RSK2<sup>Ser227</sup> at N-terminal kinase domain is a potential therapeutic target for multiple myeloma

Yuji Shimura<sup>1</sup>, Junya Kuroda<sup>1</sup>, Masaki Ri<sup>2</sup>, Hisao Nagoshi<sup>1</sup>, Mio Yamamoto-Sugitani<sup>1</sup>, Tsutomu Kobayashi<sup>1</sup>, Miki Kiyota<sup>1</sup>, Ryuko Nakayama<sup>1</sup>, Shinsuke Mizutani<sup>1</sup>, Yoshiaki Chinen<sup>1</sup>, Natsumi Sakamoto<sup>1</sup>, Yosuke Matsumoto<sup>1</sup>, Shigeo Horiike<sup>1</sup>, Yukimasa Shiotsu<sup>3</sup>, Shinsuke Iida<sup>2</sup>, Masafumi Taniwaki<sup>1</sup>

1. Division of Hematology and Oncology, Department of Medicine, Kyoto Prefectural University of Medicine, 465, Kajii-cho, Kamigyo-ku, Kyoto, 602-8566, Japan
2. Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi, 467-8601, Japan
3. Kyowa Hakko Kirin Co., Ltd. Inc., 3-6-6 Asahi-cho, Machida, Tokyo, 194-0023, Japan

Running title: RSK2 as therapeutic target for myeloma

Keywords: RSK2, myeloma, apoptosis, c-MYC, Cyclin D

Grant Support

This work was partly supported by Grants-in-Aid for Scientific Research from Grants-in-Aid for Scientific Research from the Ministry of Education, Culture,
RSK2 as therapeutic target for myeloma

Sports, Science and Technology of Japan (J. Kuroda (23591403)), the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Hoansha Foundation and the Award in Aki’s Memory from the International Myeloma Foundation (J. Kuroda).

Corresponding author: Junya Kuroda M.D., Ph.D,
Division of Hematology and Oncology, Department of Medicine, Kyoto Prefectural University of Medicine. 465 Kajii-cho, Kamigyo-ku, Kyoto, 602-8566, Japan.
Tel: +81-75-251-5740, Fax:+81-75-251-5743, E-mail: junkuro@koto.kpu-m.ac.jp

Word count: 231 words (abstract), 3910 words (all)
Total number of figures: 6
Supplementary Tables: 2
References: 48
RSK2 as therapeutic target for myeloma

Abstract

Multiple myeloma (MM) is an entity of cytogenetically and genetically heterogeneous plasma cell neoplasms. Despite recent improvement in the treatment outcome of MM by novel molecular targeted chemotherapeutics, MM remains incurable. The identification of a therapeutic target molecule where various signalings for cell survival converge is a core component for the development of new therapeutic strategies against MM. RSK2 is an essential mediator of the ERK1/2 signaling pathway for cell survival and proliferation. In this study, we discovered that RSK2\textsuperscript{Ser227}, 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 MM-derived cell lines examined and six of nine patient-derived CD138-positive primary myeloma cells. The chemical inhibition of RSK2\textsuperscript{Ser227} by BI-D1870 or gene knockdown of RSK2 inhibits myeloma cell proliferation through apoptosis induction, and this anti-MM effect was accompanied by downregulation of c-MYC, Cyclin D, p21\textsuperscript{WAF1/CIP1} and MCL1. RSK2\textsuperscript{Ser227} 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 anti-proliferative effects in conjunction with the mTOR, histone deacetylase, and BH3-mimicking BCL2/BCLX\textsubscript{L} inhibitors. These results suggest that RSK2\textsuperscript{Ser227} is a potential therapeutic target not only for newly diagnosed but also for later phase MM patients who are resistant or refractory to currently available anti-MM therapies.

Shimura Y et al.
Introduction

Despite the marked improvement of treatment outcomes for multiple myeloma (MM) during the last decade through the development of new therapeutic modalities such as bortezomib (BTZ) and immunomodulatory derivatives (IMiDs) (1), MM 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), while 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 where various types of signaling for myeloma cell survival converge is essential for the development of a more effective therapy for MM.

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, while 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 demonstrated that RSK2 acts as the key regulator for cellular transformation and metastasis by mediating signaling through oncogenic tyrosine kinases (TKs) (10-12). As for myeloma, previous studies have suggested the importance of RSK2 signaling associated with fibroblast growth factor 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 MM therapy. In this study, we investigated the activation of RSK2, especially of RSK2\textsuperscript{Ser227} 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 MM.

**Materials and Methods**

**Cells and reagents**

This study used the human myeloma cell lines NCI-H929, OPM-2, LP-1, PRMI8226, IM9, AMO-1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), KMS-11, KMS-18, KMS-12-BM, KMS-28-PE, KMS-34, KMS-20 (kind gifts from Dr. Ohtsuki T, 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, 18).
respectively) (19). Cells were maintained in RPMI-1640 containing 10% fetal calf serum (FCS), 2mM L-glutamate and penicillin/streptomycin. Subclones of OPM-2 and KMS-11 (OPM-2/BTZ and KMS-11/BTZ), which are resistant to BTZ, were developed under continuous exposure to BTZ in the same medium over half a year. These cells acquire BTZ 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. BM mononuclear cells were labeled with anti-CD138 MicroBeads and positively isolated with the MiniMACS separator (Miltenyi Biotec KK, Tokyo, Japan) (21). Isolated myeloma cells were cultured with 50 ng/ml interleukin-6 (IL-6). BI-D1870, an RSK2 inhibitor, was purchased from Symansis NZ Limited (Auckland, New Zealand). Z-VAD-FMK, a pan-caspase inhibitor, from Calbiochem (San Diego, CA, USA). MS-275, a histone deacetylase inhibitor (DACI), from Selleck Chemicals (Houston, TX, USA), and ABT-263, a BH3-mimic inhibitor for BCL2/BCLX<sub>L</sub>, from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lenalidomide (LEN) was supplied by Celgene Corporation (Summit, NJ, USA), and BTZ, adriamycin (ADR), melphalan (L-PAM), U0126, an inhibitor for MEK, and everolimus (RAD001), an inhibitor for mTOR, by the Screening Committee of Anticancer Drugs (Tokyo, Japan) (Fig. 1).

**RNA interference (RNAi)**

RNAi for RSK2 was performed by transfecting small interfering RNA
siRNA) into NCI-H929 cells. The sequence of the sense strand for siRNA for RSK2 was 5-GUAUCAGUCCAGUUAATT-3 and that of the antisense siRNA was 5-UUUAAGACUGGACUGAUACTT-3. The corresponding sequences for control were 5-UCUUAAUCGCGUAUAAGGCTT-3 and 5-GCCUUAUACGCGAUUAAGATT-3. Briefly, 2.5×10^6 NCI-H929 cells were resuspended with 100 μl of transfection buffer and transfected with siRNA for RSK2 or with control siRNA (Takara Bio Inc., Otsu, Japan) by means of the CLB-Transfection Kit (Lonza AG, Basel, Switzerland) using protocol 9.

**Drug combination assays**

Cells were treated with various concentrations of BTZ, LEN, L-PAM, ADR, RAD001, MS-275, or ABT-263 alone or combined with BI-D1870 for 48 hours. The fractional effect concentrations (ie, a fractional effect of 0.25 equals a 25% growth inhibitory effect) and the combination index (CI) were calculated with CalcuSyn (Biosoft, Cambridge, UK). This method facilitates the quantification of synergism (CI < 1) and antagonism (CI > 1) at different doses and effect levels. CI calculations were performed on the assumption that the drug mechanisms were not mutually exclusive.

**Western blot analysis**

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

**Fluorescence in situ hybridization (FISH)**

Shimura Y et al.
Cytogenetic abnormalities were examined by means of interphase FISH analysis (23). Probes used were described in Supplementary Information.

Assays for growth inhibition and apoptosis

Assays for growth inhibition and apoptosis were performed as previously described (21, 22) (Supplementary information).

Results

**RSK2<sup>Ser227</sup> is phosphorylated regardless of type of cytogenetic or upstream molecular signaling in MM cell lines**

We first identified that RSK2<sup>Ser227</sup> was phosphorylated in all 12 MM cell lines examined (Fig. 2A). Normal peripheral lymphocytes were utilized as the positive control (7, 8). Among 12 cell lines, t(4;14) involving IGH/FGFR3 was detected in only seven cell lines (NCI-H929, OPM-2, LP-1, KMS-18, KMS-28-PE, KMS-34 and AMU-MM1) and high FGFR3 expression in only six 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 RSK2<sup>Ser227</sup> phosphorylation. Our study also did not show any positive relationship between RSK2<sup>Ser227</sup> phosphorylation and other so-called “high-risk” cytogenetic abnormalities (Supplementary Table S1). Also, RSK2<sup>Ser227</sup> was phosphorylated even in the absence of ERK1/2 phosphorylation in several MM cell lines, indicating that ERK1/2 activation is not mandatory for RSK2<sup>Ser227</sup> phosphorylation in MM cell lines. Indeed, although a MEK inhibitor U0126 completely inhibited the phosphorylation of ERK1/2 and

Shimura Y et al.
inhibited the cell proliferation in NCI-H929 cells with t(4;14) and active ERK1/2, it only partly inhibited RSK2$^{\text{Ser227}}$ phosphorylation. Also, U0126 failed to inhibit the cell growth and RSK2$^{\text{Ser227}}$ phosphorylation in KMS-12-BM which lacks t(4;14) or ERK1/2 phosphorylation (Fig. 2B). Moreover, RSK$^{\text{Tyr529}}$ phosphorylation at CKTD was observed only in five of twelve cell lines examined in our study, indicating that phosphorylation of RSK$^{\text{Tyr529}}$ seems to be irrelevant for the phosphorylation of Ser227 in MM cell lines. Collectively, RSK2$^{\text{Ser227}}$ 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 MM cell lines.

**Inhibition of RSK2$^{\text{Ser227}}$ prevents the proliferation of MM cell lines via induction of apoptosis**

Next, we examined the effect of RSK2$^{\text{Ser227}}$ inactivation on MM cell lines using BI-D1870, a selective RSK2 NTKD inhibitor (24, 25). As shown in Fig. 3A, treatment with 7.5$\mu$M BI-D1870 resulted in complete dephosphorylation of RSK2$^{\text{Ser227}}$, but not of RSK2$^{\text{Tyr529}}$, within 3 hours, while BI-D1870 did not affect the phosphorylation status of ERK1/2 or of related signaling kinases, suggesting that BI-D1870 dephosphorylated RSK2$^{\text{Ser227}}$ directly. This inhibitory effect of BI-D1870 on RSK2$^{\text{Ser227}}$ resulted in a dose-dependent growth inhibition of 6 MM-derived cell lines with IC$_{50}$ values of 3.0-5.4 $\mu$M through the cell death induction by apoptosis (Figs. 3B and 3C). In addition, this cell death induction by BI-D1870 was accompanied by the activation of caspase-8, caspase-9 and caspase-3 (Fig. 3D), while the blockade of caspase activity by pretreatment

*Shimura Y et al.*
with z-VAD-FMK did not diminish the anti-MM effect of BI-D1870 on NCI-H929 cells (Fig. 3E). These findings indicate that RSK2<sup>Ser227</sup> inhibition by BI-D1870 causes both caspase-related and -unrelated apoptosis in NCI-H929 cells.

**RSK2<sup>Ser227</sup> is phosphorylated in primary MM cells with and without t(4;14)**

We also examined the phosphorylation status of RSK2<sup>Ser227</sup> and the effect of RSK2<sup>Ser227</sup> inhibition on patient-derived primary MM cells (Supplementary Table S2). RSK2<sup>Ser227</sup> was activated in six of nine primary myeloma cells (all but one sample were from newly diagnosed MM patients) regardless of the presence or absence of t(4;14) (Fig. 4A). In addition, BI-D1870 treatment was found to induce cell death in MM cells from all four patients examined in this study (Fig. 4B).

**Molecular sequelae following RSK2<sup>Ser227</sup> inactivation by BI-D1870 in MM cell lines**

We next tried to identify the downstream target molecules of the blockade of RSK2<sup>Ser227</sup> phosphorylation with BI-D1870. We first examined the effect of RSK2<sup>Ser227</sup> inhibition on anti-apoptotic BCL2 proteins, BCL2, BCLXL and MCL1, as well as BIM, a central member of the pro-apoptotic BH3-only protein, of which degradation by phosphorylation is mediated by RSK2 (26-28). BI-D1870 treatment resulted in downregulation of MCL1 and BIM upregulation, but did not cause any alterations in the expression level of BCL2 or BCLXL in NCI-H929 cells (Figs. 5A and 5B). As for cell cycle regulators, we focused on their association with RSK2<sup>Ser227</sup> inhibition and CCNDs, because abnormal
CCND expression is associated with myelomagenesis and response to mitogens in the BM milieu (29, 30). Also, because a previous study had demonstrated molecular links between RSK2 and cyclin-dependent kinase inhibitors (CDKIs) (6), we examined the effects of BI-D1870 on expressions of CDKIs. BI-D1870 treatment caused reduction in CCND2 and p21^{WAF/CIP1} expression in NCI-H929 cells (Fig. 5C). We also investigated the relationship between RSK2^{Ser227} inhibition and transcription factors which are critical for myelomagenesis, i.e., 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^{WAF/CIP1} and c-MYC by BI-D1870 treatment was also observed in two other MM 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^{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^{WAF/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^{Ser227} but by its off-target effects (BI-D1870 inhibits polo-like kinase 1 at approximately four-folds higher concentrations than RSK2 (24)), we next performed experiments involving gene knockdown of RSK2. Transient gene knockdown of RSK2 by means of RNAi caused downregulation of CCND2, p21^{WAF/CIP1} and c-MYC (Fig. 5G), while it induced cell death in NCI-H929 cells.
RSK2 as therapeutic target for myeloma

(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).

Utility of targeting RSK2<sup>Ser227</sup> for the treatment of MM

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 LEN, the most powerful IMiD which has a direct cytotoxic effect on MM cells (33), was partly prevented by IL-6 in NCI-H929 cells, co-administration of BI-D1870 (0.2 μM, a concentration low enough not to affect cell viability when used alone) overcame the resistance to LEN induced by IL-6 (Fig. 6A). The development of a therapeutic approach which can overcome resistance to BTZ is still being awaited. OPM-2/BTZ and KMS-11/BTZ were highly resistant to BTZ-induced cell death; by 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 BTZ and RSK2<sup>Ser227</sup> inhibition by BI-D1870 (Fig. 6B). We finally examined the combinatory effects of BI-D1870 and various anti-MM drugs, both clinically available and currently being developed, on NCI-H929 cells and KMS-34 cells. Although the combinatory use of BI-D1870 combined with clinically available agents, such as BTZ, ADR, or L-PAM, showed limited additive or synergistic effects on either cell line, it showed synergistic or additive effects when combined with forthcoming new anti-MM agents, such as RAD001, 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 RSK2$^{\text{Ser227}}$, 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 MM. A couple of previous studies have indicated that RSK2 is a critical signaling effector of FGFR3-induced myelomagenesis, while 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 RSK2$^{\text{Ser227}}$ 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 prior to Ser227 phosphorylation (13, 14). Moreover, previous studies examined the functional roles of RSK2 utilizing fmk as an inhibitor for RSK2; however, fmk is active only in the CTKD, but does not directly affect the NTKD of RSK2. Based on our findings that phosphorylation of RSK2 CTKD is not a prerequisite for RSK2$^{\text{Ser227}}$ phosphorylation in myeloma cells, the experimental settings in previous studies might not be suitable for investigating the status and roles of RSK2$^{\text{Ser227}}$ in myeloma cells.

Shimura Y et al.
Inhibition of RSK2<sup>Ser227</sup> by BI-D1870 or RSK2 repression by means of siRNA transfection caused downregulation of c-MYC, p21<sup>WAF1/CIP1</sup>, 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 MM, such as acquisition of multidrug resistance or extra-medullary 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. p21<sup>WAF1/CIP1</sup> plays an essential role in G1/S cell cycle arrest after genotoxic damage, while it also promotes cell protection from apoptosis (37). In addition, p21<sup>WAF1/CIP1</sup> stabilizes cyclins by promoting interaction between cyclin dependent kinase 4 and cyclins (37). Thus, the repression of p21<sup>WAF1/CIP1</sup> by RSK2 may contribute to both cytotoxicity and inhibition of cell proliferation as a result of cyclin destabilization. MCL1 is a member of the anti-apoptotic 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 MM (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 RSK2<sup>Ser227</sup> is a rational molecular target for the development of a new therapy for MM.

Currently, combinatorial use of anti-MM drugs is the gold-standard for...
RSK2 as therapeutic target for myeloma

When used simultaneously, BI-D1870 showed limited additive effect in combination with currently available agents, such as BTZ, ADR, L-PAM. Because c-MYC is required to realize cell death by BTZ (40, 41), it may be preferable to administer RSK2 inhibitor following BTZ for effective myeloma cell killing. In this connection, our data showed no cross-resistance between BTZ and RSK2 inhibitor, thus suggesting that drugs that target RSK2^Ser227 may be beneficial for eliminating residual myeloma cells following BTZ exposure. Conversely, RSK2^Ser227 inhibition rescued the cytotoxic effect of LEN from the IL-6-mediated cytoprotection. Therefore, simultaneous treatment may be preferable when RSK2^Ser227 inhibitor is used in combination with IMiD.

RSK2 inhibitor BI-D1870 showed additive and/or synergistic anti-MM effects when combined with several drugs currently being developed, such as RAD001 (42), MS-275 (43-45) and ABT-263 (23, 46-48). RAD001 exerts its cytotoxic effect by inhibiting the phosphatidylinositol 3-Kinase (PI3K)/AKT signaling pathway, so that co-administration of RAD001 and BI-D1870 can be expected to exert powerful anti-proliferative effects on myeloma cells through simultaneous inhibition of both the RAS/ERK1/2 and PI3K/AKT signaling pathways (17). DACI has demonstrated its anti-myeloma effects both in vitro and in vivo, while p21^{WAF1/CIP1} induction by DACI has been shown to be one of the mechanisms for DACI resistance (43-45). p21^{WAF1/CIP1} repression by RSK2^Ser227 inhibition may therefore enhance the anti-MM effect of DACI. ABT-263 (formerly ABT-737) also possesses anti-MM potency (46, 47), and we have already demonstrated 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 RSK2\textsuperscript{Ser227} inhibition is a rational strategy for attacking MM. Considering that (i) RSK2\textsuperscript{Ser227} phosphorylation was observed in treatment-naïve primary myeloma cells and in all of the myeloma cell lines examined which were derived from MM patients in the aggressive phase, (ii) RSK2\textsuperscript{Ser227} inhibition is effective for BTZ-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 MM patients but also for late-phase MM patients who are resistant/refractory to conventional anti-MM 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 N-terminal kinase domain is a potential general therapeutic target for MM.

\textbf{Disclosure of Potential Conflicts of Interest}

The authors declare no conflict of interest.

\textbf{Acknowledgements}

We wish to express our appreciation for the support by the Screening Committee of Anticancer Drugs to obtain a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from The Ministry of Education, Culture, Sports,
RSK2 as therapeutic target for myeloma

Science and Technology, Japan, which provided the SCADS inhibitor kit.

References


15. Lentzsch S, Chatterjee M, Gries M, Bommert K, Gollasch H, Dörken B, et al. PI3-K/AKT/FKHR and MAPK signaling cascades are redundantly stimulated by a variety of cytokines and contribute independently to proliferation and

Shimura Y et al.


Shimura Y et al.


30. Glassford J, Rabin N, Lam EW, Yong KL. Functional regulation of D-type


Shimura Y et al.
Rev Cancer 2009;9:400-14


43. Rosato RR, Almenara JA, Grant S. The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of p21\textsuperscript{CIP1/WAF1}. Cancer Res 2003;63:3637-45.


Legends

Figure 1. **Structures of anticancer agents utilized in this study.** ADR: adriamycin, L-PAM: melphalan, RAD001: everolimus.

Figure 2. **RSK2**\(^{\text{Ser227}}\) is phosphorylated regardless of type of cytogenetic or upstream molecular signaling in MM cell lines. A. Expression of RSK2, FGFR3 and ERK1/2 in 12 myeloma-derived cell lines. p: phosphorylated. B. Effects of U0126 on the activities of ERK-1/2 and RSK2\(^{\text{Ser227}}\) in NCI-H929 cells with t(4;14) and KMS-12-BM without t(4;14). Cells were treated with 80μM of U0126 for 6 hours (IC\(_{50}\) for NCI-H929).

Figure 3. **Effects of BI-D1870, an inhibitor for RSK2-NTKD, on NCI-H929 cells.** A. BI-D1870 specifically inhibits phosphorylation of RSK2\(^{\text{Ser227}}\), but not of RSK2\(^{\text{Tyr529}}\), AKT or upstream MAPKs in myeloma cells. NCI-H929 cells were treated with 7.5 μM BI-D1870 for the indicated periods. B. BI-D1870 inhibits the growth of various myeloma cell lines in a dose-dependent manner. Myeloma cell lines were exposed to various concentrations of BI-D1870 for 48 hours and the cell proliferation was determined by modified MTT assay. IC\(_{50}\)s for NCI-H929, AMO-1, LP-1, OPM-2, RPMI8226 and IM9 were 3.0, 4.7, 3.7, 3.7, 5.4 and 4.9 μM, respectively. Results are shown as mean +/- S.D. C. Induction of apoptosis by BI-D1870 in NCI-H929 cells. NCI-H929 cells were treated with 7.5 μM BI-D1870 for the indicated periods and were subjected to flow cytometric analysis (left panel). Fraction I: Annexin-V (AV)(-)/Propidium Iodide (PI) (-) (viable cells), fraction II: AV(+)/PI(-) (cells undergoing early apoptosis), fraction...
III: AV(+)/PI(+) (cells undergoing late apoptosis) and fraction IV: AV(-)/PI(+) populations (necrotic cells). Cell ratios of fractions II and III are shown according to the incubation periods (right panel). **D.** Activation of caspases by BI-D1870 treatment in NCI-H929 cells. NCI-H929 cells were treated with 7.5 μM BI-D1870 for the indicated periods. **E.** Caspase-independent cytotoxicity of BI-D1870 (BI) in NCI-H929 cells. NCI-H929 cells were pre-incubated with 50 μM z-VAD-FMK (fmk) for 1 hour, and then treated with 7.5 μM BI-D1870 for 48 hours. Viable cell ratio of vehicle cells was assumed to be 1.0. Despite the inhibition of caspase activation, BI-D1870 induced cell death to the same extent as in BI-D1870-treated cells without z-VAD-FMK pretreatment. Triplicate experiments were performed for each condition, and viable cell ratios are shown as mean +/- S.D.

**Figure 4.** RSK2 in patient-derived primary myeloma cells. **A.** Phosphorylation status of RSK2\textsuperscript{Ser227} in patient-derived primary myeloma cells isolated by CD138-positive selection. N.A.: not available. **B.** *In vitro* cytotoxic effect of BI-D1870 on patient-derived primary myeloma cells. Isolated cells were exposed to 10 or 20 μM 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 performed for each condition, and viable cell ratios are shown as mean +/- S.D.

**Figure 5.** Molecular sequelae following RSK2\textsuperscript{Ser227} inhibition by

Shimura Y et al.
**RSK2 as therapeutic target for myeloma**

**BI-D1870 in myeloma cells.** NCI-H929 cells were treated with 7.5 μM BI-D1870 for the indicated periods for examination of the effects on the expression of A: anti-apoptotic BCL2 family proteins, B: BIM, a central pro-apoptotic BH3-only protein, C: cell cycle regulators, and D: major transcription factors associated with myelomagenesis. E. Effects of BI-D1870 on MCL1, p21\(^{WAF1/CIP1}\), CCND2 and c-MYC were also examined in KMS-34 cells and AMO-1 cells. Cells were treated with 7.5 μM BI-D1870 for the indicated periods. F. z-VAD-FMK pretreatment did not diminish the effects of BI-D1870 on MCL1, p21\(^{WAF1/CIP1}\), CCND2 and c-MYC. G. Gene knockdown of RSK2 by RNAi caused the downregulation of MCL1, p21\(^{WAF1/CIP1}\), CCND2 and c-MYC proteins in NCI-H929 cells. Expression levels of target proteins relative to those treated with control siRNA were calculated with Image-J software and described below bands. H. Gene knockdown of RSK2 by RNAi caused the loss of cell viability in NCI-H929 cells. The viable cell number of mock transfected cells was assumed to be 1.0. Triplicate experiments were performed for each condition, and viable cell ratios are shown as mean +/- S.D. si-cntl.: control siRNA, si-RSK2: siRNA for RSK2.

**Figure 6. Utility of RSK2\(^{Ser227}\) inhibition of MM.** A. NCI-H929 cells were treated with 100 μM LEN with or without IL-6 (50 ng/ml) for 48 hours. IL-6 partly protected against cell death of NCI-H929 cells by LEN, while the addition of a subtoxic concentration of BI-D1870 (BI) restored the cytotoxic activity of LEN. B. BI-D1870 showed no cross resistance with BTZ in BTZ-resistant KMS-11/BTZ cells and OPM-2/BTZ cells. Cells were treated with the indicated concentrations of either BI-D1870 or BTZ for 48 hours. The viable cell number of untreated cells

*Shimura Y et al.*
was assumed to be 1.0. Triplicate experiments were performed for each condition, and viable cell ratios are shown as mean +/- S.D. C. The combinatory effects of BI-D1870 and various anti-MM agents (ADR, L-PAM, BTZ, MS-275, RAD001, 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 demonstrated as CI +/- 1.96 S.D. Shaded areas show the synergistic or additive effects of two agents and the areas below the arrows in particular show synergism of two agents.
Figure 1

ADR  L-PAM  MS-275  RAD-001

ABT-263  BTZ  BI-D1870
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>NCI-H929</th>
<th>OPM-2</th>
<th>LP-1</th>
<th>KMS-18</th>
<th>RPMI8266</th>
<th>KMS-12-BM</th>
<th>KMS-28-PE</th>
<th>KMS-34</th>
<th>KMS-20</th>
<th>IM9</th>
<th>AMO-1</th>
<th>AMU-MM1</th>
<th>Normal Lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-RSK2^{Ser227}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-RSK2^{Tyr529}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSK2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>NCI-H929</th>
<th>KMS-12-BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>U0126 (80µM)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK1/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-RSK2^{Ser227}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSK2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3

A

<table>
<thead>
<tr>
<th>BI-D1870 (7.5 μM)</th>
<th>-</th>
<th>15 min.</th>
<th>30 min.</th>
<th>1h</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-AKT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-p38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-JNK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JNK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGFR3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-RSK2&lt;sup&gt;Y245&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-RSK2&lt;sup&gt;Ser227&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSK2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Cell viability vs. BI-D1870 (μM)

C

Propidium Iodide

Annexin V

D

BI-D1870 (7.5 μM) - 15 min. 30 min. 1h 3h 6h

Caspase-9 cleaved

Caspase-8 cleaved

Caspase-3 cleaved

E

Cell viability with inhibitors

<table>
<thead>
<tr>
<th></th>
<th>BI</th>
<th>fmk</th>
<th>BI + fmk</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI-D1870</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>z-VAD-fmk</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>cleaved</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Downloaded from mct.aacrjournals.org on January 27, 2018. © 2012 American Association for Cancer Research.
Figure 4

A

<table>
<thead>
<tr>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
<th>#5</th>
<th>#6</th>
<th>#7</th>
<th>#8</th>
<th>#9</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(4;14)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>N.A.</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

p-RSK2<sup>Ser227</sup>  
RSK2  
β-Actin

B

Graph showing the effect of BI-D1870 (µM) on viable cell ratio. Points represent different patients (Pt.A, Pt.B, Pt.C, Pt.D).
**Figure 5**

A. BI-D1870 (7.5μM) - 15 min. 30 min. 1h 3h 6h
- BCLXL
- BCL2
- MCL1
- β-Actin

B. BI-D1870 (7.5μM) - 15 min. 30 min. 1h 3h 6h
- BimEL
- BimL
- Bims

C. BI-D1870 (7.5μM) - 15 min. 30 min. 1h 3h 6h
- CCND1
- CCND2
- p27kip1
- p21WAF1/CIP1
- TP53
- β-Actin

D. BI-D1870 (7.5μM) - 15 min. 30 min. 1h 3h 6h
- IRF4
- c-MYC
- β-Actin

E. BI-D1870 KMS34 - 3h 6h AMO-1
- MCL1
- p21WAF1/CIP1
- CCND2
- c-MYC
- RSK2
- β-Actin

F. BI-D1870 z-VAD-fmk
- CCND2
- p21WAF1/CIP1
- MCL1
- c-MYC
- p-RSK2Ser227
- β-Actin

G. p21WAF1/CIP1
- CCND2
- MCL1
- c-MYC
- p-RSK2Ser227
- RSK2
- β-Actin

H. 36hr after transfection
- Cell viability
  - si-cntl.
  - si-RSK2
Figure 6

A

![cystotoxicity bar graph](image)

B

![cell viability graphs](image)

C

![NCI-H929 and KMS34 graphs](image)
Molecular Cancer Therapeutics

RSK2Ser227 at N-terminal kinase domain is a potential therapeutic target for multiple myeloma

Yuji Shimura, Junya Kuroda, Masaki Ri, et al.

Mol Cancer Ther Published OnlineFirst September 25, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0605

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/10/15/1535-7163.MCT-12-0605.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/early/2012/09/25/1535-7163.MCT-12-0605.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.