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LETTER TO THE EDITOR

Rifamycin O as a novel CRBN ligand for targeted degradation of IKZF1/3 proteins in hematopoietic malignancies

KEY WORDS

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To the Editor:

Cereblon (CRBN) has emerged as a promising therapeutic target, enabling the design of proteolysis-targeting chimeras (PROTACs) and molecular glues (MGs) for targeted protein degradation[1]. The immunomodulatory imide drugs (IMiDs), such as thalidomide, lenalidomide and pomalidomide, represent classical CRBN ligands and have been widely used in the treatment of hematopoietic malignancies. These IMiDs exert their immunomodulatory and anti-inflammatory effects by binding to CRBN and triggering the degradation of neosubstrates, including IKZF1 and IKZF3, which are crucial for B and T cells biology[2]. However, the glutarimide motif of IMiDs induces off-target degradation of other key zinc finger-containing proteins that may lead to side effects including increased risks of hematological toxicity, teratogenicity and cardiovascular events[3]. Although current efforts focus on modifying the isoindolinone or phthalimide moieties of IMiDs[4-5], these approaches cannot fully avoid the aforementioned side effects. Therefore, the discovery of CRBN ligands with novel structural scaffolds is of critical importance. In this study, we identify Rifamycin O as a novel CRBN ligand and elucidate the molecular mechanisms that mediate targeted protein degradation.

1. Rifamycin O is a novel CRBN ligand

We developed a fluorescence polarization (FP) screening platform using a novel FITC-probe derived from thalidomide (Fig. 1A, Supporting Information Fig. S1A and S1B, Supporting Information Scheme S1) and purified recombinant hsCRBN-hsDDB1. Screening over 1300 approved drugs from our in-house library identified Rifamycin O, which exhibited an IC_{50} value of 1.47 μmol/L in the FP binding assay (Fig. 1B), compared to 0.15 μmol/L for lenalidomide and 0.56 μmol/L for thalidomide (Fig. S1C). Subsequent microscale thermophoresis (MST) assay and isothermal titration calorimetry (ITC) analysis collectively validated the effective binding affinity of Rifamycin O for CRBN, yielding K_d values of 3.55 and 3.03 μmol/L, respectively (Fig. 1C and D). Consistently, thermal stability assay (TSA) demonstrated that Rifamycin O enhanced CRBN thermal stability in a temperature-dependent manner compared with DMSO control (Fig. S1D). Besides, molecular docking revealed that Rifamycin O formed four hydrogen bonds with Asp292, Lys324 and Asn127 in CRBN, which is separate from the canonical thalidomide-binding site (Fig. S1E). These results collectively indicated that Rifamycin O had a direct interaction with CRBN and might be a novel ligand of CRBN.

2. Rifamycin O is a potent anti-tumor agent

The discovery that Rifamycin O effectively hijacked CRBN was very interesting, because neither Rifamycin O nor structurally similar Rifamycin analogs had ever been reported to be CRBN effective. FP binding determination for commercially available

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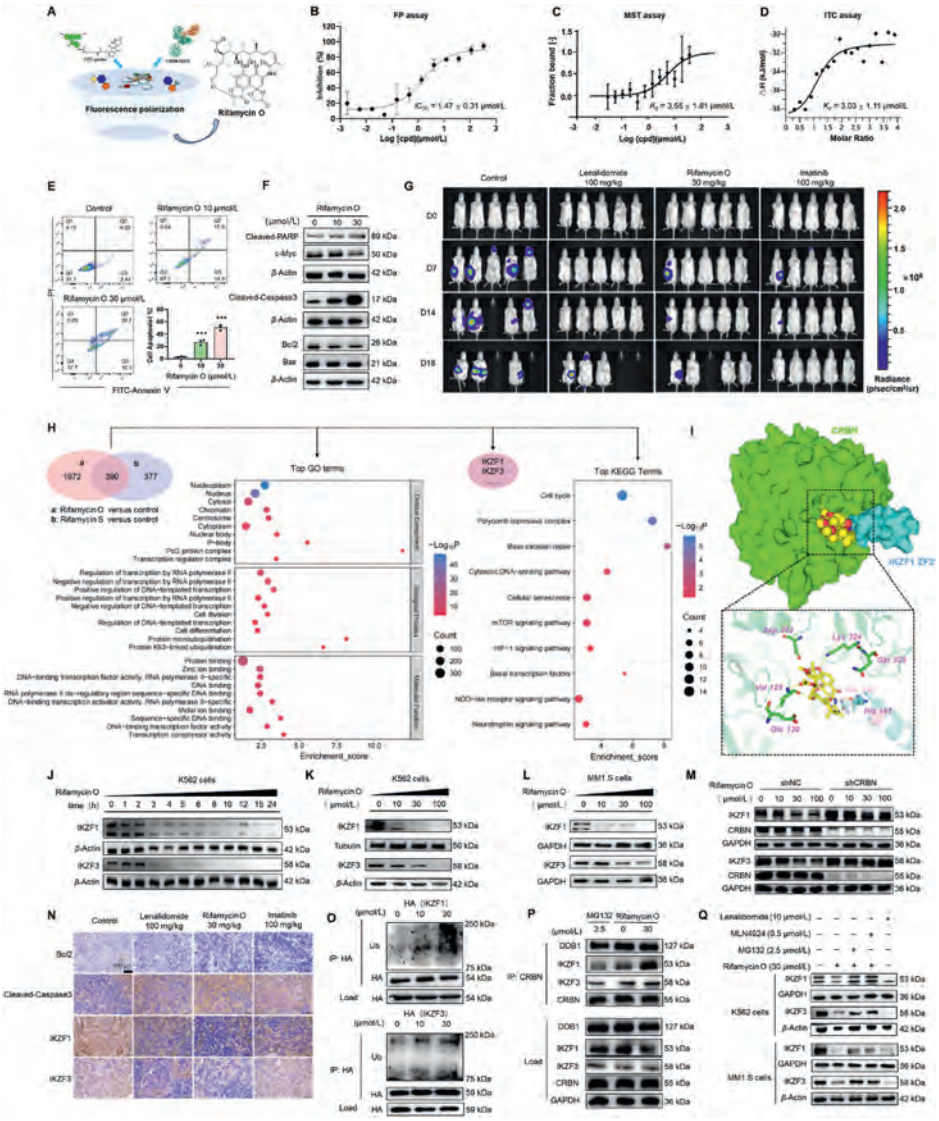

Figure 1 Rifamycin O functions as a novel CRBN ligand and induces degradation of IKZF1 and IKZF3. (A) FP screening identified Rifamycin O. (B) Binding affinity of Rifamycin O to CRBN shown in the FP experiments. (C) Binding affinity of Rifamycin O to CRBN is shown in the MST assay. (D) ITC assay demonstrating the binding of Rifamycin O with purified CRBN. (E) Flow cytometry analysis of cell populations in various quadrants following Rifamycin O treatment. (F) Western blot analysis of the apoptosis-related proteins expression with increasing concentrations of Rifamycin O. (G) Representative bioluminescence imaging of mice at different time points in different groups. (H) Global proteomic profiling of Rifamycin O and Rifamycin S. (I) Overview of the docking results for Rifamycin O with CRBN and IKZF1. (J) Time-course analysis of IKZF1 and IKZF3 protein levels in K562 cells after treatment with Rifamycin O. (K) Dose-dependent effects of Rifamycin O on endogenous IKZF1 and IKZF3 protein expression in K562 cells. (L) Dose-dependent effects of Rifamycin O on endogenous IKZF1 and IKZF3 protein expression in MM1.S cells. (M) Effects of CRBN knockdown on the expression of IKZF1 and IKZF3 proteins. (N) IHC analysis of spleen tissue sections for Bcl2, Cleaved-caspase 3, IKZF1 and IKZF3 in the different groups. Scale bar: 100 μm. (O) *In vivo* ubiquitination analysis of HA-tagged IKZF1 and IKZF3 expressed in 293T cells treated for 6 h with the indicated concentrations of Rifamycin O. (P) Immunoprecipitation of endogenous CRBN in K562 cells treated for 2 h with the indicated drugs. (Q) K562 and MM1.S cells were treated with DMSO and Rifamycin O in the absence or presence of MLN4924 or MG132, and endogenous IKZF1 and IKZF3 levels were analyzed by Western blot. Data are presented as mean ± SD, $n = 3$ independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, by two-way ANOVA.

rifamycin derivatives found that only Rifamycin O, Rifamycin S, and Rifabutin effectively bound to CRBN (Supporting Information Fig. S2A, IC_{50} of 1.47, 3.09, and 15.81 μmol/L, respectively). The antiproliferative activities of Rifamycin O and Rifamycin S against hematological tumor cells were consistent with their CRBN binding activities, and both exhibited low toxicity towards normal PBMC cells (Fig. S2B). Consistently, the flow cytometry and Western blot analysis exhibited an apoptosis phenotype in K562 cells after Rifamycin O and S treatment (Fig. 1E and F, Fig. S2C). Besides, the K562 tumor progression was significantly inhibited after Rifamycin O administration in two *in vivo* xenograft leukemia models, without any significant change in the average body weight and organ functions (Fig. 1G, Supporting Information Fig. S3). Collectively, these data suggest that Rifamycin O is a novel anti-tumor agent through its binding with CRBN. *In vivo* efficacy studies were conducted with the approval

of the Institutional Animal Care and Use Committee (IACUC) of ClinBridge Biotech Co., Ltd. (Nanjing, China) (Animal Protocol No: AP-C250310).

3. Rifamycin O decreases IKZF1 and IKZF3 abundance

We hypothesized that the pleiotropic anti-tumor effects of Rifamycin O might be caused by altered ubiquitination degradation of neosubstrates, since CRBN functions as the substrate recognition adapter in the $CRL4^{CRBN}$ E3 ubiquitin ligase complex. Proteomics data showed that 390 differentially expressed proteins (DEPs), including the canonical CRBN neo-substrates IKZF1 and IKZF3, were downregulated in Rifamycin-treated groups. GO analysis highlights enrichment in transcriptional regulation, cell division, differentiation, and protein ubiquitination processes, supporting a degradation-dependent mechanism *via* CRBN E3 ligase. Enriched molecular functions of DNA-binding transcription factor activity and sequence-specific DNA binding further support transcriptional regulation. KEGG analysis demonstrated that Rifamycin O and Rifamycin S may mediate these cellular functions through cell cycle, polycomb repressive complex (PRC), and mTOR signaling pathways, which are in line with the IKZF1 and IKZF3 functions (Fig. 1H). A comprehensive network analysis with STRING and Cytoscape helped pinpoint IKZF1 and IKZF3 as the proteins most strongly associated with CRBN (Supporting Information Fig. S4A). RNA-seq and RT-PCR data confirm that Rifamycin O does not alter the transcription levels of IKZF1 and IKZF3 (Fig. S4B). Molecular docking results demonstrated the formation of a stable ternary CRBN−Rifamycin O−IKZF1 complex, in which Rifamycin O forms three hydrogen bonds with Val128 and Lys324 in CRBN, and establishes an additional hydrogen bond with His167 in IKZF1 (Fig. 1I). Besides, no significant changes of SALL4 were observed by Western blot, indicating a high target specificity of Rifamycin O (Fig. S4C). These findings collectively suggest IKZF1 and IKZF3 to be candidate neo-substrates for further investigation. While no other DEPs have yet been identified as potential neo-substrates of CRBN (such as RIPK2, a previously established CRBN interactor), an in-depth analysis of these additional DEPs is currently underway.

Cellular Western blot analysis revealed that Rifamycin O decreased abundance of IKZF1 and IKZF3 in a time- and dose-dependent manner (Fig. 1J−L, Fig. S4D and S4E). However, the expression of IKZF1 and IKZF3 remained unperturbed in CRBN knockdown conditions (Fig. 1M), and the antiproliferative activity of Rifamycin O against K562 cells was also attenuated (Fig. S4F). Immunohistochemical (IHC) analysis of the mouse spleen section demonstrated that Rifamycin O effectively downregulated Bcl2 while upregulating cleaved-caspase3. Moreover, the expression levels of IKZF1 and IKZF3 in spleen were significantly reduced in Rifamycin O treatment group (Fig. 1N). These findings strongly underscore the pivotal role of CRBN in mediating the antitumor efficacy of Rifamycin O, specifically through IKZF1 and IKZF3 downregulation.

4. Rifamycin O induces IKZF1 and IKZF3 ubiquitination degradation

The ubiquitination levels of IKZF1 and IKZF3 were examined following transfection of HA-tagged IKZF1 and IKZF3 plasmids into 293T cells, revealing a dose-dependent increase in ubiquitination after Rifamycin O treatment (Fig. 1O). Immunoprecipitation analysis demonstrated enhanced co-immunoprecipitation of IKZF1 and IKZF3 with endogenous CRBN and the CUL4 adaptor protein DDB1 in cells treated with Rifamycin O (Fig. 1P), supporting the promotion of IKZF1 and IKZF3 ubiquitination disturbance by Rifamycin O. The potential rescue of Rifamycin O-induced IKZF1 and IKZF3 degradation by proteasome inhibition was assessed using the proteasome inhibitor MG132 and cullin RING E3 ligase inhibitor MLN4924, which effectively prevented the decrease in IKZF1 and IKZF3 levels in K562 and MM1.S cells (Fig. 1Q). These observations indicate that the degradation of IKZF1 and IKZF3 by Rifamycin O involves ubiquitination mediated by a cullin-based E3 ubiquitin ligase. Moreover, Rifamycin S was also shown to downregulate the intracellular abundance of IKZF1 and IKZF3 through a ubiquitination-dependent degradation pathway akin to that of Rifamycin O (Supporting Information Fig. S5).

Targeting the degradation of specific proteins through the ubiquitin−proteasome system has garnered significant interest within industry and academia as a novel therapeutic modality for cancer treatment. Notable examples of this strategy include PROTACs and MGs. To address the limitations associated with IMiDs and expand the scope of developing CRBN-recruiting degraders, we identified Rifamycin O from our in-house drug library as an alternative CRBN-binding chemotype. A combination of fluorescence polarization, MST, ITC, and TSA analysis collectively substantiates the robust binding affinity of Rifamycin O for CRBN. Molecular docking studies further disclose that the naphthalene ring and its associated furan, lactone, and amide moieties within Rifamycin O can form multiple hydrogen bonds with CRBN and IKZF1. The distinct binding profile of Rifamycin O, which deviates from the canonical IMiDs binding site, implies a mechanism that may bypass the teratogenic and hematotoxic side effects of IMiDs. This is corroborated by *in vivo* evaluation in a xenograft leukemia model, where Rifamycin O exhibits comparable efficacy and enhanced safety to lenalidomide at lower doses.

An integrative approach involving proteomics, RNA sequencing, molecular docking, ubiquitination assays, and Co-IP experiments, elucidating its role of Rifamycin O in recruiting CRBN to ubiquitinate degradation of IKZF1 and IKZF3. The well-established role of IKZF1 and IKZF3 degradation as a potent anti-tumor strategy not only fortifies the therapeutic efficacy of IMiDs but also extends to Rifamycin O. Our studies indicate that Rifamycin O and Rifamycin S, with minor chemical modifications, can confer gain-of-function glue properties to an inhibitor, suggesting a broader strategy for converting target-binding molecules into molecular glues. Hence, by leveraging the existing array of chemical, enzymatic, and biotransformation techniques widely used in the rifamycin family, selectively retaining functional segments such as the naphthalene ring and its substituents that occupy the CRBN cavity, and optimizing other substituents of rifamycin, such as removing the acetyl group and extending the flexible chain, to enhance the interactions between functional groups and CRBN as well as substrates, the development of rifamycin-based PROTACs or MGs appears to be readily achievable.

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Author contributions

Jian Li and Jinlian Wei contributed to the design of the research, revised the manuscript and provided financial support. Liang Liao, Qiang Wang and Zhengyang Gao performed the experiments and analyzed the data. Jiayao Liu, and Yuxuan Tong helped to perform the experiments. Xin Chen and Jin Zhu helped to revise it critically for important intellectual content. All the authors have approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2025.10.033.

References

1. Bekes M, Langley DR, Crews CM. PROTAC targeted protein degraders: the past is prologue. *Nat Rev Drug Discov* 2022;**21**:181−200.
2. Lu G, Middleton RE, Sun H, Naniong M, Ott CJ, Mitsiades CS, et al. The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. *Science* 2014;**343**:305−9.
3. Sievers QL, Petzold G, Bunker RD, Renneville A, Slabicki M, Liddicoat BJ, et al. Defining the human C2H2 zinc finger degrome targeted by thalidomide analogs through CRBN. *Science* 2018;**362**: eaat0572.
4. Min J, Mayasundari A, Keramatnia F, Jonchere B, Yang SW, Jarusiewicz J, et al. Phenyl-glutarimides: alternative cereblon binders for the design of PROTACs. *Angew Chem Int Ed Engl* 2021;**60**: 26663−70.
5. Steinebach C, Bricelj A, Murgai A, Sosic I, Bischof L, Ng YLD, et al. Leveraging ligand affinity and properties: discovery of novel benzamide-type cereblon binders for the design of PROTACs. *J Med Chem* 2023;**66**:14513−43.

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