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RESEARCH ARTICLE

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Discovery of first-in-class PROTACs targeting maternal embryonic leucine zipper kinase (MELK) for the treatment of Burkitt lymphoma†

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Maternal embryonic leucine zipper kinase (MELK) is a novel target for the treatment of various kinds of B-cell malignancies. However, the toxicity of inhibitors of MELK has led to clinical failures in cancer treatments. Moreover, inactivation of MELK catalytic domain is insufficient for achieving cancer cell apoptosis. To further confirm the role of MELK in Burkitt lymphoma treatment, we describe herein a structure-guided design of PROTACs targeting MELK. Through design, computer-assisted optimization and SAR studies, we developed the first-in-class MELK-targeting PROTAC **MGP-39**, which promoted a rapid and potent degradation of MELK in RAMOS cells. Additionally, the newly designed MELK degrader induced significant cell cycle arrest and apoptosis in cancer cells. Notably, compared to MELK inhibitors, **MGP-39** has better anti-cancer activity and lower toxicity, indicating the practical role of PROTACs in avoiding the side effects of traditional inhibitors. More importantly, our results show that the use of a PROTAC can be adopted as a general and effective strategy for targeted cancer therapy.

Introduction

Burkitt's lymphoma (BL), a hematologic malignancy, is the most common non-Hodgkin's lymphoma (NHL) in children,[1] with approximately 30 000 people diagnosed worldwide each year.[2] BL is a rapidly proliferating lymphoma derived from germinal-center B cells.[3,4] BL typically has a dramatic clinical presentation, and rapidly spreads to intra-abdominal organs and the central nervous system (CNS).[5] Therefore, immediate evaluation is warranted. Although intensive chemoimmunotherapy has appeared,[6] relapse may occur shortly with dismal prognosis, and the survival rates are always lower than 30%.[5,7]

Maternal embryonic leucine zipper kinase (MELK) is an enzyme encoded by the *MELK* gene. As an important member of the AMP-activated Ser/Thr kinase family, MELK plays a crucial role in cancer progression and drug resistance.[8] MELK promotes cancer progression through the mTOR pathway.[9,10] As mentioned, BL is a rapidly proliferating NHL. The MELK/mTOR pathway plays a crucial role in development, differentiation and signalling of BL and other NHLs.[11–13] Meanwhile, MELK has

proven to be a novel and practical target for treatment of B cell malignancies such as BL and MCL. Inhibition or degradation of MELK has potential benefits on the prognosis of BL.[14,15] However, currently reported inhibitors of MELK display serious toxicity, which leads to clinical failures.[16–18] Hence there is an urgent need to develop a new strategy to overcome these shortcomings.

Proteolysis-targeting chimeras (PROTACs) have recently garnered considerable research interest in academia and in the pharmaceutical industry.[19,20] PROTACs constitute a series of bifunctional molecules each capable of bringing E3 ligase into the proximity of a target protein of interest (POI), inducing non-natural ubiquitin degradation of the target protein.[21] Unlike traditional protein inhibitors, PROTACs achieve inactivation of the whole target protein, including its non-enzymatic functions, rather than inhibiting the kinase pocket only.[22] Moreover, a typical advantage of PROTACs is their excellent selectivity.[23] Based on the novel ubiquitination mechanism, selective degradation of a POI can be achieved without potential toxicity from protein inhibitors.[24,25]

Herein, for the first time, we describe the development of a novel degrader of MELK using the PROTAC strategy. The degrader, denoted as **MGP-39**, induced potent and rapid degradation of MELK in Burkitt lymphoma cells. In addition, our newly designed PROTAC also induced significant G2/M cell cycle arrest and apoptosis in RAMOS cells, indicating the therapeutic importance of MELK in B-cell malignancies. More

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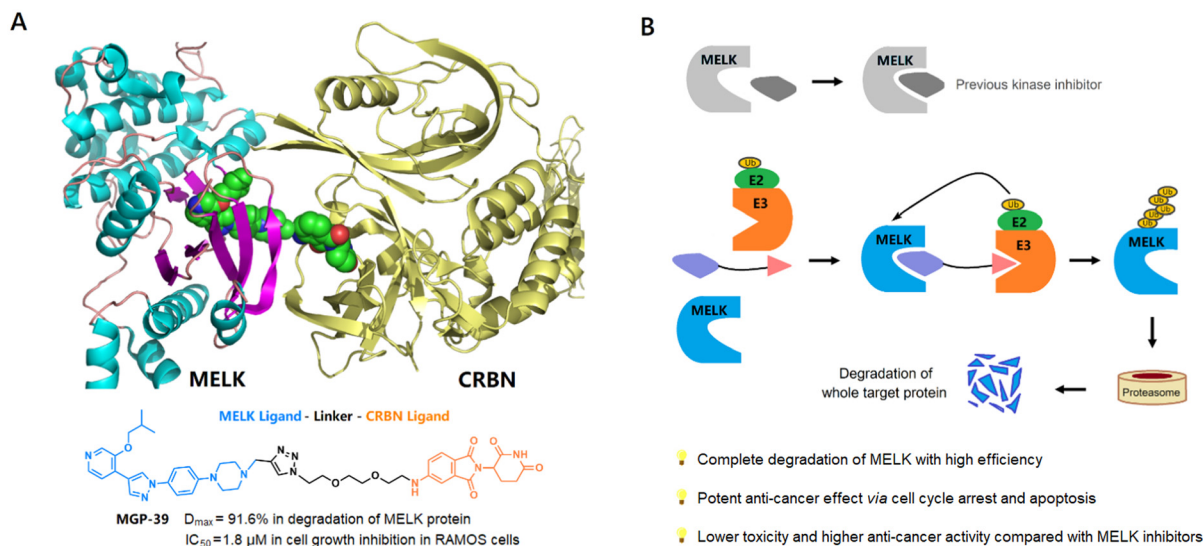
Fig. 1 Development of novel PROTACs targeting the MELK protein. (A) Structural representation of MELK degrader design. (B) Mechanism of the newly developed PROTACs targeting MELK.

importantly, compared to MELK inhibitors, **MGP-39** showed better anti-cancer activity and lower toxicity (Fig. 1A and B). Therefore, the use of degradation, a novel method in medicinal chemistry, has been demonstrated to be a targeted and effective strategy in cancer therapy.

Results and discussion

### The screening of novel MELK degraders

In order to develop MELK-targeting PROTAC-designed degraders, a MELK-targeting arm was conjugated to a MELK-degradation arm *via* linkers with variable lengths. As a result, a

MELK ligand and pomalidomide were employed as corresponding MELK and E3 ligase binding partners. According to the design principles, PROTAC molecules in different combinations were prepared (Fig. 2A).

The RAMOS cell line, being common for BL, was utilized for the evaluations. **MGP-39**, a bifunctional molecule with a triethylene glycol linker and pomalidomide ligand, was demonstrated from the results to have the strongest MELK-degrading ability and anti-cancer activity (Fig. 2A). Compound **MPG-42**, in which polyethylene glycol linker was replaced by an aliphatic chain with the same length, showed relatively low efficiency, indicating a significant impact of the identity of the
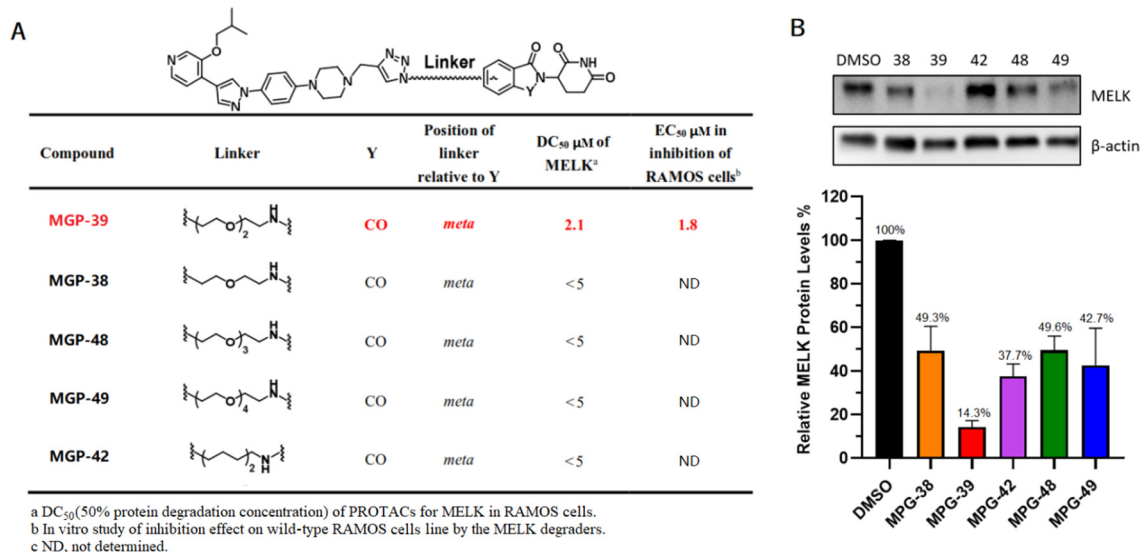
Fig. 2 Chemical structure and characterizations of novel MELK degraders. (A) SAR studies for MELK degrader evaluations. In a cell viability assay, 3000 cells per well in 96-well plates were incubated at 37 °C for 72–96 h. The final EC_{50} was calculated using CCK-8. (B) Immunoblotting analysis of novel PROTACs targeting MELK. RAMOS cells treated with compounds (5 μM) for 48 h; 2×10^5 cells per well in 12-well plates were incubated at 37 °C. Grayscale analysis data were generated using ImageJ.
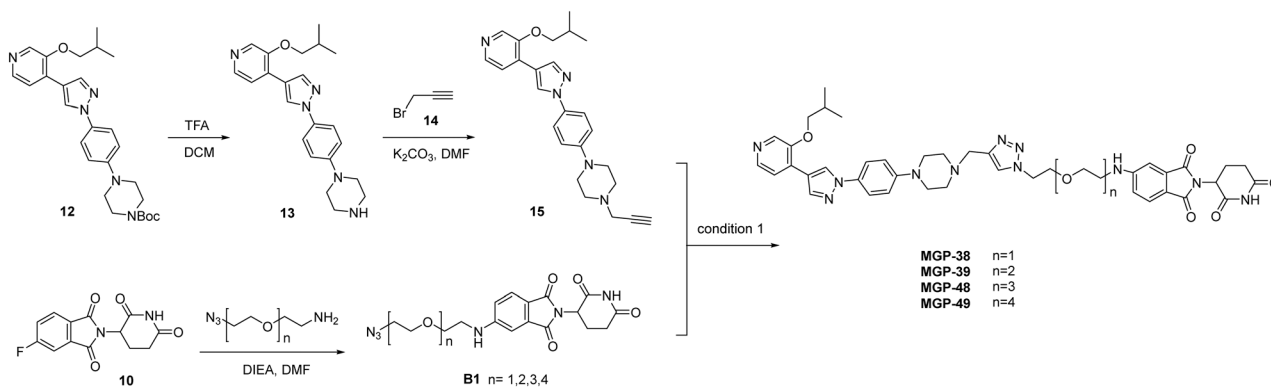
Scheme 1 Synthesis of compound **MGP-39** analogues.

linker on the bioactivity of the PROTAC (Fig. 2B). On the basis of a statistical analysis of western blots, **MGP-39** was selected for further experiments.

The synthesis of MGP-39

The synthesis of the MELK degrader **MGP-39** is shown in Scheme 1. Briefly, a substitution reaction was conducted with compound **13** and propargyl bromide as substrates to prepare intermediate **15**. Compound **10** and amine derivatives were then utilized in substitution reactions to prepare intermediates **B1**. Finally, precursors **15** and **B1** were transformed to **MGP-39** and its analogues *via* a click reaction in the last synthetic step (for details of condition 1, please see ESI†).[17] Note the relative

simplicity of the **MGP-39** synthetic route, convenient for any large-scale preparations to be considered in the future.

Identification of MGP-39 as a MELK PROTAC degrader

PROTAC-induced degradation of MELK occurred rapidly and completely. The half-life of the MELK protein in RAMOS cells was <3 h in the presence of **MGP-39** (Fig. 3A and ESI† Fig. S1). MLN4924 and MG132—as an NAE inhibitor and proteasome inhibitor, respectively—could effectively disable the function of **MGP-39** in degrading MELK (Fig. 3B). These results confirmed that the MELK degradation was mediated by the ubiquitin-proteasome system (UPS), consistent with the PROTAC mechanism. To further demonstrate the
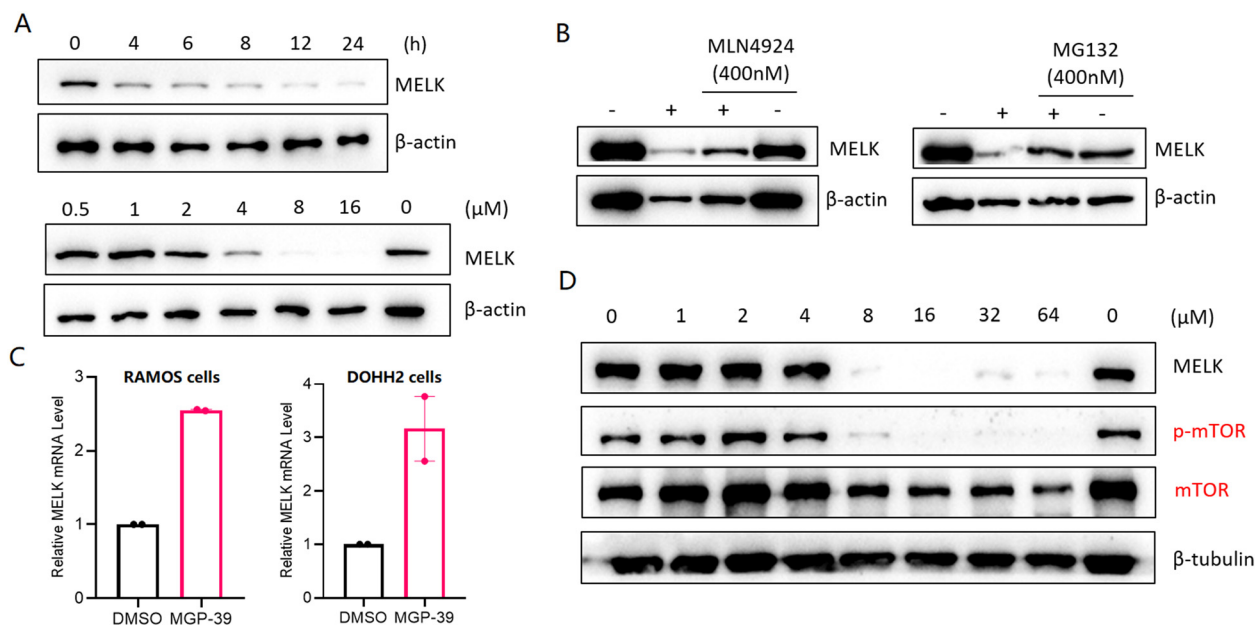
Fig. 3 Efficacy and mechanism studies of **MGP-39** in degrading MELK. (A) Western blotting analysis for MELK in RAMOS cells with 20 μM **MGP-39** for the indicated exposure times (top) and western blotting analysis for MELK in RAMOS cells with indicated concentrations of **MGP-39** for 48 h. (B) Immunoblot for MELK and β-actin after a pretreatment for 4 h with MLN4924 or MG132 (400 nM), followed by a treatment with **MGP-39** (20 μM) for 24 h in RAMOS cells. "+" refers to treatment with **MGP-39**, "−" refers to no treatment with **MGP-39**. (C) Relative MELK mRNA levels in RAMOS cells or DOHH2 cells treated with **MGP-39** (20 μM) for 24 h. (D) Western blotting analysis for MELK, p-mTOR, mTOR, and β-tubulin proteins after 24 h of treatment of RAMOS cells with indicated concentrations of **MGP-39**.
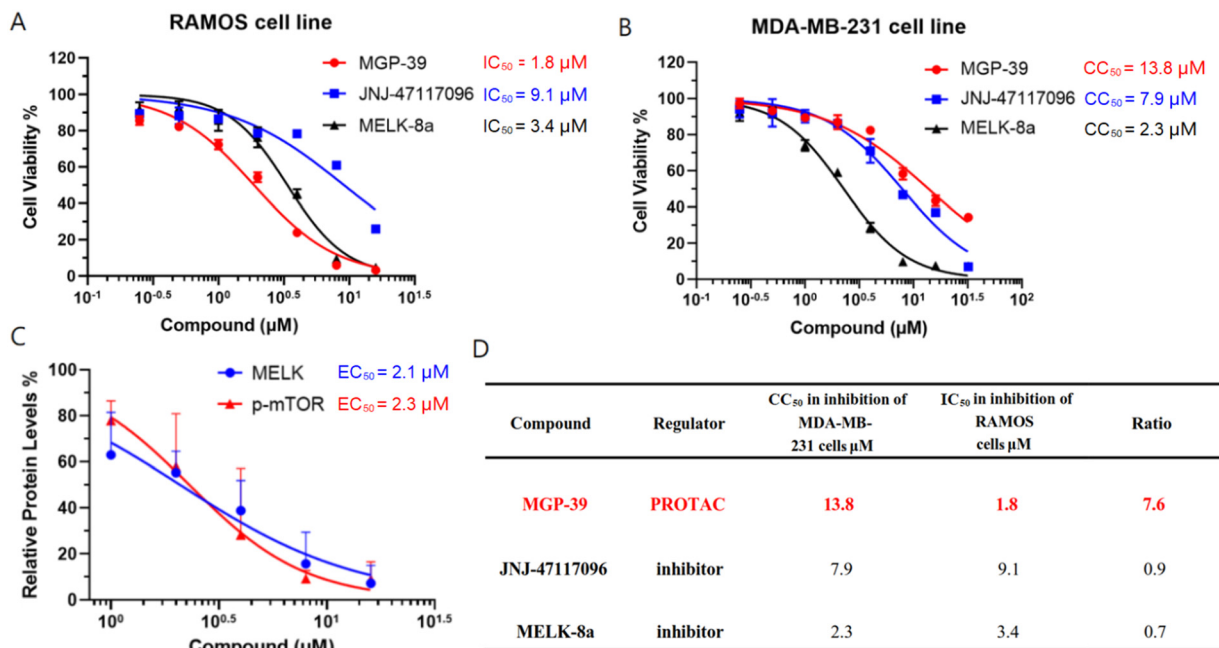
Fig. 4 Cell viability assays of MELK regulators. (A) Anti-cancer effect of MELK degrader **MGP-39** and MELK inhibitors **JNJ-47117096/MELK-8a** against the RAMOS cell line. (B) Cytotoxicity of MELK degrader **MGP-39** and MELK inhibitors **JNJ-47117096/MELK-8a**, specifically toward the MDA-MB-231 cell line. (C) Relative protein levels of MELK and mTOR in RAMOS cells after being treated with **MGP-39** for 24 h. (D) EC_{50}s of MELK regulators in inhibiting RAMOS cells and MDA-MB-231 cells.

degradation pathway used by **MGP-39**, relative MELK mRNA levels in RAMOS cells and DOHH2 cells treated with PROTACs were determined. Significantly increased levels of MELK mRNA were observed when **MGP-39** was included (Fig. 3C), with the increase achieved through a negative feedback loop.

To evaluate the detailed anti-cancer effects of **MGP-39** in BL cells, we examined the activity of the downstream signaling molecule mTOR upon MELK degradation. Indeed, **MGP-39** potently inhibited the phosphorylation of mTOR in accordance with MELK downregulation (Fig. 3D).

MGP-39 demonstrates higher efficacy and lower toxicity than do MELK inhibitors

As mentioned above, MELK is a novel and practical target for B-cell malignancy therapy. However, currently reported MELK inhibitors show serious toxicity, which leads to clinical failures. Although MELK is generally expressed in human cells, it is not necessary for the proliferation of basal-like breast cancer cells.[26] Therefore, a cell viability assay was conducted using the MDA-MB-231 cell line to determine the toxicity of the novel MELK degrader **MGP-39**. As shown in
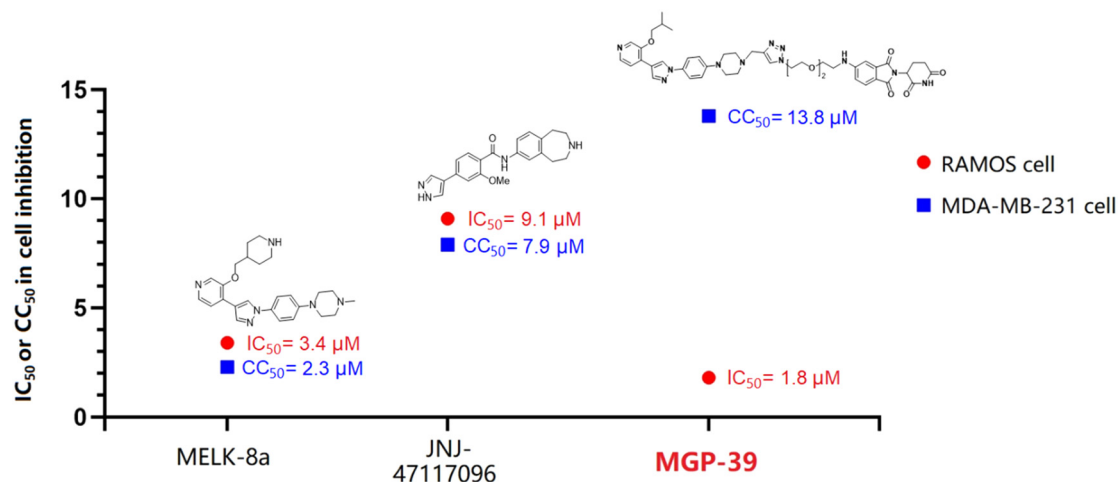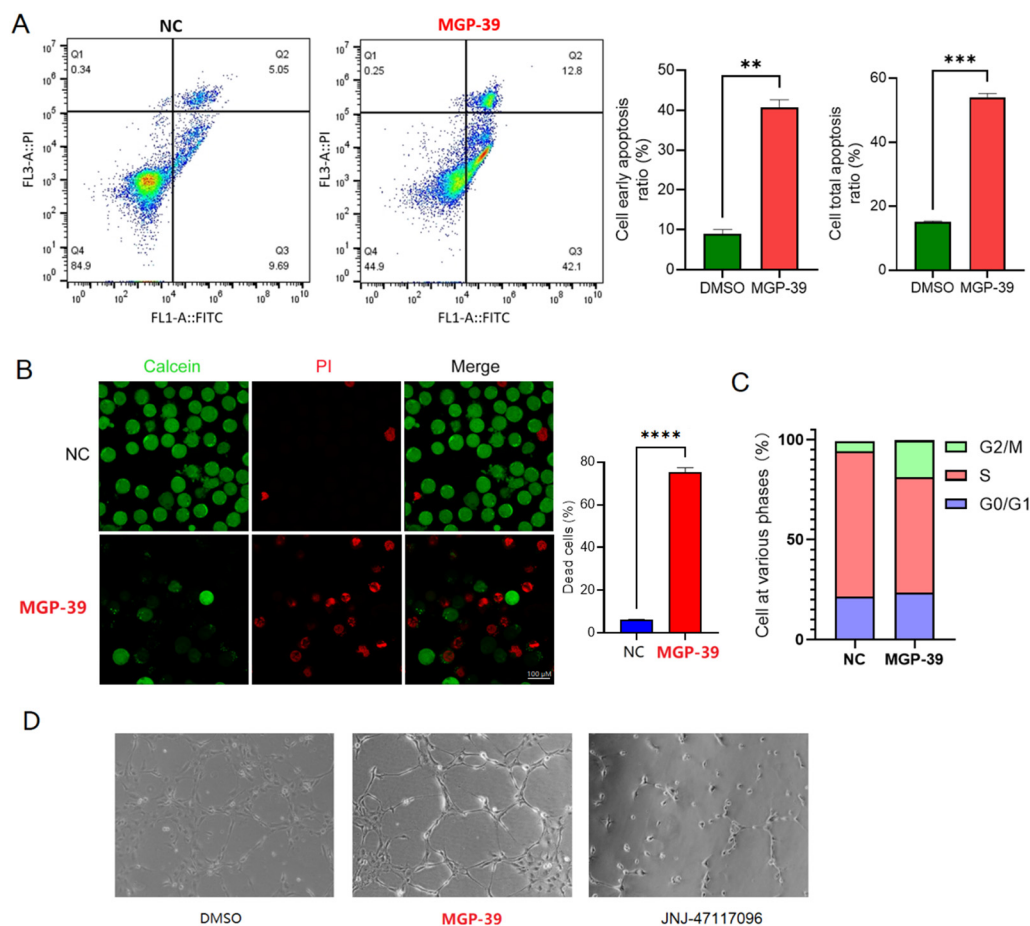

Fig. 5 Therapeutic windows of MELK regulators.

Fig. 6 Apoptosis and cell cycle arrest triggered by **MGP-39**. (A) Flow cytometry quantification of early/late apoptotic cells (RAMOS) treated with 20 μM of **MGP-39** for 24 h. **$P < 0.01$, ***$P < 0.001$. (B) Calcein AM/PI staining of RAMOS cells after being treated for 24 h with 20 μM **MGP-39**. ****$P < 0.0001$. (C) **MGP-39** arrested the cell cycle at the G2/M phase in the RAMOS cell line. Cells were treated with 10 μM of the compound for 24 h and stained with propidium iodide. The cell cycle phases were determined using flow cytometry. (D) Tube formation assays for HUVEC cells treated with the indicated compounds (4 μM) for 24 h.

Fig. 4A, **MGP-39** exhibited better anti-cancer activity than did the two tested MELK inhibitors, and exhibited the lowest cytotoxicity against MDA-MB-231 cells (Fig. 4B). Furthermore, **MGP-39** exhibited an obvious therapeutic window, while MELK inhibitors showed obvious general toxicity (Fig. 4D and 5), indicating the strong potential for developing a PROTAC-based therapeutic MELK regulator.

MGP-39 induces cell cycle arrest and apoptosis in BL cells

To further investigate the anti-cancer mechanism of **MGP-39**, detections of apoptosis and cell cycle arrest were performed using flow cytometry. The results showed that **MGP-39**, as a MELK degrader, could effectively induce both early and total apoptosis of RAMOS cells (Fig. 6A). Meanwhile, the apoptosis function of compound **MGP-39** was confirmed using a calcein AM/PI staining assay (Fig. 6B). The G2/M-phase checkpoint was established to prevent cells with damaged DNA from entering mitosis and allows for the repair of DNA. Therefore, cell cycle arrest at the G2/M phase is a promising therapeutic approach in the battle against cancer.[27] Indeed, the MELK-

targeting PROTAC **MGP-39** can potently arrest the cell cycle at G2/M in the RAMOS cell line (Fig. 6C and ESI† Fig. S2). Moreover, a tube formation assay on HUVEC cells confirmed the lower general cytotoxicity of **MGP-39** compared with MELK inhibitor **JNJ-47117096** (Fig. 6D and ESI† Fig. S3), indicating that **MGP-39**, as a new type of MELK PROTAC, could be of clinical significance in the treatment of B-cell malignancies.

Conclusions

In summary, we have developed a first-in-class MELK-targeting PROTAC strategy for Burkitt lymphoma treatment. Remarkably, the representative PROTAC molecule **MGP-39** could degrade the MELK protein with high efficacy. Furthermore, downstream signaling activities were strongly blocked by **MGP-39**, indicating a clear anti-cancer signaling pathway of the novel degrader. Moreover, **MGP-39** arrested the cell cycle at G2/M and significantly induced early/late apoptosis in the RAMOS cell line. More importantly, compared with MELK inhibitors, the MELK degrader **MGP-39** not only had better anti-cancer activity

against Burkitt lymphoma, but also showed lower general cytotoxicity. Therefore, these findings indicated that **MGP-39**, a novel PROTAC molecule targeting MELK, may be used as part of a safe and effective method for cancer therapy and may have the potential to overcome the clinical failures of MELK inhibitors in treating NHL.

Author contributions

The project and experiments were designed by Y. S. and X. L. The chemistry was performed by Y. S., Q. H., X. L., Y. W. and N. Z. The biological research was carried out by Y. S., X. L. and W. Y. All authors contributed to the manuscript writing and review process.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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