Introduction

Diffuse Large B-cell Lymphoma (DLBCL) is one of the most common malignant tumors in the hematopoietic system, belonging to invasive non-Hodgkin lymphoma (NHL), accounting for about 30% -40% of all NHL[1, 2], with significant biological heterogeneity. According to the characteristics of gene expression profiles, DLBCL can be divided into three major molecular subtypes: activated B-cell like (ABC), germinal center B-cell like (GCB), and unclassified[3-5].

At present, the first-line R-CHOP standard regimen consisting of anti-CD20 monoclonal antibody, Rituximab (RTX), combined with CHOP regimen (cyclophosphamide, doxorubicin, vincristine, prednisone)[4, 6, 7] can cure about 60% -70% of patients, and about 30% -40% of patients still experience disease recurrence or progression[5]. Among them, ABC subtype is a high-risk subtype with drug resistance and poor prognosis [6, 8], which is also the core reason for this study to focus on exploring the mechanism of this subtype.

Previous researches have confirmed that downregulation of CD20 expression and depletion of target internalization are considered important mechanisms for treatment failure [9-11].In addition to target related factors, the failure of ABC subtype DLBCL against CD20 therapy also involves various mechanisms such as abnormal signaling pathways within tumor cells and weakened immune response[12]. ABC subtype DLBCL commonly exhibits sustained activation of the B-cell receptor (BCR) signaling pathway, as well as abnormal activation of key downstream pathways such as nuclear factor kappa B (NF-κB), signal transduction and transcription activator 3 (STAT3) [2, 13], and overexpression of IRF4 [10], which can upregulate the expression of anti apoptotic genes and drive sustained malignant proliferation of tumor cells. The sustained abnormal activation of the NF-κB pathway is a critical carcinogenic event, defined as the "survival addiction" mechanism of tumor cells, and is a key pathway driving tumor malignant proliferation, apoptosis resistance, and treatment resistance[14, 15]. Gene expression profiling analysis showed that the basal expression levels of NF-κB target genes in ABC-DLBCL were significantly higher than those in GCB subtypes, accompanied by sustained phosphorylation degradation of IκBα and nuclear accumulation of p65 heterodimers[16]. Functional studies

have further confirmed that blocking NF-κB signaling through Ik B ultrasound inhibitors or small molecule inhibitors can specifically induce apoptosis in ABC-DLBCL cells, with little effect on GCB subtypes, suggesting that abnormally activated NF-kB is a key dependency for the survival of ABC subtype tumor cells [14]. Currently, it is widely believed that abnormal activation of the NF-κ B pathway is an important molecular basis for the failure and recurrence/resistance of R-CHOP immunotherapy[17]. Therefore, targeting BTK has become a key therapeutic strategy for inhibiting NF-κB abnormal activation in ABC-DLBCL.

IRF4 is a member of the interferon regulatory factor family and an important regulatory factor in normal immune responses, including germinal center development, lymphocyte activation, and plasma cell differentiation. The increased expression of IRF4 in ABC DLBCL cell line is associated with its unique molecular phenotype and is considered a part of the malignant features of this subtype [18, 19]. STAT3 is a core member of the STAT protein family, which can be activated by various cytokines such as IL-6 and IL-10. After phosphorylation, it enters the nucleus to regulate the transcription of target genes and widely participates in biological processes such as cell proliferation and apoptosis. It is a key downstream effector molecule of the IRF4/NF-κB/STAT3 signaling axis [20]. Clinical studies have shown that abnormal activation of STAT3 is closely related to poor prognosis in DLBCL patients receiving R-CHOP treatment, with more prominent prognostic value in the ABC subtype [21, 22]. Previous research has confirmed that the abnormal activation of STAT3 is closely related to the decreased sensitivity of DLBCL to rituximab and BTK inhibitors [23, 24].

Current research has found that sustained abnormal activation of the IRF4/NF-κB/STAT3 signaling axis is considered one of the core molecular features of ABC-DLBCL, and is closely related to poor response and resistance to rituximab based treatment regimens in this subtype [24-26]. Clinical and basic research has shown that abnormal activation of this signaling axis is closely related to the adverse reactions of ABC DLBCL to R-CHOP treatment [24]. In summary, the IRF4/NF-κB/STAT3 signaling axis is the core molecular mechanism underlying the pathogenesis of ABC DLBCL, providing a theoretical basis for subsequent research.

Previous researches have confirmed that lenalidomide can downregulate IRF4 expression by degrading transcription factors Ikaros (IKZF1)/Iolos (IKZF3), thereby inhibiting BCR dependent NF- κB activation and exerting a proliferative inhibitory effect on DLBCL cells [27, 28]. Zanubrutinib specifically blocks the downstream transmission of BCR signals, thereby inhibiting abnormal activation of the NF-κB pathway and downregulating the expression of anti apoptotic and pro proliferative target genes [29]. Currently, clinical studies have shown that the combination of Zebutinib with lenalidomide and rituximab (ZR2) demonstrates controllable safety and effective response rates in ABC-DLBCL subtypes [30, 31]. The combination of rituximab and lenalidomide has shown certain efficacy in the treatment of DLBCL patients [32]. The ZR2 regimen has shown certain efficacy and controllable safety in the treatment of relapsed/refractory DLBCL (especially in elderly and non GCB subtypes) patients [33, 34]. However, the synergistic effect and mechanism of ZR2 regimen are still not fully elucidated. This study will explore the synergistic inhibition of IRF4/NF-κB/STAT3 pathway by lenalidomide combined with BTK inhibitor through in vitro experiments to enhance the sensitivity of rituximab to ABC type diffuse large B-cell lymphoma.

2. Materials

2.1. Cell Lines

This study selected the ABC type human diffuse large B-cell lymphoma cell line NU-DUL-1 as the experimental object and purchased it from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. All experiments were conducted using cells in the exponential growth phase to ensure the stability and reproducibility of the results. This study was approved by the Medical Ethics Committee (Ethics Review Number: Suihong Hospital Medical Ethics Review 2025-063-01).

Table 1. All agents were listed as follows.

Reagent	Manufacturer
Rituximab	Shanghai Fuhong Hanlin Biopharmaceutical Co., Ltd
Zanubrutinib	BeiGene Corporation
Lenalidomide	Selleck Biotechnology Co., Ltd
Annexin V-FITC/PI Double Staining Kit	BD Pharmingen Company in the United States
CCK-8 reagent kit	Biyun Tian Company
Anti human IRF4 antibody	Cell Signaling Technology
Rabbit derived anti human phosphorylated NF-κB-p65 antibody	Cell Signaling Technology
Rabbit derived anti human Cleaved caspase-3 antibody	Cell Signaling Technology
Rabbit derived anti human phosphorylated STAT3 (Tyr705) antibody	Cell Signaling Technology
Rabbit derived anti human GAPDH reference antibody	Cell Signaling Technology
HRP labeled goat anti rabbit secondary antibody	Cell Signaling Technology

3. Methods

3.1. Determination of Drug Concentrations of Lenalidomide and Zanubrutinib

Firstly,we determine the concentration of lenalidomide and Zanubrutinib, and then establish a culture system without immune cells and serum. Collect logarithmic growth stage NU-DUL-1 cells, adjust the cell suspension density to 5×10^4 cells/mL, and inoculate 100μL system per well into a 96 well culture plate to ensure a cell count of 5×10^3 per well. Subsequently, the experiment was divided into groups and different concentration gradients of Zanubrutinib, Lenalidomide monotherapy. And then,the combination administration systems were added. Three parallel wells were set up in each group, and blank controll wells containing only culture medium and negative control wells containing only cells without drugs were also set up. After 48 hours of continuous drug intervention, 10μL of CCK-8 detection reagent was added to each well, gently shaken and mixed, and then cultured for 4 hours. The absorbance values (OD values) of each well at a wavelength of 450 nm were measured using a full wavelength enzyme-linked immunosorbent assay (ELISA) reader. The cell survival rate of each group was calculated according to the formula: Cell survival rate (%)=(experimental group OD value - blank well OD value)/(control group OD value - blank well OD value)×100%.

Based on the concentration effect curve, the half maximal inhibitory concentration (IC50) of Zanubrutinib was fitted, and combined with the results of the Bliss synergistic index analysis of the two drugs, the drug working concentration for subsequent core experiments was finally determined. The Bliss independent model is a classic model for analyzing the combined effects of anti-tumor drugs in vitro. Its criterion rule

is: when there is no interaction between the two drugs, the theoretical cell survival rate of the combination group=the cell survival rate of drug A monotherapy × the cell survival rate of drug B monotherapy. The synergy index CI=measured survival rate/theoretical survival rate of the joint group as a quantitative indicator. CI<1 is considered a synergistic effect, CI=1 is considered an additive effect, and CI>1 is considered an antagonistic effect.

3.2. Determination of Rituximab Concentration

The working concentration of rituximab was determined based on previous literature reports. We used 100μg/mL of rituximab [35, 36].

3.3. Three Drug Treatment for Tumor Cell Culture

Based on our previous research findings, Peripheral blood mononuclear cells (PBMNCs) do not exert a direct effect on lymphoma cells, but rather on secreted cytokines. We employed a culture system: mononuclear cells and tumor cells were cultured using Transwell inserts (CORNING, New York, USA) for tumor cell apoptosis and the expression of proteins related to the IRF4/NF-κB/STAT3 pathway and apoptotic proteins. The peripheral blood mononuclear cells, NU-DUL-1 cells, and drugs AZA, LEN, RTX were cultured for 72 hours, with a tumor cell to mononuclear cell ratio of 1:1,in combination with human serum (dilution 1:4). Pooled human serum collected from healthy donors was used as a source of complement. [36, 37].

The concentrations of the three drugs selected are as follows: 100μg/mL of Rituximab,10μg/mL of Lenalidomide and 3μg/mL of Zanubrutinib. This experiment was divided four groups as follows: blank control group (Control) ; Rituximab monotherapy group; Zanubrutinib and Lenalidomide two drug group; triple drug group (Zanubrutinib + Lenalidomide + Rituximab). The following experiment explores the effects of different drug treatments on apoptosis, IRF4/NF-κB/STAT3 pathway, and related protein expression in DLBCL cells

3.4. Flow Cytometry Detection of Cell Apoptosis Levels

Collect tumor cells from the three drug culture system and quantitatively detect the induction effect of different drug treatments on cell apoptosis using Annexin V^-FITC/PI double staining method. Collect treated cells from each group and divide them into four subgroups based on double staining parameters: live cells (Annexin V^+/PI^+), early apoptotic cells (Annexin V^+/PI^+), and late apoptotic/necrotic cells (Annexin V^+/PI^+). Calculate the total apoptosis rate of each group by adding the early and late apoptosis rates, and complete the statistical comparison between groups.

3.5. Western Blot Detection of Protein Expression Levels

Collect tumor cells from a three-drug culture system, and use protein immunoblotting to extract, quantify, and detect the expression of target proteins. Imaging and quantitative analysis: Mix ECL chemiluminescence substrates A and B in equal volumes, evenly drop them onto the surface of PVDF membrane, and react in the dark for 1-2 minutes. Then, use a chemiluminescence imaging system to complete exposure and image acquisition. Using GAPDH and β-actin as internal reference proteins, the grayscale values of each target protein band were measured using ImageJ (1.54f) software to calculate the relative expression level of the target protein and complete inter group comparison.

3.6. Statistical Methods

The in vitro cell experiments of this study were independently repeated three times, and representative results are shown in the corresponding figures. GraphPad Prism 8 software was used for plotting and statistical analysis. t-test was used for comparison between two groups, and one-way ANOVA was used for comparison between multiple groups of data ($n \geq 3$. * $P<0.05$, * * $P<0.01$, * * $P<0.001$ indicate statistically significant differences.

4. Results

4.1. Screening and Determination of Combined Drug Concentrations

To screen for suitable combination drug concentrations, this study used the CCK-8 method to detect the inhibitory effects of Zanubrutinib, Lenalidomide monotherapy, and dual drug combination on the proliferation of NU-DUL-1 cells. The final experimental concentration was determined based on pharmacological characteristics, Bliss independent model synergy analysis, and literature review.

Using an in vitro system without complement and immune cells, Zanubrutinib exhibited a concentration dependent inhibitory effect on the proliferation of NU-DUL-1 cells. The concentration effect curve was fitted (Figure 3-1), and the half maximal inhibitory concentration (IC_{50}) was 8.98μg/mL (95% CI: 7.90-10.17μg/mL). In order to reduce the interference of high concentration drug strong single drug cytotoxicity on the subsequent joint effect judgment, this study intends to select a concentration of 3μg/mL lower than IC_{50} as the experimental working concentration of Zanubrutinib.

Using the Synergy Finder software, a synergistic effect analysis was conducted on the combined treatment of Zanubrutinib and Lenalidomide cross concentration gradients based on the Bliss independent model (Figure 2A-2C). The results showed that the combined effect of the two drugs exhibited concentration dependent characteristics: at low concentrations (<=1μg/mL) of Zanubrutinib, the additive effect was predominant, with no significant antagonistic effect; When the concentration of Zanubrutinib is >=3μg/mL, multiple combinations show a synergistic trend, and the overall average synergistic index of the full concentration combination is 0.71. The combination of Zanubrutinib (3μg/mL) and Lenalidomide (10μg/mL) selected in this study had a synergistic index CI of 0.92 calculated by the Bliss model, showing a mild synergistic trend and avoiding non-specific cytotoxic interference from high concentration drugs. This suggests that this concentration combination has certain advantages in synergistic effects and is suitable for the pharmacological requirements of subsequent mechanism studies.

In summary, subsequent experiments will use 3μg/mL of Zanubrutinib and 10μg/mL of Lenalidomide as the basic working concentrations for the dual drug combination, and will be conducted in combination with Rituximab at a concentration of 100μg/mL.

4.2. Effects of Different Drug Treatments on Apoptosis of DLBCL Cells

Collect tumor cells cultured in a three-drug culture system, and use Annexin V-FITC/PI double staining combined with flow cytometry to detect the apoptosis of NU-DUL-1 cells treated with different drugs and explore the pro-apoptotic effect of combination therapy.

The results showed (Figure 3A-3B) that compared with the blank control group, the apoptosis rate of cells in the RTX monotherapy group was slightly increased ($p<0.05$), and the dual drug group (ZAN+LEN) could significantly improve the apoptosis rate ($P<0.001$).The apoptosis rate of the combination group (ZAN+LEN+RTX) was the highest among all groups, significantly higher than that of the dual drug group (ZAN+LEN)($p<0.01$) and the RTX monotherapy group ($P<0.001$), indicating that the combination of Zanubrutinib and Lenalidomide can effectively induce apoptosis in DLBCL cells, and the induction of apoptosis by the three drug combination is the most significant.

4.3. Different Drug Treatments Regulate Apoptosis and Expression of IRF4/NF-k B/STAT3 Pathway Related Proteins in DLBCL Cells

To verify the regulatory effect of combination therapy on the IRF4/NF-κ B/STAT3 signaling axis, this study used Western Blot to detect the expression changes of key proteins and their phosphorylation levels in this pathway, including IRF4, p-NF-κB (p65), p-STAT3, and the activation level of apoptotic protein Cleaved caspase-3.

The results showed (Figure 4A-4E) that compared with the blank control group, the overall expression of IRF4/NF-κB/STAT3 pathway related proteins showed a downward trend. The expression levels of phosphorylated p65(p-p65), phosphorylated STAT3 (p-STAT3), and IRF4 proteins gradually decreased with drug combination treatment. The changes in the ZAN+LEN dual drug group and three drug combination group were more significant than those in the RTX monotherapy group and ZAN monotherapy group, and the three drug combination group showed an overall trend of further enhancement. In addition, each drug treatment group can upregulate the expression level of apoptosis executing protein Cleaved caspase-3 to varying degrees, with the three drug combination group showing a more significant trend of change. The above results suggest that combination therapy may exert a combined anti-tumor effect on DLBCL cells by regulating the IRF4/NF-k B/STAT3 signaling pathway and affecting the expression of apoptosis related proteins.

5. Discussion

DLBCL is the most common invasive non Hodgkin lymphoma in the hematological system, with ABC subtype being the core high-risk subtype with drug resistance and poor prognosis. The downregulation of CD20 target expression[9-12, 38, 39] and abnormal activation of IRF4/NF-κB/STAT3 signaling axis [24-26] are the two core driving factors for ABC subtype rituximab resistance. This study revealed that BTK inhibitor and immune modulator lenalidomide synergistically inhibit abnormal activation of the IRF4/NF-κB/STAT3 signaling axis, and synergistically enhance the apoptosis of rituximab inABC-DLBCL cell, NUL-DUL-1 .

To screen for suitable combination drug concentrations, this study used the CCK-8 method to detect the inhibitory effects of Zanubrutinib, Lenalidomide monotherapy, and dual drug combination on the proliferation of NU-DUL-1 cells. Based on pharmacological characteristics and the synergistic effect of the Bliss independent model, 3μg/mL was determined as the experimental working concentration of Zanubrutinib (3μg/mL) and Lenalidomide(10μg/mL), indicating that this concentration combination has certain advantages in combined effects and is suitable for the pharmacological requirements of subsequent mechanism studies.

The subsequent experiments selected Zanubrutinib (3μg/mL)and Lenalidomide (10μg/mL) as the basic working concentrations for the dual drug combination, and combined with Rituximab (100μg/mL) for in vitro studies.

The results showed that the apoptosis rate of the triple drug group was the highest among all groups, significantly higher than the dual drug group (ZAN+LEN) and the RTX monotherapy group, indicating that the combination of Zanubrutinib and Lenalidomide can effectively enhance apoptosis of Rituximab in DLBCL cells.

In ABC-DLBCL, IRF4, NF-κB, and STAT3 do not function independently, but rather form a continuously abnormally activated oncogenic signaling axis through a multi-level transcriptional regulation coupled with cytokine signaling (Figure 1) [24-26, 40]. The upstream BCR signal activates NF-κB through BTK, and NF-κB transcription activates IRF4, forming a positive feedback loop. In addition, STAT3 can be activated through the IL-6/IL-10/JAK pathway [40]. At the midstream level, IRF4 and STAT3 form bidirectional regulation, jointly upregulating survival promoting and anti apoptotic genes such as MYC, BCL 2, and BCL-XL, enhancing tumor cell proliferation and survival ability[40]. Meanwhile, recent studies have shown that cytokines such as IL-6/IL-10 maintain sustained activation of STAT3 through an autocrine loop, which enhances the resistance phenotype of ABC-DLBCL [41-43]. NF-κB synergizes with STAT3 through positive feedback and cross activation loops, further enhancing the oncogenic function of the signaling axis [17].

Clinical and basic research has shown that abnormal activation of this signaling axis is closely related to the adverse reactions of ABC-DLBCL to R-CHOP treatment [24]. In summary, the IRF4/NF-κB/STAT3 signaling axis is the core molecular mechanism underlying the pathogenesis of ABC DLBCL, providing a theoretical basis for subsequent research.

Although previous clinical studies have preliminarily shown that the combination of Zanubrutinib with Lenalidomide and Rituximab (ZR2 regimen) has shown good efficacy and controllable safety in patients with newly diagnosed and relapsed/refractory DLBCL [33, 34].In order to verify the regulatory effect of the combination therapy on the IRF4/NF-κB/STAT3 signaling axis, this study used Western Blot to detect the expression changes of key proteins and their phosphorylation levels in this pathway. The results of this study showed that after treatment with rituximab monotherapy, there was no significant change in the total protein expression of IRF4 and the phosphorylation levels of NF-κB p65 and STAT3 in NU-DUL-1 cells, indicating limited inhibitory effect on this key survival signaling pathway. This can partly explain the weak anti-tumor effect of rituximab monotherapy in the ABC subtype. After treatment with Zanubrutinib combined with Lenalidomide, IRF4 expression decreased, while NF-κB-p65 and STAT3 phosphorylation

levels were significantly reduced, indicating that this dual drug regimen can significantly inhibit the sustained activation of the BCR downstream signaling pathway in ABC-DLBCL cells. In the three drug combination group, the inhibitory effect of this signaling axis showed a further enhanced trend, while the expression of Cleaved caspase-3 increased, indicating an increase in cell apoptosis levels. Based on the results of apoptosis experiments, the combination of three drugs promoted cell apoptosis more significantly than dual or single drug treatment, and the overall anti-tumor effect showed a gradually increasing trend. This result indicates that the combination of Zanubrutinib and Lenalidomide can inhibit IRF4/NF-κB/STAT3 signaling, reduce the anti - apoptotic ability of tumor cells, and to some extent enhance the anti-tumor effect of rituximab, providing a molecular mechanism basis for further research on the three drug combination regimen (ZR2).

In this study,on the one hand,the combination therapy of Zanubrutinib and Lenalidomide can synergically kill lymphoma cells, on the other hand, they synergistically enhance apoptosis of rituximab on lymphoma cells by inhibiting the abnormal activation of the IRF4/NF-κB/STAT3 signaling axis.Previous studies have revealed that lenalidomide and BTK inhibitors upregulate CD20 expression and optimize structure in diffuse large B-cell lymphoma cells [33, 37]. Take together,integrate previous research findings,we explore that Zanubrutinib and Lenalidomide synergically enhance the apoptotic ability and the anti-tumor effect of rituximab on tumor cells (Figure 5)

Nonetheless, this study has certain limitations: (1) The research only employed the single ABC-DLBCL cell line NU-DUL-1 for in vitro experiments, without investigating drug-resistant strains or establishing an in vivo animal model to validate the efficacy of the triple-drug combination. Future studies could further verify the antitumor effects and immune regulatory mechanisms of this regimen in vivo using DLBCL humanized mouse models. (2) Based on in vitro cell models, this study primarily focused on intrinsic tumor cell signaling pathways and target expression regulation mechanisms, without accounting for the influence of the tumor microenvironment (e.g., immune cells such as T cells and dendritic cells). As a result, it can not comprehensively reflect the complex immune regulatory processes in vivo.

This study provides a direct molecular explanation at the intrinsic level of tumor cells. However, current research suggests that its mechanism may not only rely on the intrinsic regulation of tumor cells revealed in this study,but also closely relate to the remodeling of the tumor microenvironment[34]. The proposal of the ZR2 "chemotherapy-free" treatment model indicates that the therapeutic strategy for aggressive lymphoma is shifting from traditional cytotoxic therapy to a precise immune regulation model based on molecular mechanisms [44].

In summary, this study supplements the molecular mechanism of the zanubrutinib combined with lenalidomide and rituximab regimen (ZR2) from the intrinsic level of tumor cells, providing a more comprehensive theoretical perspective for interpreting the clinical efficacy of this regimen.

6. Figures

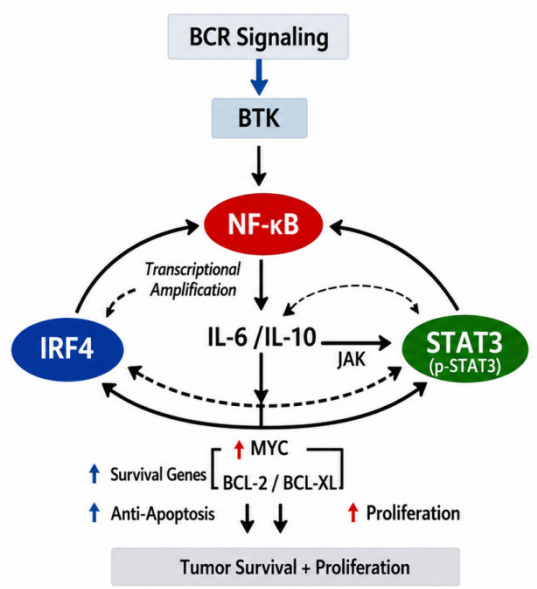

Figure 1. Schematic diagram of the pathogenic mechanism of IRF4/NF-κB/STAT3 signaling pathway in ABC-DLBCL

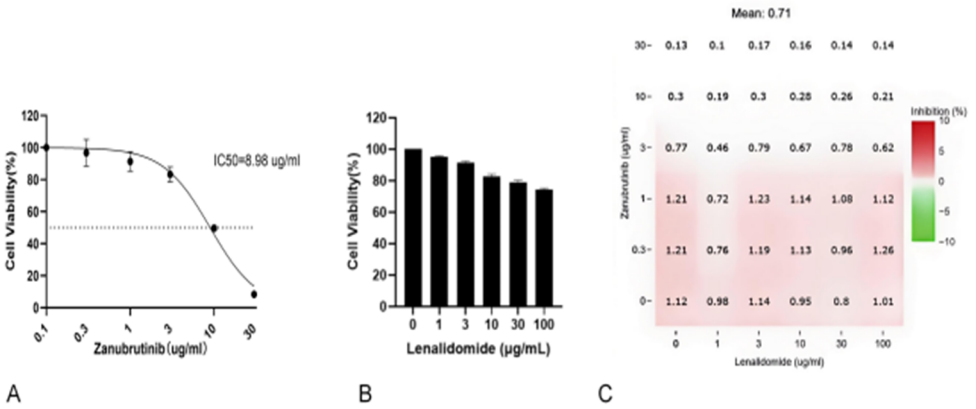

Figure 2. Synergistic effect of zanubrutinib and Lenalidomide cross concentration gradient combined treatment on the proliferation inhibition of NU-DUL-1 cells

A: Effects of different concentrations of zanubrutinib on the proliferation activity of NU-DUL-1 cells

B: The effect of different concentrations of lenalidomide on the proliferation activity of NU-DUL-1 cells

C: Synergistic effect of Zanubrutinib and Lenalidomide cross concentration gradient combined treatment on the proliferation inhibition of NU-DUL-1 cells

Zanubrutinib (Zan), Lenalidomide (Len), Rituximab (RTX)

Availability of Data and Materials

All the data can be found in this manuscript or received from the corresponding author upon reasonable request.

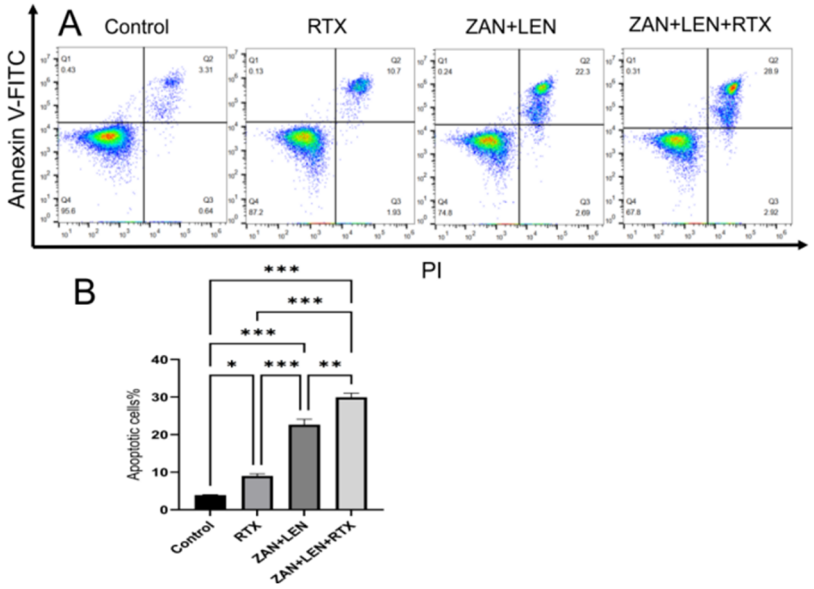

Figure 3. The effect of different drug treatments on inducing apoptosis in NU-DUL-1 cells

A: Representative cell apoptosis results of flow cytometry; B: Statistics of total apoptosis rate of cells (* P<0.05, * * P<0.01, * * * P<0.001, ns indicates no statistical difference). Zanubrutinib (Zan), Lenalidomide (Len), Rituximab (RTX)

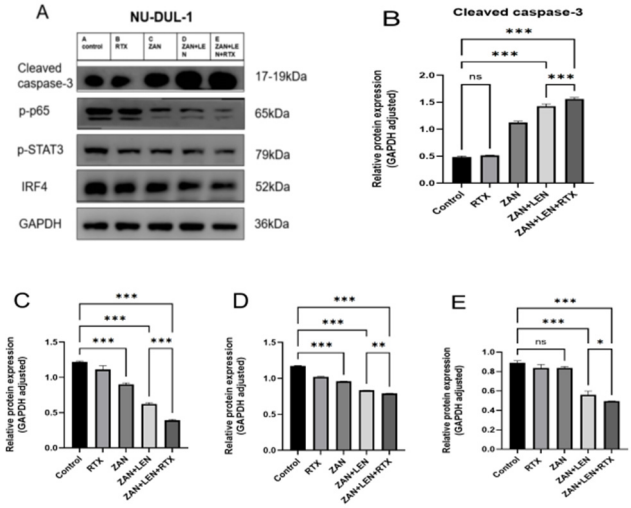

Figure 4. The regulatory effects of different drug treatment groups on the IRF4/NF-κB/STAT3 signaling axis and apoptosis related proteins in NU-DUL-1 cells

A: Western blot original bands of Cleaved caspase-3, p-NF-κB (p65), p-STAT3, IRF4, and reference proteins in NU-DUL-1 cells;

B-E: Quantitative analysis results of Cleaved caspase-3, p-NF-kB (p65), p-STAT3, and IRF4 proteins, respectively (standardized with blank control group as reference, * P<0.05, * * P<0.01, * * P<0.001, ns indicates no statistical difference)

Zanubrutinib (Zan), Lenalidomide (Len), Rituximab (RTX)

Contributions of the Authors

LQS conceived the study, provided overall supervision. LQS, WT and GGL obtained fundins. WT designed and performed the experiments, obtained funding, analyzed the data, and curated the supporting materials. GGL, WT, WY and CHY performed additional experiments and provided technical support, LXT prepared the figures and analyzed the data. WT and LXT drafted the initial manuscript; LQS critically revised it. All authors reviewed and approved the final version.

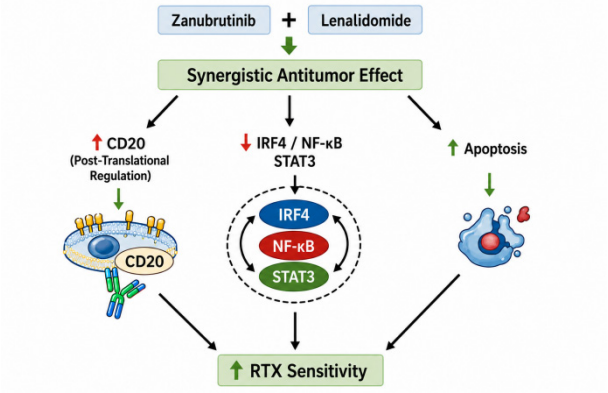

Figure 5. Mechanism diagram of synergistic antitumor effects with Zanubrutinib, Lenalidomide and Rituximab

Conflict of Interest

No conflict of interest was disclosed by the authors.

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