RSK2<sub>Ser227</sub> at N-Terminal Kinase Domain Is a Potential Therapeutic Target for Multiple Myeloma

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Abstract

Multiple myeloma is an entity of cytogenetically and genetically heterogenous plasma cell neoplasms. Despite recent improvement in the treatment outcome of multiple myeloma by novel molecular-targeted chemotherapeutics, multiple myeloma remains incurable. The identification of a therapeutic target molecule in which various signaling for cell-survival converge is a core component for the development of new therapeutic strategies against multiple myeloma. RSK2 is an essential mediator of the ERK1/2 signaling pathway for cell survival and proliferation. In this study, we discovered that RSK2<sub>Ser227</sub>, which is located at the N-terminal kinase domain and is one site responsible for substrate phosphorylation, is activated through phosphorylation regardless of the type of cytogenetic abnormalities or upstream molecular signaling in all 12 multiple myeloma–derived cell lines examined and 6 of 9 patient-derived CD138-positive primary myeloma cells. The chemical inhibition of RSK2<sub>Ser227</sub> by BI-D1870 or gene knockdown of RSK2 inhibits myeloma cell proliferation through apoptosis induction, and this anti-myeloma effect was accompanied by downregulation of c-MYC, cyclin D, p21<sup>WAF1/CIP1</sup>, and MCL1. RSK2<sub>Ser227</sub> inhibition resulting from BI-D1870 treatment restored lenalidomide-induced direct cytotoxicity of myeloma cells from interleukin-6–mediated cell protection, showed no cross-resistance to bortezomib, and exerted additive/synergistic antiproliferative effects in conjunction with the mTOR, histone deacetylase, and BH3-mimicking BCL2/BCLX<sub>L</sub> inhibitors. These results suggest that RSK2<sub>Ser227</sub> is a potential therapeutic target not only for newly diagnosed but also for patients with later phase multiple myeloma who are resistant or refractory to currently available anti-myeloma therapies.

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Introduction

Despite the marked improvement of treatment outcomes for multiple myeloma during the last decade through the development of new therapeutic modalities such as bortezomib (BTZ) and immunomodulatory derivatives (IMiD; ref. 1), multiple myeloma remains mostly incurable due to both cell-intrinsic and -extrinsic drug-resistant mechanisms. The former is induced by highly complex and heterogeneous molecular abnormalities (2, 3), whereas the latter is generated by various myeloma microenvironment factors, such as soluble factors, or adjacent bone marrow (BM) stromal cells (4, 5). Therefore, the search for a therapeutic target molecule in which various types of signaling for myeloma cell survival converge is essential for the development of a more effective therapy for multiple myeloma.

RSK2 is a member of the 90 kDa ribosomal S6 kinase family of serine/threonine (Ser/Thr) kinases that regulates diverse cellular processes, such as cell proliferation, cell motility, or cell survival (6). In hematopoietic system, RSK2 is phosphorylated in B and T lymphocytes in physiologic condition, whereas its further phosphorylation has been shown to be essential in their activation (7, 8). It is located downstream of the RAS/ERK1/2 signaling cascade, which is activated in response to oncogenic signalings and/or growth factor stimuli (6, 9). In this process, ERK1/2 activates RSK2 by phosphorylating Thr577 of its carboxy-terminal kinase domain (CTKD) and Thr365 and Ser369 in the linker region. Subsequently, CTKD phosphorylation leads to autophosphorylation of Ser386 in the linker region of RSK2 to generate the PDK1 binding site, after which PDK1 binds and phosphorylates RSK2 at Ser227 of the N-terminal kinase domain (NTKD), which
is responsible for phosphorylation of various downstream substrates, including transcriptional regulators and molecules associated with cell survival, cell cycle, or cell metabolism (6). Recent studies have shown that RSK2 acts as the key regulator for cellular transformation and metastasis by mediating signaling through oncogenic tyrosine kinases (TK; refs. 10–12). As for myeloma, previous studies have suggested the importance of RSK2 signaling associated with fibroblast growth factor receptor 3 (FGFR3) activation by t(4;14) (13, 14). Considering that the activation of RAS/ERK1/2 cascades may occur through various cell-intrinsic molecular abnormalities and extracellular microenvironmental signals regardless of t(4;14) status (15–17), we hypothesized that RSK2 could be a possible general therapeutic target for multiple myeloma therapy. In this study, we investigated the activation of RSK2, especially of RSK2Ser227 at NTKD, in myeloma-derived cell lines and patient-derived primary myeloma cells with various cytogenetic abnormalities, and assessed the importance of RSK2 as a potential therapeutic target for multiple myeloma.

Materials and Methods

Cells and reagents

This study used the human myeloma cell lines NCI-H929, OPM-2, LP-1, PRM80226, IM9, AMO-1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), KMS-11, KMS-18, KMS-12-BM, KMS-28-PE, KMS-34, KMS-20 (kind gifts from Dr. Ohitsuji, Kawasaki Medical School, Okayama, Japan), and AMU-MM1 (18). OPM-2, KMS-11, and KMS-18 have been shown to express variants of FGFR3 (K650E, Y373C, and G384D, respectively; ref. 19). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mmol/L-glutamate, penicillin/streptomycin. Subclones of OPM-2 and KMS-11 (OPM-2/BTZ and KMS-11/BTZ), which are resistant to bortezomib, were developed under continuous exposure to bortezomib in the same medium over half a year. These cells acquire bortezomib resistance by a mutation of gene encoding proteasome beta5 subunit, which suppresses apoptotic signals through the inhibition of unfolded protein accumulation and subsequent excessive ER stress (20). No further authentication was carried out. Studies using patient samples were approved by the Ethics Review Board of our institute. Studies using patient samples were approved by the Ethics Review Board of our institute.

RNA interference

RNA interference (RNAi) for RSK2 was conducted by transfecting small-interfering RNA (siRNA) into NCI-H929 cells. The sequence of the sense strand for siRNA for RSK2 was 5-GUAUCAGUCCAGUCUUAATT-3 and that of the antisense siRNA was 5-UUUAAGACUGGA-CUGAUACCTT-3. The corresponding sequences for control were 5-UCUUAAUCGGAUAGCCCTT-3 and 5-GCCUUAAUCGGAGAUAAGATT-3. Briefly, 2.5 × 10⁶ NCI-H929 cells were resuspended with 100 μL of transfection buffer and transfected with siRNA for RSK2 or with control siRNA (Takara Bio Inc.) by means of the CLB-Transfection Kit (Lonzza AG) using protocol 9.

Drug combination assays

Cells were treated with various concentrations of bortezomib, lenalidomide, melphalan, adriamycin, everolimus, MS-275, or ABT-263 alone or combined with BI-D1870 for 48 hours. The fractional effect concentrations (i.e., a fractional effect of 0.25 equals a 25% growth inhibitory effect) and the combination index (CI) were calculated with CalcuSyn (Biosoft). This method facilitates the quantification of synergism (CI < 1) and antagonism (CI > 1) at different doses and effect levels. CI calculations were conducted on the assumption that the drug mechanisms were not mutually exclusive.

Western blot analysis

Western blot analysis was conducted as described elsewhere (21, 22). Primary antibodies used were described in Supplementary Information.

Fluorescence in situ hybridization

Cytogenetic abnormalities were examined by means of interphase fluorescence in situ hybridization (FISH) analysis (23). Probes used were described in the Supplementary Information.

Assays for growth inhibition and apoptosis

Assays for growth inhibition and apoptosis were conducted as previously described (21, 22; Supplementary Information).

Results

RSK2Ser227 is phosphorylated regardless of type of cytogenetic or upstream molecular signaling in multiple myeloma cell lines

We first identified that RSK2Ser227 was phosphorylated in all 12 multiple myeloma cell lines examined (Fig. 2A). Normal peripheral lymphocytes were used as the positive control (7, 8). Among 12 cell lines, t(4;14) involving IGH/FGFR3 was detected in only 7 cell lines (NCI-H929, OPM-2, LP-1, KMS-18, KMS-28-PE, KMS-34, and AMU-MM1).
and high-FGFR3 expression in only 6 cell lines (NCI-H929, OPM-2, KMS-18, KMS-12-BM, KMS-28-PE, and KMS-34). These findings indicate that, in contrast to previous studies (13, 14), FGFR3 activation is not always a prerequisite for RSK2Ser227 phosphorylation. Our study also did not show any positive relationship between RSK2Ser227 phosphorylation and other so-called high-risk cytogenetic abnormalities (Supplementary Table S1). Also, RSK2Ser227 was phosphorylated even in the absence of ERK1/2 phosphorylation in several multiple myeloma cell lines, indicating that ERK1/2 activation is not mandatory for RSK2Ser227 phosphorylation in multiple myeloma cell lines. Indeed, although a MEK inhibitor U0126 completely inhibited the phosphorylation of ERK1/2 and inhibited the cell proliferation in NCI-H929 cells with t(4;14) and active ERK1/2, it only partly inhibited RSK2Ser227 phosphorylation. Also, U0126 failed to inhibit the cell growth and RSK2Ser227 phosphorylation in KMS-12-BM, which lacks t(4;14) or ERK1/2 phosphorylation (Fig. 2B). Moreover, RSK2Tyr529 phosphorylation at CKTD was observed only in 5 of 12 cell lines examined in our study, indicating that phosphorylation of RSK2Tyr529 seems to be irrelevant for the phosphorylation of Ser227 in multiple myeloma cell lines. Collectively, RSK2Ser227 may exert its activity as a site responsible for substrate phosphorylation through constitutive phosphorylation regardless of the type of cytogenetic abnormalities or upstream molecular signaling status in most multiple myeloma cell lines.

Inhibition of RSK2Ser227 prevents the proliferation of multiple myeloma cell lines via induction of apoptosis

Next, we examined the effect of RSK2Ser227 inactivation on multiple myeloma cell lines using BI-D1870, a selective RSK2 NTKD inhibitor (24, 25). As shown in Fig. 3A, treatment with 7.5 μmol/L BI-D1870 resulted in complete dephosphorylation of RSK2Ser227, but not of RSK2Tyr529, within 3 hours, whereas BI-D1870 did not affect the phosphorylation status of ERK1/2 or of related signaling kinases, suggesting that BI-D1870 dephosphorylated RSK2Ser227 directly. This inhibitory effect of BI-D1870 on RSK2Ser227 resulted in a dose-dependent growth inhibition of 6 multiple myeloma–derived cell lines with IC50 values of 3.0 to 5.4 μmol/L through the cell death induction by apoptosis (Figs. 3B and C). In addition, this cell death induction by BI-D1870 was accompanied by the activation of caspase-8, -9, and -3 (Fig. 3D), whereas the blockade of caspase activity by pretreatment with z-VAD-FMK did not diminish the antimultiple myeloma effect of BI-D1870 on NCI-H929 cells (Fig. 3E). These findings indicate that RSK2Ser227 inhibition by BI-D1870 causes both caspase-related and -unrelated apoptosis in NCI-H929 cells.

RSK2Ser227 is phosphorylated in primary multiple myeloma cells with and without t(4;14)

We also examined the phosphorylation status of RSK2Ser227 and the effect of RSK2Ser227 inhibition on
patient-derived primary multiple myeloma cells (Supplementary Table S2). RSK2\textsuperscript{Ser227} was activated in 6 of 9 primary myeloma cells (all but 1 sample were from newly diagnosed patients with multiple myeloma) regardless of the presence or absence of t(4;14) (Fig. 4A). In addition, BI-D1870 treatment was found to induce cell death in multiple myeloma cells from all 4 patients examined in this study (Fig. 4B).

**Molecular sequelae following RSK2\textsuperscript{Ser227} inactivation by BI-D1870 in multiple myeloma cell lines**

We next tried to identify the downstream target molecules of the blockade of RSK2\textsuperscript{Ser227} phosphorylation with BI-D1870. We first examined the effect of RSK2\textsuperscript{Ser227} inhibition on antiapoptotic BCL2 proteins, BCL2, BCLXL, and MCL1, as well as BIM, a central member of the proapoptotic BH3-only protein, of which degradation by phosphorylation is mediated by RSK2 (26–28). BI-D1870 treatment resulted in downregulation of MCL1 and BCLXL expression in NCI-H929 cells (Figs. 5A and B). As for cell-cycle regulators, we focused on their association with RSK2\textsuperscript{Ser227} inhibition and CCNDs, because abnormal CCND expression is associated with myelomagenesis and response to mitogens in the bone marrow milieu (29, 30). Also, because a previous study had shown molecular links between RSK2 and cyclin-dependent kinase inhibitors (CDKIs; ref. 6), we examined the effects of BI-D1870 on expressions of CDKIs. BI-D1870 treatment caused reduction in CCND2 and p21\textsuperscript{WAF1/CIP1} expression in NCI-H929 cells (Fig. 5C). We also investigated the relationship between RSK2\textsuperscript{Ser227} inhibition and transcription factors that are critical for myelomagenesis, that is, IRF4 and c-MYC (31). BI-D1870 treatment caused rapid downregulation of c-MYC, but did not modulate expression levels of IRF4 in NCI-H929 cells (Fig. 5D). Importantly, the downregulation of MCL1, CCND2, p21\textsuperscript{WAF1/CIP1}, and c-MYC by BI-D1870 treatment was also observed in 2 other multiple myeloma cell lines (Fig. 5E). Also, because the blockade of caspase activation by z-VAD-FMK pretreatment did not affect the changes in the expression of any of the molecules affected by BI-D1870 (Fig. 5F), the downregulation of those molecules by RSK2\textsuperscript{Ser227} inhibition was found to be not merely the consequence of protein degradation by caspase activation.

To exclude the possibility that the downregulation of MCL1, CCND2, p21\textsuperscript{WAF1/CIP1}, and c-MYC, upregulation of BIM, and cell death induction by BI-D1870 in NCI-H929 cells were not caused by the inhibitory effect of RSK2\textsuperscript{Ser227} but by its off-target effects [BI-D1870 inhibits polo-like kinase 1 at approximately 4-folds higher concentrations than RSK2 (24)], we next carried out experiments involving gene knockdown of RSK2. Transient gene knockdown of RSK2 by means of RNAi caused downregulation of...
CCND2, p21WAF1/CIP1, and c-MYC (Fig. 5G), whereas it induced cell death in NCI-H929 cells (Fig. 5H). These results indicate that most of the effects of BI-D1870 and RNAi on RSK2 overlapped in NCI-H929 cells. Unfortunately, the effect of RNAi against RSK2 on the expression of BIM was not evaluable, as even the transfection with control siRNA resulted in BIM induction in our study (data not shown).

**Use of targeting RSK2Ser227 for the treatment of multiple myeloma**

IL-6–mediated cell protection is one of the major cell-extrinsic mechanisms for multidrug resistance in myeloma cells (5, 32). While cell death induction by lenalidomide, the most powerful IMiD that has a direct cytotoxic effect on multiple myeloma cells (33), was partly prevented by IL-6 in NCI-H929 cells, coadministration of...
agents, such as everolimus, MS-275, and ABT-263, at a when combined with forthcoming new anti-myeloma either cell line, it showed synergistic or additive effects lan, showed limited additive or synergistic effects on able agents, such as bortezomib, adriamycin, or melpha-


cytotoxic effect of BI-D1870 on patient-derived primary myeloma cells. Isolated cells were exposed to 10 or 20 μmol/L BI-D1870 for 48 hours in IL-6-containing medium, and viable cells were counted with the Trypan-blue dye exclusion method. The viable cell number for untreated cells was assumed to be 1.0. Triplicate experiments were carried out for each condition, and viable cell ratios are shown as mean ± SD.

BI-D1870 (0.2 μmol/L, a concentration low enough not to affect cell viability when used alone) overcame the resistance to lenalidomide induced by IL-6 (Fig. 6A). The development of a therapeutic approach that can overcome resistance to bortezomib is still being awaited. OPM-2/BTZ and KMS-11/BTZ were highly resistant to bortezomib-induced cell death; in contrast, both cell lines showed similar sensitivity to BI-D1870-induced cell death when compared with that of their parental cells. Thus, our results indicate that there was no cross-resistance between bortezomib and RSK2Ser227 inhibition by BI-D1870 (Fig. 6B). We finally examined the combinatorial effects of BI-D1870 and various anti-myeloma drugs, both clinically available and currently being developed, on NCI-H929 cells and KMS-34 cells. Although the combinatorial use of BI-D1870 combined with clinically available agents, such as bortezomib, adriamycin, or melphalan, showed limited additive or synergistic effects on either cell line, it showed synergistic or additive effects when combined with forthcoming new anti-myeloma agents, such as everolimus, MS-275, and ABT-263, at a wide range of fractional effect concentrations (Fig. 6C).

Discussion

We initially hypothesized that RSK2 may be activated by various cell-intrinsic and -extrinsic stimuli, such as genetic and/or molecular oncogenic abnormalities or different growth factor-mediated signals; however, we unexpectedly found that RSK2Ser227, which is a site responsible for substrate phosphorylation, is generally activated through phosphorylation regardless of the type of cytogenetic abnormalities or upstream molecular signaling in multiple myeloma. A couple of previous studies have indicated that RSK2 is a critical signaling effector of FGFR3-induced myelomagenesis, whereas RSK2 activation was detected only in myeloma cells with dysregulated FGFR3, but not in myeloma cells with functionally normal FGFR3 (13, 14). Those results were different from ours, which showed no relationship between RSKcSer227 activation and the status of upper signaling molecules. A possible reason for this discrepancy may be that we focused on the phosphorylation status of Ser227 at NTKD, whereas previous studies focused on Tyr529 and Ser386 residues, which are phosphorylated by upper signals before Ser227 phosphorylation (13, 14). Moreover, previous studies examined the functional roles of RSK2 using fmk as an inhibitor for RSK2; however, fmk is active only in the CTKD, but does not directly affect the NTKD of RSK2. On the basis of our findings that phosphorylation of RSK2 CTKD is not a prerequisite for RSK2Ser227 phosphorylation in myeloma cells, the experimental settings in previous studies might not be suitable for investigating the status and roles of RSK2Ser227 in myeloma cells.

Inhibition of RSK2Ser227 by BI-D1870 or RSK2 repression by means of siRNA transfection caused downregulation of c-MYC, p21WAF1/CIP1, CCND2, and MCL1. c-MYC is a key regulator of tumorigenesis in various cancers, and its overexpression results in progression to a more advanced stage of multiple myeloma, such as acquisition of multidrug resistance or extramedullary tumor formation (34–36). The transcription factor IRF4 is thought to be one of the crucial factors for myelomagenesis, and is reportedly interdependent with c-MYC (31). However, our result indicates that IRF4 expression was not changed by RSK2 inhibition, which suggests the presence of an RSK2-related but IRF4-unrelated alternative pathway for c-MYC expression. p21WAF1/CIP1 plays an essential role in G1–S cell-cycle arrest after genotoxic damage, whereas it also promotes cell protection from apoptosis (37). In addition, p21WAF1/CIP1 stabilizes cyclins by promoting interaction between cyclin-dependent kinase 4 and cyclins (37). Thus, the repression of p21WAF1/CIP1 by RSK2 may contribute to both cytotoxicity and inhibition of cell proliferation as a result of cyclin destabilization. MCL1 is a member of the antipoptotic BCL2 family of proteins and, as the downstream effector molecule of various signal pathways such as IL-6 or nuclear factor kappa-B, promotes cell survival and drug resistance in multiple myeloma (38, 39). MCL1 repression caused by RSK2 inhibition may thus be involved in apoptosis induction and may also be beneficial for overcoming drug resistance and for preventing disease progression. Taken together, these results suggest that RSK2Ser227 is a rational molecular target for the development of a new therapy for multiple myeloma.

Currently, combinatorial use of anti-myeloma drugs is the gold-standard for multiple myeloma. When used simultaneously, BI-D1870 showed limited additive
effect in combination with currently available agents, such as bortezomib, adriamycin, and melphalan. Because c-MYC is required to realize cell death by bortezomib (40, 41), it may be preferable to administer RSK2 inhibitor following bortezomib for effective myeloma cell killing. In this connection, our data showed no cross-resistance between bortezomib and RSK2 inhibitor, thus suggesting that drugs that target RSK2Ser227 may be beneficial for eliminating residual myeloma cells following bortezomib exposure. Conversely, RSK2Ser227 inhibition rescued the cytotoxic effect of lenalidomide from the IL-6–mediated cytoprotection. Therefore, simultaneous treatment may be preferable when RSK2Ser227 inhibitor is used in combination with IMiD.

RSK2 inhibitor BI-D1870 showed additive and/or synergistic anti-myeloma effects when combined with several drugs currently being developed, such as everolimus (42), MS-275 (43–45), and ABT-263 (23, 46–48). Everolimus exerts its cytotoxic effect by inhibiting the phosphatidylinositol 3-Kinase (PI3K)/AKT signaling...
pathway, so that coadministration of everolimus and BI-D1870 can be expected to exert powerful antiproliferative effects on myeloma cells through simultaneous inhibition of both the RAS/ERK1/2 and PI3K/AKT signaling pathways (17). HDACi has shown its antimyeloma effects both in vitro and in vivo, whereas p21WAF1/CIP1 induction by HDACi has been shown to be one of the mechanisms for HDACi resistance (43–45). p21WAF1/CIP1 repression by RSK2Ser227 inhibition may therefore enhance the anti-myeloma effect of HDACi. ABT-263 (formerly ABT-737) also possesses anti-myeloma potency (46, 47), and we have already shown that MCL1 repression or c-MYC inhibition dramatically enhances apoptosis induction by ABT-737 (47, 48). In view of these considerations, the combination of ABT-263 and RSK2Ser227 inhibition is a rational strategy for attacking multiple myeloma. Considering that (i) RSK2Ser227 phosphorylation was observed in treatment-naïve primary myeloma cells and in all of the myeloma cell lines examined which were derived from patients with multiple myeloma in the

Figure 6. Use of RSK2Ser227 inhibition of multiple myeloma. A, NCI-H929 cells were treated with 100 μmol/L lenalidomide (Len) with or without IL-6 (50 ng/mL) for 48 hours. IL-6 partly protected against cell death of NCI-H929 cells by lenalidomide, whereas the addition of a subtoxic concentration of BI-D1870 (BI) restored the cytotoxic activity of lenalidomide. B, BI-D1870 showed no cross-resistance with bortezomib in bortezomib-resistant KMS-11/BTZ cells and OPM-2/BTZ cells. Cells were treated with the indicated concentrations of either BI-D1870 or bortezomib for 48 hours. The viable cell number of untreated cells was assumed to be 1.0. Triplicate experiments were carried out for each condition, and viable cell ratios are shown as mean ± SD. C, the combinatory effects of BI-D1870 and various antimultiple myeloma agents [adriamycin (ADR), melphalan (L-PAM), bortezomib, MS-275, everolimus, or ABT-263] were also examined. Cells were treated with various concentrations of the combinatory agents for 48 hours. x-axis, fractional effects concentrations; y-axis, CI. CI was shown as CI ± 1.96 SD. Shaded areas show the synergistic or additive effects of 2 agents and the areas below the arrows in particular show synergism of 2 agents.
aggressive phase, (ii) RSK2\textsuperscript{Ser227} inhibition is effective for bortezomib-resistant myeloma cells, and (iii) RSK2\textsuperscript{Ser227} inhibition proved to be an effective partner for several agents under development, we speculate that RSK2\textsuperscript{Ser227} is a suitable therapeutic target not only for newly diagnosed patients with multiple myeloma but also for patients with late-phase multiple myeloma who are resistant/refractory to conventional anti-myeloma therapies. One of the limitations of this study is the lack of experiments for examining the \textit{in vivo} effect of RSK2\textsuperscript{Ser227} inhibition. This was due to the absence of an \textit{in vivo} bioavailable inhibitor, which specifically targets RSK2\textsuperscript{Ser227}. Future works are urgently needed to develop a novel RSK2 NTKD-specific inhibitor which can be used for a preclinical study for myeloma.

In conclusion, the findings of this study suggest that RSK2\textsuperscript{Ser227} in an NTKD is a potential general therapeutic target for multiple myeloma.

**Disclosure of Potential Conflicts of Interest**

Y. Shimura is Group Head in Kyowa Hakko Kirin. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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References


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