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ST2825, independent of MyD88, induces reactive oxygen species-dependent apoptosis in multiple myeloma cells

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ABSTRACT

Myeloid differentiation factor 88 (MyD88), which is a key regulator of nuclear factor kappa B (NF-κB), plays an important role in tumorigenesis in lymphoid malignancies such as Waldenstrom's macroglobulinemia (WM). However, its biological function in multiple myeloma (MM), which is a malignant plasma cell disorder like WM, remains unexplored. In this article, we first demonstrated that higher expression *MyD88* was significantly correlated with poor survival in patients with MM using multiple publicly available datasets. Interestingly, bioinformatic analysis also revealed that *MyD88* gene alteration, which is recognized in nearly 80% of patients with WM, was extremely rare in MM. In addition, ST2825 (a specific inhibitor of MyD88) suppressed cell growth followed by apoptosis. Furthermore, ST2825 induced intracellular reactive oxygen species (ROS) in MM cells, and N-acetyl-L-cysteine, which is known as a ROS scavenger, significantly decreased the number of apoptotic MM cells evoked by ST2825 treatment. Taken together, our results indicated that ST2825 leads to ROS-dependent apoptosis in MM cells and could be an attractive therapeutic candidate for patients with MM. By highlighting the pathological mechanism of MyD88 in MM, this study also provides novel treatment strategies to conquer MM.

1. Introduction

Multiple myeloma (MM), which is characterized by clonal evolution of plasma cells in the bone marrow, accounts for approximately 10% of all hematologic malignancies [1]. The global incidence of MM reportedly amounted to over 176,000 cases and mortality was over 117,000 patients in 2020 [2]. The overall survival (OS) has improved for patients with MM because of the advent of novel treatment strategies including proteasome inhibitors, immunomodulatory imide drugs (IMiDs), and immunostimulatory monoclonal antibodies. More recently, B-cell maturation antigen-directed chimeric antigen receptor T-cell therapy has also been administered in patients with relapsed and refractory MM. However, in spite of the advance of treatment strategies, MM is still considered to be an incurable malignancy especially in subsets of patients with genetic abnormalities such as t(4; 14), t(14; 16), t(14,20), del 17p, *p53* mutation, and gain 1q [3–5]. Thus, there remains a strong need for further new treatment strategies to combat MM.

Myeloid differentiation factor 88 (MyD88), which was first identified as a myeloid differentiation primary response gene in 1990, is an essential adaptor molecule for toll-like receptors and mediates the induction of inflammatory cytokines via nuclear factor kappa B (NF-κB) [6,7]. Constitutive activation of NF-κB signaling is related to progression of various malignancies including lymphoid neoplasms [8]. In fact, 29% of patients with activated B-cell-like subtype of diffuse large B-cell lymphoma harbored the L256P gain-of-function mutation in *MyD88*, implicating the mutation in disease pathogenesis by regulating NF-κB signaling, JAK kinase activation of STAT3, and secretion of IL-6, IL-10, and interferon-β [9]. Furthermore, *MyD88* L265P mutation was acquired in 79% of Waldenstrom's macroglobulinemia (WM), which was considered the first genetic hit in WM [10]. However, little is known about the pathological role of MyD88 in MM although both WM and MM have monoclonal gammopathy as a common feature.

ST2825, an inhibitor of MyD88 dimerization, reportedly interferes with the recruitment of interleukin 1 receptor associated kinase 1 and 4 by MyD88. That resulted in the inhibition of interleukin 1 beta-mediated activation of NF-κB activity [11]. Preclinical studies have demonstrated the antitumor activity of ST2825 in multiple types of malignancies such as lymphoma, leukemia, and osteosarcoma [12,13]. However, neither the pathological role of MyD88 nor the treatment effect of ST2825 is currently under investigation in MM.

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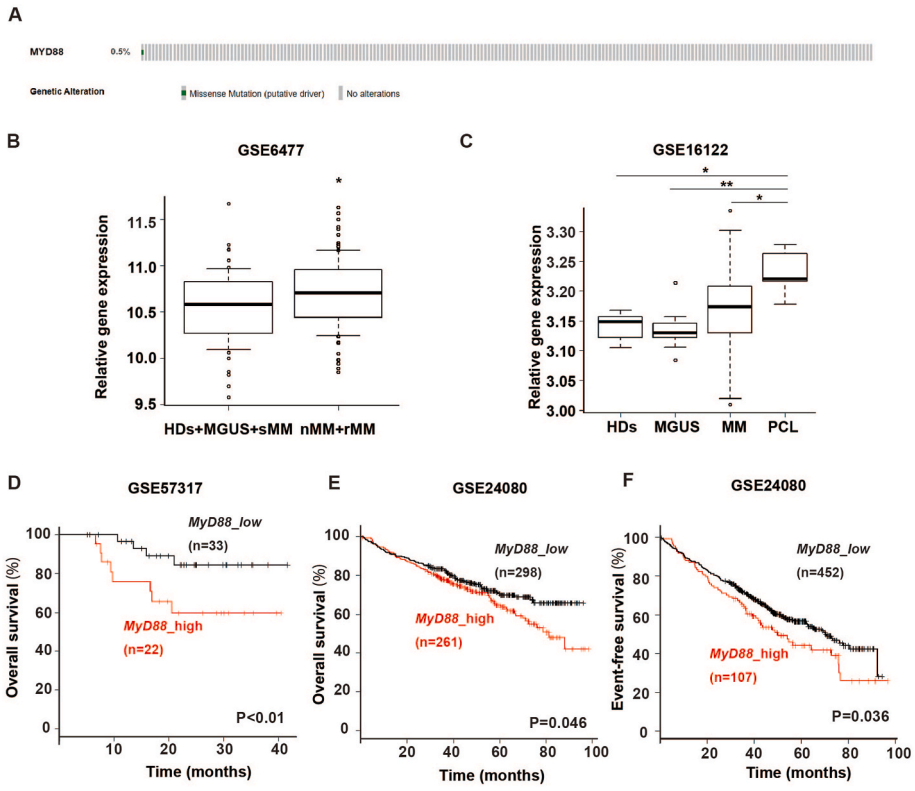

Fig. 1. *MyD88* is upregulated in symptomatic MM and associated with poor survival despite the low alteration frequency. A. The frequency of myeloid differentiation factor 88 (*MyD88*) alteration in multiple myeloma (MM) using the publicly accessible gene expression profiling dataset obtained from the cBioPortal. B, C. Publicly accessible gene expression profiling datasets GSE6477 (B) and GSE16122 (C) were used to evaluate the expression of *MyD88* in patients with MM. $^{*}P < 0.05$, $^{**}P < 0.01$. D-F. Kaplan Meier analysis was used to evaluate the clinical significance of the relationship of *MyD88* to survival time. Overall survival was analyzed with GSE57317 (D), GSE24080 (E), and event-free survival was analyzed with GSE24080 (F). HDs, healthy donors; MGUS, monoclonal gammopathy of undetermined significance; sMM, smoldering MM; nMM, newly diagnosed MM; rMM, relapsed MM.

In the present study, we initially demonstrated that patients with MM overexpressing *MyD88* had poor prognosis. This finding suggested that MyD88 has a potential to be a reliable prognosis predictor in patients with MM. Furthermore, ST2825 has anti-myeloma activity in vitro settings, which suggests that it could be a promising therapeutic agent.

2. Materials and methods

2.1. Databases and gene expression data analysis

Gene expression levels of *MyD88* in MM were evaluated using publicly available datasets (GSE6477 and GSE16122) from the Gene Expression Omnibus (GEO) database. The correlation between *MyD88* mRNA levels and clinical outcome was also assessed using publicly accessible datasets (GSE57317 and GSE24080) from the GEO database. The cutoff value was determined using a receiver operating characteristic curve. In brief, 55 patients in GSE57317 and 559 patients in GSE24080 were categorized as presenting high or low levels of *MyD88*. Kaplan-Meier analysis of OS in GSE57317, and OS and event-free survival in GSE24080 were performed to determine the significance of the difference in survival between two groups: one expressing high levels of *MyD88* and one expressing low levels of *MyD88*.

2.2. Reagents and human cell lines

ST2825 was purchased from MedChem Express (Princeton, NJ, USA) and dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10 mM or 100 mM depending on the experiments. RPMI8226, U266, and KMS11 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in RPMI 1640 (Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 2 μM L-glutamine, and 1% penicillin-streptomycin. Cell lines also underwent authentication testing using short tandem repeat DNA profiling within the last five years.

2.3. Quantitative reverse transcription-PCR

RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed with a SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific). We performed quantitative reverse transcription–PCR (qRT–PCR) with an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The analysis of target genes was conducted in quadruplicate, and transcript levels were normalized to β-actin expression.

2.4. Western blot

Cells were cultured, then harvested, washed, lysed, and stained using the following primary antibodies: anti-cleaved PARP (#5625; Cell Signaling Technology, Danvers, MA, USA), anti-cleaved caspase 3 (#9664S; Cell Signaling Technology), and anti-actin–horse radish peroxidase (HRP; sc1615; Santa Cruz Biotechnology).

2.5. Evaluation of cell growth

MM cells were seeded at densities of 1×10^4 cells/well into 96-well plates. After treatment with ST2825 for 48 h, cell viability was evaluated using a WST-1 assay (Premix WST-1 Cell Proliferation Assay; Takara Bio, Otsu, Japan) and an Infinite M1000 PRO microplate reader (Tecan

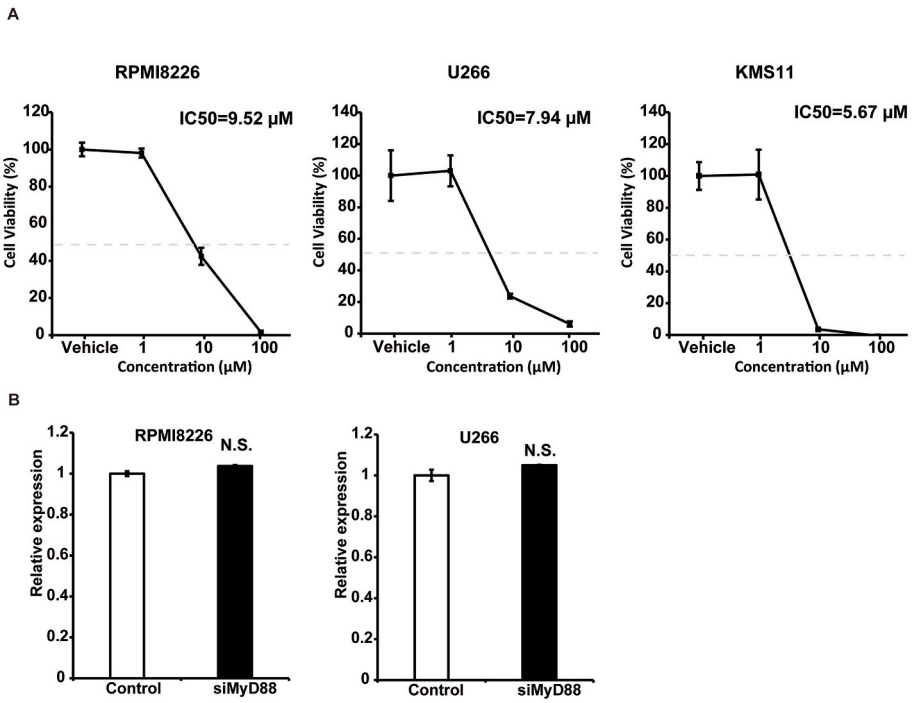

Fig. 2. ST2825, independent of MyD88, inhibits multiple myeloma cell proliferation in a dose dependent manner. A. RPMI8226, U266, and KMS11 cells were treated with the indicated dose of ST2825 for 48 h. Cell viability was assessed with the WST-1 assay. Data are represented as percentage of cell viability normalized to the control group. Data are the mean of triplicate measurements. Error bars represent the standard deviation. B. RPMI8226 and U266 cells were transfected with non-targeting siRNA (Control) and siRNA targeting myeloid differentiation factor 88 (*MyD88*). Cell viability was evaluated using a WST-1 assay at 48 h after siRNA transfection. Data are the mean of triplicate measurements. Error bars represent the standard deviation. N.S.; not significant.

Japan, Kawasaki, Japan).

2.6. Flow cytometry assays

MM cells were seeded at densities of 1×10^6 cells/well into 6-well plates and treated with ST2825 for 48 h. Subsequently, apoptosis was measured using an Annexin V/7-AAD staining kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol, followed by analysis on a BD FACS Canto II instrument with FACSDiva software (BD Biosciences) as previously described [14]. To assess the anti-apoptotic activity of pan-caspase inhibitor Z-VAD-FMK (Promega, Madison, WI, USA) and IL-6 (FUJIFILM Wako Chemicals, Richmond, VA, USA), RPMI8226 cells were seeded at densities of 1×10^6 cells/well into 6-well plates, treated with ST2825 for 24 h, and apoptotic cells were measured using an annexin V/7-AAD staining kit as mentioned above.

Intrinsic ROS levels were evaluated with a CellROX Deep Red Flow Cytometry Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RPMI8226 cells were seeded at densities of 1×10^6 cells/well into 6-well plates with or without the pretreatment of N-acetyl-L-cysteine (NAC; 10 mM) for 2 h and treated with ST2825 for 48 h. Cells were stained with the CellROX Deep Red reagent for 30 min at 37 °C. Cells were washed and suspended in phosphate buffered saline and analyzed using a BD Canto II instrument with FlowJo software (BD Bioscience). *Tert*-butyl hydroperoxide (T-BHP; Sigma–Aldrich) was used as an oxidizer to evaluate ROS-mediated cytotoxicity in MM cells. RPMI8226 cells were seeded at densities of 1×10^6 cells/well into 6-well plates, pretreated with or without NAC for 2 h, treated with t-BPH, and evaluated for apoptotic characteristics using an annexin V/7-AAD staining kit as mentioned above.

2.7. Statistical analysis

For statistical analyses, the Student *t*-test or Mann-Whitney *U* test were utilized. Statistical significance was defined as P values < 0.05. EZR software version 1.33 was used for the statistical analyses [15].

3. Results

3.1. Overexpression of MyD88 is significantly associated poor overall survival and event-free survival in MM patients

As *MyD88* is frequently altered in patients with WM, we hypothesized that *MyD88* would also be altered in patients with an allied disease, MM. To explore it, we investigated the gene alteration status of 205 patients with MM using a cBioPortal (https://www.cbioportal.org/) database. Interestingly, out of the 205 patients only one, who had L265P mutation, had altered *MyD88* contradictory to our hypothesis (Fig. 1A). Using the GSE6477 dataset, we next proceeded to evaluate the expression of *MyD88* in patients with symptomatic MM (newly diagnosed MM [nMM] and relapsed MM [rMM]) in comparison with normal healthy donors (HDs), patients with monoclonal gammopathy of undetermined significance (MGUS), and patients with smoldering MM (sMM). *MyD88* in patients with symptomatic MM was significantly upregulated compared to that in HDs, and those with MGUS and sMM (Fig. 1B). Similarly, analysis of GSE16122 revealed that *MyD88* expression was higher in patients with MM than in MGUS patients and HDs although there was no statistically significant difference. Furthermore, *MyD88* expression was significantly higher in PCL patients than in MM patients, MGUS patients, and HDs (Fig. 1C). These data indicate that *MyD88* expression seems to be increasingly elevated as MM progresses. We also investigated the clinical impact of *MyD88* expression on survival in patients with MM using the publicly available gene expression datasets GSE57317 and GSE24080. The cut-off value was determined using a ROC curve. *MyD88* transcript levels were statistically significantly negatively correlated with OS in both datasets and with EFS in GSE24080 (Fig. 1D–F).

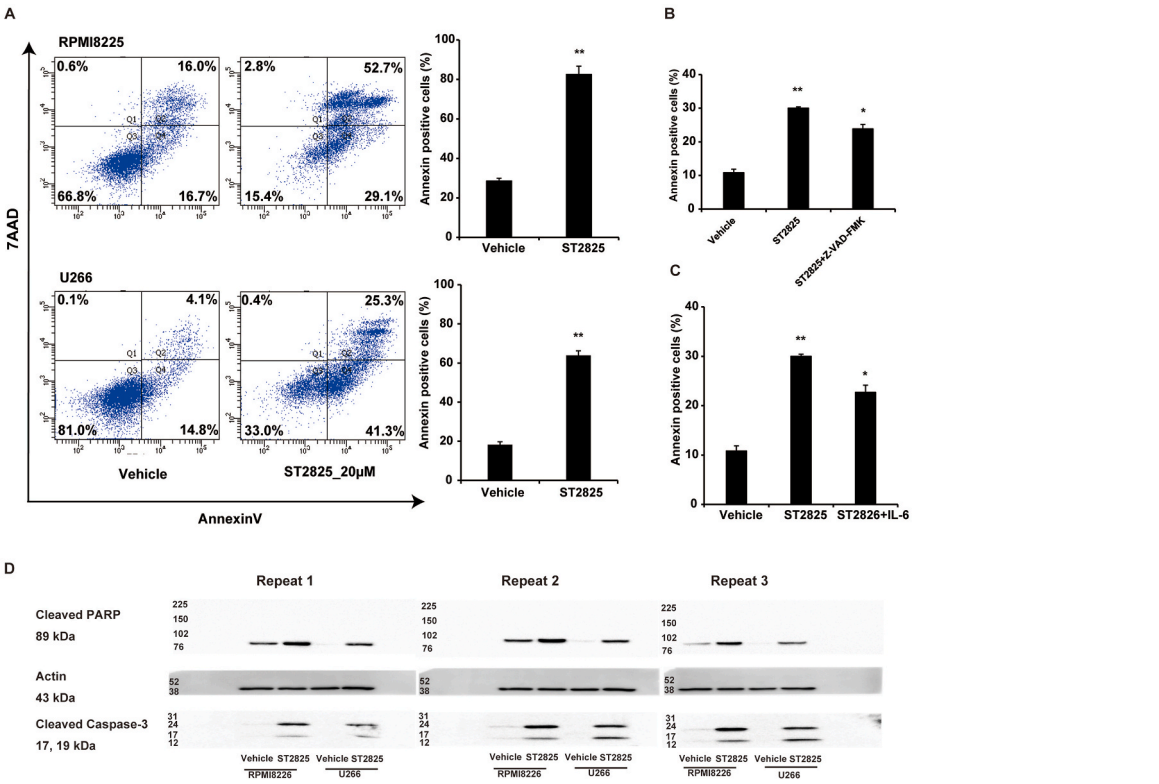

Fig. 3. ST2825 induces apoptosis in multiple myeloma cell lines. A. RPMI8226 and U266 cells were treated with ST2825 at the concentration of 20 μM for 48 h. Cells were collected, and apoptosis was measured by flow cytometry using Annexin/7AAD staining. Bar plots represent the percentage of annexin-positive cells. Data are the mean of triplicate measurements. Error bars represent the standard deviation. $^{**}P < 0.01$. B, C. To assess the anti-apoptotic activity of pan-caspase inhibitor Z-VAD-FMK (B) and IL-6 (C), RPMI8226 cells were seeded at densities of 1×10^6 cells/well into 6-well plates, treated with ST2825 for 24 h, and apoptotic cells were measured using an annexin V/7-AAD staining kit. Error bars represent the standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$. D.We performed immunoblot analysis, using anti-cleaved PARP, anti-cleaved caspase 3, and β-actin antibodies, of total protein extracts obtained from RPMI8226 and U266 cells treated with 20 μM ST2825 for 48 h.

3.2. ST2825, an inhibitor of MyD88, dose-dependently inhibits growth of MM cells

As overexpression of *MyD88* was correlated with poor prognosis in MM, we proceed to verify the anti-myeloma activity of ST2825, which is a MyD88 dimerization inhibitor [16]. ST2825 significantly reduced the proliferation of three different cell lines, RPMI8226, U266, and KMS11, in a dose-dependent manner. The IC50 values were 9.52 μM for RPMI8226, 7.94 μM for U266, and 5.62 μM for KMS11 cells (Fig. 2A). Next, to investigate the impact of MyD88 on MM, we conducted knock-down of *MyD88* using small interfering RNA (siRNA) in MM cell lines. qRT-PCR and Western blot were used to evaluate the knock-down efficiency. *MyD88* was successfully down regulated in both cell lines (Supplementary Figs. S1A and B). However, contrary to our expectations, knock-down of *MyD88* did not affect the proliferation of either MM cell line (Fig. 2B). Furthermore, ST2825 treatment did not down-regulate the mRNA expression of TNF receptor-associated factor 6 (*TRAF6*), which is reportedly essential for MyD88-dependent signaling [17] (Supplementary Fig. S2). These results indicate that the anti-myeloma activity of ST2825 was mediated by a MyD88 independent pathway.

3.3. ST2825 induces apoptosis in MM cells

To explore the mechanism of cytotoxicity triggered by ST2825 in MM cells, we performed flow cytometry analysis. Annexin/7AAD staining showed significantly increased percentage of apoptotic RPMI8226 and U266 cells treated with 20 μM of ST2825 for 48 h (Fig. 3A). The percentage of apoptotic cells was significantly reduced when treated with pan-caspase inhibitor Z-VAD-FMK at the concentration of 50 μg/mL for 24 h. These results provided strong evidence of apoptosis evoked by ST2825 treatment in MM cells (Fig. 3B). Furthermore, co-culture with ST2825 and IL-6, which is produced by marrow stromal cells and plays a crucial role as a growth factor in MM, also resulted in the significant decrease of apoptotic cells compared to ST2825 single treatment (Fig. 3C). We also investigated caspase activity of MM cells treated with ST2825 by immunoblotting. ST2825 increased proteolytic cleavage of PARP and caspase 3 which is a classic characteristic of apoptosis (Fig. 3D).

3.4. ST2825 induces reactive oxygen species in MM cells

We next measured intracellular ROS levels to evaluate whether ST2825 stimulated ROS generation in MM cells, as several anti-cancer drugs reportedly stimulate the production of ROS in cancer cells [18, 19]. First, RPMI8226 cells were treated with t-BPH for 24 h to evaluate the cytotoxicity of ROS to MM cells. The number of apoptotic cells was significantly increased when treated with t-BPH and the increase was completely cancelled by pre-treatment with NAC (Fig. 4A). Next, flow cytometry analysis using CellROX Deep Red revealed that levels of intracellular ROS in RPMI8226 cells treated with ST2825 for 24 h were dose-dependently increased. Furthermore, NAC significantly decreased the level of ST2825-induced ROS (Fig. 4B) and also significantly suppressed the percentage of ST2825-induced apoptotic cells (Fig. 4C). Based on these findings, we conclude that ST2825 inhibits proliferation of MM cells by stimulating ROS-dependent apoptosis.

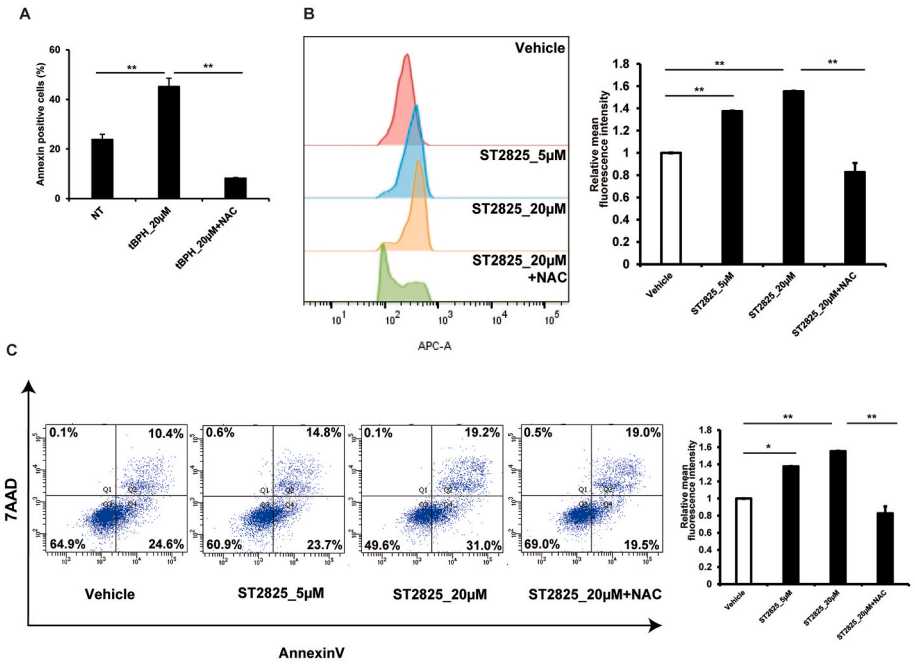

Fig. 4. ROS induction was increased by ST2825 treatment in multiple myeloma cell lines. A. RPMI8226 cells were seeded at densities of 1×10^6 cells/well into 6-well plates, pretreated with or without NAC for 2h, treated with *tert*-butyl hydroperoxide, and evaluated for apoptotic characteristics using an annexin V/7-AAD staining kit. Error bars represent the standard deviation. $^{**}P < 0.01$. B. RPMI8226 cells were treated with ST2825 at the concentration of 20 μM for 48 h. Cells were collected, and flow cytometry analysis using CellROX Deep Red reagent was conducted to detect the reactive oxygen species (ROS) levels after treatment with ST2825. Bar plots represent the percentage of annexin-positive cells. Data are the mean of triplicate measurements. Error bars represent the standard deviation. $^{**}P < 0.01$. C. RPMI8226 cells were pre-treated with N-acetyl-L-cysteine (NAC) at the concentration of 10 mM for 2 h. Subsequently, the cells were collected, and then treated with 20 μM ST2825 for 24 h, collected again, and measured by flow cytometry using Annexin/7AAD staining to determine the extent of apoptosis. Bar plots represent the percentage of annexin-positive cells. Data are the mean of triplicate measurements. Error bars represent the standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

4. Discussion

In the current study, we first demonstrated that *MyD88* transcript levels were up regulated in MM and negatively correlated with OS in patients with MM. Although these results prompted us to conduct loss-of-function analysis using siRNA to evaluate the biological significance of MyD88 in MM cells, *MyD88* silencing did not affect the proliferation of MM cells. Furthermore, we revealed that ST2825 exerted anti-tumor activity through induction of apoptosis in MM cells. These results indicate that the anti-tumor activity evoked by ST2825 is independent of MyD88. Therapeutic advances have dramatically extended the OS of patients with MM in the last several years. However, MM is still an incurable and dismal disease as most patients are refractory or relapse after one or more treatment regimens. Thus, new treatment strategies are strongly desired to improve the prognosis of patients with MM. Our study demonstrated that ST2825 has the potential to be a new therapeutic candidate for treating MM.

In this study, we also revealed that apoptosis evoked by ST2825 treatment was dependent on ROS generation. ROS have a dual role in cell metabolism. At low to moderate levels, ROS activate cell proliferation, migration, invasion, and angiogenesis. In contrast, excessive levels of ROS cause cell death [20]. Manipulating ROS is regarded as a promising treatment strategy [21]. Indeed, proteasome inhibitors such as bortezomib and carfilzomib, which are current key drugs for the treatment of MM, cause cell death via stimulating overproduction of ROS [22]. Also, combinations of bortezomib and clinically relevant HDAC inhibitors synergistically induce apoptosis in MM cells through a ROS-dependent mechanism [23]. Based on these findings, ROS induction with ST2825 could be a reasonable treatment for MM in common with well-established therapeutic agents such as proteasome inhibitors.

Interestingly, however, it was reported that ROS were decreased after ST2825 treatment in murine microglia cells, which led to suppression of neuroinflammation [24]. Although these results seem inconsistent, it implies that ST2825 works differently depending on the cell types. Cancer cells normally exhibit higher basal levels of ROS compared to normal cells because of an imbalance between oxidants and antioxidants. Redox therapy with antioxidants is usually considered to be beneficial for cancer treatment. In contrast, several investigations revealed that representative antioxidants NAC and vitamin E increased tumor progression and cancer metastasis [25,26]. As mentioned above, many chemotherapeutics increase the production of ROS in cancer cells. The induction of ROS is a promising strategy to treat cancer. Conversely, antioxidant therapy has represented an effective strategy for the treatment of inflammatory diseases such as inflammatory bowel disease, cardiovascular disease, and brain diseases [27]. ST2825 is a unique compound as it induces or reduces ROS depending on the cell type. Further studies are needed to clarify the exact mechanism of ST2825-induced ROS production for the purpose of manipulation.

In summary, ST2825 induces ROS-dependent apoptosis in MM cells. Although there is still a room for further investigation about the mechanism of ROS production for the purpose of manipulation, our study highlights ST2825 as a promising novel therapeutic candidate to combat MM.

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CRediT authorship contribution statement

Hajime Nakamura: Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yohei Arihara:** Investigation, Formal analysis, Data curation. **Makoto Usami:** Investigation, Formal analysis, Data curation. **Kohichi Takada:** Writing – review & editing, Supervision, Project administration, Investigation,

Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Kohichi Takada reports financial support was provided by Daiichi Sankyo.

Data availability

Data will be made available on request.

Acknowledgments

We dedicate our efforts in memory of Satoshi Suzuki who passed away in 2021 from POEMS syndrome.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101681.

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