Small Molecule Therapeutics

Delineating the mTOR Kinase Pathway Using a Dual TORC1/2 Inhibitor, AZD8055, in Multiple Myeloma

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Abstract

Despite promising preclinical results with mTOR kinase inhibitors in multiple myeloma, resistance to these drugs may arise via feedback activation loops. This concern is especially true for insulin-like growth factor 1 receptor (IGF1R), because IGF1R signaling is downregulated by multiple AKT and mTOR feedback mechanisms. We have tested this hypothesis in multiple myeloma using the novel selective mTOR kinase inhibitor AZD8055. We evaluated p-mTOR S2481 as the readout for mTORC2/Akt activity in multiple myeloma cells in the context of mTOR inhibition via AZD8055 or rapamycin. We next validated AZD8055 inhibition of mTORC1 and mTORC2 functions in multiple myeloma cells alone or in culture with bone marrow stroma cells and growth factors. Unlike rapamycin, AZD8055 resulted in apoptosis of multiple myeloma cells. AZD8055 treatment, however, induced upregulation of IGF1R phosphorylation in p-Akt S473-expressing multiple myeloma cell lines. Furthermore, exposure of AZD8055-treated cells to IGF1 induced p-Akt S473 and rescued multiple myeloma cells from apoptosis despite mTOR kinase inhibition and TORC2/Akt blockage. The addition of blocking IGF1R antibody resulted in reversing this effect and increased AZD8055-induced apoptosis. Our study suggests that combination treatment with AZD8055 and IGF1R-blocking agents is a promising strategy in multiple myeloma with potential IGF1R/Akt signaling–mediated survival.

Introduction

Multiple myeloma is the second most common hematologic malignancy that is characterized by clonal proliferation of malignant plasma cells in the bone marrow (BM) microenvironment. Despite significant development of novel drugs in the last decade, multiple myeloma still remains incurable in the majority of patients (1). Current opinion is that molecularly informed targeted combination therapies will be required for long-term disease control (2).

Aberrant activation of the PI3K–protein kinase B (PKB/AKT) signaling in myeloma cells in the context of BM and cytokines led to the prediction that mTOR, a key protein of the PI3K/Akt pathway, may be a useful target in multiple myeloma. The serine/threonine kinase mTOR interacts with several proteins to form two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 phosphorylates p70 ribosomal protein S6 kinase (p70S6K) and translation initiation regulator 4E binding protein (4E-BP1), and regulates protein translation and cell size; mTORC2 phosphorylates Akt and SGK1, and regulates survival and cytoskeletal organization (3). mTOR is regulated via phosphorylation (4). There are several characterized phosphorylation sites in mTOR. T2446 is phosphorylated by adenosine monophosphate kinase and is regulated by nutrient availability. S2448 is phosphorylated by p70S6K, reflecting the amino acid and nutrient status. S2481 is the autophosphorylation site, which directly represents intrinsic mTOR-specific catalytic activity (5).

Initial studies targeting mTOR in multiple myeloma have focused on the inhibition of mTORC1 with rapamycin (6–8). The observed mTORC2 activation of Akt following rapamycin treatment prompted the rapid development of mTOR kinase inhibitors (9). On the basis of recent preclinical testing of mTOR kinase in multiple myeloma (10), it was predicted that reduction in phosphorylated (p) 4E-BP1 and p-Akt, both refractory to rapamycin, may provide preliminary evidence of mTOR kinase target inhibition and may overcome rapamycin-related resistance mechanisms.
However, cumulative evidence suggests that Akt activation may occur even in the context of mTOR kinase inhibition and blockage of TORC2/Akt signaling as a consequence of disinhibition of feedback signaling (11). Specifically, relief of feedback inhibition of receptor tyrosine kinases [RTK; e.g., EGFR, HER2, -3, -4, insulin-like growth factor 1 receptor (IGF1R), insulin receptor, and FGFR1-3] leading to subsequent PI3K activation and an mTOR-independent rephosphorylation of Akt at T308 is sufficient to reactivate Akt signaling (12). Therefore, the levels of p-Akt S473 in tumors treated with mTOR kinase inhibitors may be misleading as a biomarker of either response or resistance.

To date, suggested biomarkers of response to mTOR kinase inhibitors have been proposed based on preclinical data (4). These include TORC1 and TORC2 downstream targets, specifically the phosphorylated forms of P70S6K, 4E-BP1, and Akt. Although evaluating multiple markers may offer a more robust evaluation of the inhibition of mTOR signaling, the baseline variability between mTORC1 and mTORC2 outputs in myeloma tumors may present an obstacle in defining the mTOR profile of myeloma tumors sensitive to mTOR kinase inhibitors. Moreover, pathway inhibition may not be a useful marker of response because different components of downstream signaling have differing thresholds for inhibition. Recent studies have proposed that p-mTOR S2481 can be used to monitor mTOR kinase inhibition. Moreover, it has been suggested that this phosphosite is a better marker for mTORC2 activity than Akt Ser473 phosphorylation (13, 14).

We have now tested these assertions in multiple myeloma using the novel selective mTOR kinase inhibitor AZD8055 (15). Initially, we evaluated p-mTOR S2481 as the readout for mTORC2/Akt activity in multiple myeloma cells alone and in the context of mTOR inhibition via AZD8055 or rapamycin. We found that AZD8055 treatment induced upregulation of IGF1R in multiple myeloma cell lines with constitutive Akt activation, and that IGF1/IGF1R signaling mediated Akt upregulation despite TORC2 blockage. Our findings suggest that IGF1/IGF1R/Akt signaling may bypass mTORC2 when mediating Akt activation, which may reduce apoptosis induced by mTOR kinase inhibition. We believe the mTOR/Akt and IGF1R/Akt pathways cross-regulation might be of particular interest in multiple myeloma tumors with IGF1-enabled Akt activation. A better understanding of the feedback mechanisms responsible for Akt activation in multiple myeloma with or without stimulatory signals will complement the design of clinical trials for mTOR inhibitors either alone or in combination for patients with multiple myeloma.

Materials and Methods

Cell culture and reagents

**Human myeloma cell lines.** Dex-sensitive MM.1S and -resistant MM.1R cells were provided by Dr. Steven Rosen (Northwestern University, Evanston, IL). U266 and RPMI8226 cell lines were obtained from the ATCC. Doxo- rubinc-resistant RPMI-DOX40 and Melphalan-resistant RPMI-LR5 cells were kindly provided by Dr. William Dalton (Lee Moffitt Cancer Center, Tampa, FL). OPM1 and OPM2 plasma cell leukemia cell lines were kindly provided by Dr. Edward Thompson (University of Texas Medical Branch, Galveston, TX). IL6-dependent INA-6 cell line was provided by Dr. Renate Burger (University of Kiel, Germany). These lines were characterized by short tandem repeat profiling and compared with the known ATCC database and German Collection of Micro-organisms and Cell Cultures databases. The cell lines were passaged for fewer than 6 months following receipt or resuscitation from frozen stocks. All cell lines were cultured in RPMI-1640 containing 10% FBS (Sigma Chemical Co.), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (GIBCO), with 2.5 and 5 ng/mL of recombinant IL6 only in INA-6 and ANBL-6 cells, respectively. Patient multiple myeloma primary tumor cells were obtained from BM aspirates in accordance with the Declaration of Helsinki and with the approval of the Institutional Review Board of Massachusetts General Hospital. BM mononuclear cells were separated using Ficoll–Hapaque density sedimentation, and plasma cells were purified by positive selection with anti-CD138 magnetic activated cell separation microbeads (Miltenyi Biotec). BM stroma cells (BMSC) were generated from BM specimens from patients with multiple myeloma as described previously (16). BMSCs (10⁶ cells/well) were incubated in 96-well culture plates for 24 hours, multiple myeloma cells were then added to the wells (2 x 10⁶ cells/well) and incubated with media alone, or with increasing concentrations of AZD8055 or rapamycin for the specified times at 37°C. Blood samples collected from healthy volunteers were processed by Ficoll–Hipaque gradient to obtain peripheral blood mononuclear cells (PBMC).

**AZD8055.** Selective and orally bioavailable ATP-competitive mTOR kinase inhibitor was obtained from AstraZeneca. For *in vitro* studies, AZD8055 was prepared as 10 mmol/L stock solution in DMSO and stored at −20°C and diluted in culture medium (1–200 mmol/L) immediately before use. For studies in mice, AZD8055 was prepared as a suspension in sterile (autoclaved) vehicle 0.5% hydroxypropylmethylcellulose (ref. 56336; Fluka) + 0.1% polysorbate 80 (ref. 93780, Tween 80; Fluka), sterilized by autoclaving at 121°C for 20 minutes at 30 psi (1.5 Bar). AZD8055 was administered by oral gavage (0.1 mL/10 g of body weight) once or twice daily.

**Rapamycin.** Rapamycin was obtained from Calbiochem (EMD Millipore).

**Akti-1/2.** Akt 1/2 selective inhibitor was purchased from Calbiochem (EMD Millipore).

Cell viability and proliferation assays

**Colorimetric assay.** Colorimetric assays were done to assay drug activity. Cell cultures were pulsed with 10 µL of 5 mg/mL MTT (Chemicon International, Inc.) to each well, followed by incubation at 37°C for 4 hours, and addition of 100 µL isopropanol with 0.04 HCl. Absorbance
readings at a wavelength of 570 nm (with correction using readings at 630 nm) were taken on a spectrophotometer (Molecular Devices Corp.).

**Proliferation assay.** DNA synthesis was measured by tritiated thymidine uptake (3H-TdR; Perkin-Elmer) as previously described (8). Briefly, MM.1S cells (2–3 × 10^5 per well) were incubated in 96-well culture plates alone or in culture with BMSCs, IL6 (10 ng/mL), or IGF1 (50 ng/mL) in the presence of media or varying concentrations of AZD8055 or rapamycin for 48 hours at 37°C.

**Immunoblotting**

Multiple myeloma cells were cultured in media or with indicated concentrations of AZD8055 or rapamycin, harvested, and whole-cell lysates were subjected to SDS-PAGE, and transferred to nitrocellulose membrane (Bio-Rad Laboratories), as described previously (8). Immunoblotting was carried using specific antibodies: anti–p-mTOR S2481, -mTOR S2448, -p70S6K T389, -p70S6K T389/S424, -mTOR, –p-IGF1R T1135/1136, –p-4E-BP1 T37/46, –p-Akt S473, -Akt, –p-NDRG1 S330, -LC3B, -cleaved caspase-3, -7, -8, -9, -PARP, –p-IGF1R T1133/1135, and -IGF1R β (Cell Signaling Technology). Same lysates have been used for blotting total proteins and their phosphorylated forms. Blots were reprobed with anti-GAPDH antibody (Cell Signaling Technology) for equal protein control when there was unequal expression of total protein. Antigen-antibody complexes were detected by using enhanced chemiluminescence (Amershams).

**Protein synthesis assay**

Protein synthesis rate was measured using The CytoX-Red Cell Proliferation/Cytotoxicity Assay Kit (Epigentek Group Inc.) according to the manufacturer’s protocol. MM.1S cells (2 × 10^5 per well) were incubated in 96-well culture plates alone or with AZD8055 (50, 100, and 200 nmol/L) or rapamycin (100 nmol/L). After 24- or 48-hour incubation, the growth media were removed, cells washed with PBS, and 50 μL of indicator solution added to each well for 10 to 15 minutes at room temperature. The plate was washed, 100 μL of dissolving solution added to each well, and optical density (O.D.) was measured at 570 nanometers (nm).

**Detection of apoptosis by Annexin V/Propidium Iodide staining**

Detection of early apoptotic cells was performed with the Annexin V–PI (Annexin/PI) detection Kit (Immuno- tech/Beckman Coulter). Briefly, MM.1S cells (1 × 10^5) were exposed for various time points to AZD8055 or rapamycin. Cells were washed and then incubated in the dark at room temperature with Annexin V–FITC and PI for 15 minutes. Annexin V+/PI– apoptotic cells were enumerated using the Epics flow cytometer.

**Multiple myeloma xenograft murine model**

The in vitro anti–multiple myeloma activity of AZD8055 was evaluated in a previously established multiple myeloma xenograft model (8). CB-17 SCID mice obtained from Charles River Laboratories were subjected to animal studies according to the protocols approved by the Animal Ethics Committee. Forty 5- to 6-week-old male mice were irradiated [2 Gy (200 rad)] using cesium 137 ([137Cs])-iradiator source; 24 hours after irradiation, 2.5 × 10^6 MM.1S cells suspended in 100 μL of RPMI medium were inoculated subcutaneously in the upper back. When tumors were measurable with a tumor volume of approximately 150 to 200 mm³, mice were randomly assigned into three cohorts receiving daily gavages for 5 days per week for 4 consecutive weeks with either AZD8055 5 mg/kg (10 mice), 10 mg/kg (10 mice), or control vehicle alone (10 mice) on the same schedule. Animals were monitored for body weight and tumor volume by caliper measurements every day. Tumor volume was estimated using the following formula: \( \frac{1}{2} \times (\text{length}) \times (\text{width})^2 \). Animals were euthanized in accordance with institutional guidelines by CO2 inhalation in the event of tumor size >2 cm or major compromise in their quality of life, due to tumor ulceration. Survival was evaluated from the first day of treatment until death. Tumor growth was evaluated using caliper measurements from the first day of treatment until day of first sacrifice. Percentage tumor growth inhibition (TGI) was calculated as TGI on day X = 100 – T/C% on day X.

**Statistical analysis**

All in vitro experiments were performed in triplicate and repeated at least 3 times; a representative experiment was selected for figures. Statistical significance of differences was determined using the Student t test, with minimal level of significance \((P < 0.05)\). In vivo statistical tests were performed using the Student t test. Overall survival (OS) was measured using the Kaplan–Meier method, and results are presented as the median OS, with 95% confidence intervals.

**Results**

**mTOR/Akt signaling in multiple myeloma cells:**

p-mTOR S2481 as a marker for mTORC2-phosphorylatable Akt S473

We first examined whether autophosphorylation of mTOR on S2481 can be used as readout for mTORC2/Akt signaling in multiple myeloma cells. We evaluated the baseline phosphorylation of mTOR on S2481 in multiple myeloma cell lines (Fig. 1A). Normally, mTORC1 contains mainly mTOR phosphorylated at S2448, whereas mTORC2 contains predominantly mTOR phosphorylated at S2461 (13). Through Western blot analysis, we found that p-mTOR S2481 was present only in MM.1S, MM.1R, OPM1, OPM2, and U266 cells, whereas p-mTOR S2448 was expressed in all multiple myeloma cell lines. We next compared the expression of mTOR phosphorylation on S2448 and S2461 with TORC1 and TORC2 readouts, respectively. The best-characterized mTORC1 readouts are
P70S6K and 4E-BP, whereas Akt is a commonly used readout for mTORC2 activity. Our data show that baseline expression of p-mTOR S2448 correlated with TORC1-phosphorylatable P70S6K and 4E-BP, and expression of p-mTOR S2481 correlated with TORC2-phosphorylatable Akt (S473) across all multiple myeloma cell lines, except U266 in which p-mTOR S2481 is highly expressed, whereas p-AKT S473 is not significantly expressed.

Figure 1. mTOR/Akt signaling in multiple myeloma cells; p-mTOR S2481 as a marker for mTORC2-phosphorylatable Akt S473. A, baseline mTOR profile in multiple myeloma cell lines. Multiple myeloma cells grown in culture media alone were lysed and mTOR/TORC1/TORC2-related protein expression analyzed by Western blotting. B, effect of serum starvation/readdition on mTOR activity in multiple myeloma cells. MM.1S, OPM1, and RPMI multiple myeloma cells were cultured in regular (10% FBS) or serum-starved media for 72 hours; next, regular media were added to 72-hour–starved cells for 4, 8, 24, 48, or 72 hours, and whole-cell lysates subjected to Western blotting for p-mTOR S2448/S2481, mTOR, p-P70S6K T389, P70S6K, p-Akt S473, and Akt expression. GAPDH was used for loading control. C, Akt regulates p-mTOR S2481. MM.1S, OPM1, and RPMI cells exposed to media alone or with Akti-1/2 (5 μmol/L) for 30 minutes, 1 hour, 3 hours, or 6 hours were analyzed by Western blotting for the expression of p-Akt S473, Akt, p-mTOR S2481, and mTOR. D, p-mTOR S2481 correlates with p-Akt S473 expression in CD138+ multiple myeloma primary tumor cells. CD138+ primary tumor cells from patients with multiple myeloma and PBMCs from healthy donors were subjected to Western blotting, and p-mTOR S2481, mTOR, p-Akt S473, Akt, and GAPDH expression detected using specific antibodies.
Because serum starvation leads to a uniform response in the mTOR pathway (17), we next compared the kinetics of S2448/S2481 phosphorylation of mTOR with p-Akt S473 levels in multiple myeloma cell lines with relatively high (MM.1S and OPM1 cells) versus low (RPMI cells) constitutive expression of p-Akt S473 (Fig. 1B). MM.1S, OPM1, and RPMI cells cultured in serum-deprived media for 72 hours and then stimulated with serum for increasing time points (4–72 hours) were subjected to Western blotting. In RPMI cells, p-mTOR S2481 and p-Akt S473 followed similar kinetics of expression during serum withdrawal and readdition. In MM.1S cells, the initial induction of p-Akt S473 occurred before p-mTOR S2481 induction, suggesting that either low levels [not easily seen by Western blotting (WB)] of p-mTOR S2481 are sufficient to induce p-AKT S473 or that TORC2 is not the only candidate to phosphorylate Akt at S473 in this cell line. In OPM1 cells, p-mTOR S2481 and p-Akt S473 coexpression was present even in serum-starved cells, consistent with PI3K/Akt pathway constitutive activation due to PTEN deletion.

Because Akt plays a role in the regulation of mTOR, we next examined whether p-mTOR S2481 is dependent on the Akt activation status (Fig. 1C). We found that reduced expression of p-Akt S473 via treatment with the Akt inhibitor Akti-1/2 resulted in reduced mTOR phosphorylation at S2481 in MM.1S and RPMI, but not in OPM1 cells. Finally, we tested p-mTOR S2481 and p-Akt S473 baseline expression in CD138+ cells obtained from three patients with multiple myeloma, and in PBMCs from five healthy donors (Fig. 1D). We found that p-mTOR S2481 expression correlated with p-Akt S473 in both CD138+ and healthy donor mononuclear cells.

AZD8055, an mTOR kinase inhibitor, inhibits multiple myeloma cell growth in vitro and in vivo

We then evaluated the effect of targeting TORC1 and TORC2 in MM by using a novel selective mTOR kinase inhibitor, AZD8055 (15). We first assessed the effects of AZD8055 (25–200 nmol/L) treatment on cell number and metabolic activity of multiple myeloma cell lines by MTT assay at 72 hours. We noted dose-dependent differences in growth among cells with high versus low p-mTOR S2481/p-Akt S473 expression, with MM.1S, MM.1R, OPM1, and OPM2 cells being more sensitive to AZD8055 treatment (Fig. 2A). We also examined AZD8055 activity in freshly isolated CD138+ tumor cells from patients with multiple myeloma (Fig. 2B). Immunodeficient SCID-CB17 mice were inoculated subcutaneously with MM.1S cells. Oral AZD8055 was administered daily 5 days per week for 4 consecutive weeks either 5 mg/kg (10 mice), 10 mg/kg (10 mice), or control vehicle (10 mice) starting after the development of measurable tumor with a tumor volume of approximately 150 to 200 cm3. AZD8055 treatment resulted in tumor growth inhibition and prolonged survival.
myeloma. As evidenced by the MTT assays, AZD8055 (100–200 nmol/L) treatment (72 hours) inhibited the proliferation of three of eight tested samples (Fig. 2B). Increased dosing of AZD8055 (0–800 nmol/L) did not trigger significant cytotoxicity in healthy donors PBMCs (Fig. 2C). We next validated AZD8055’s anti–multiple myeloma activity in vivo in the MM.1S cell xenograft SCID mouse model (Fig. 2D). Treatment with AZD8055 significantly suppressed multiple myeloma tumor growth, with maximum TGI (%) noted at days 19 and 24 following start significantly suppressed multiple myeloma tumor growth, with maximum TGI (%) noted at days 19 and 24 following start treated cohorts, respectively (control vs. treated, maximum TGI (%) noted at days 19 and 24 following start treated). Treatment with AZD8055 was also associated with improved OS (control vs. treated, P < 0.05; Fig. 2D, left). No toxic deaths occurred during this study: maximum percentage of body weight loss was observed on day 5 (1.3%) at 5 mg/kg dosage schedule and on day 8 (6%) after 10 mg/kg dosing, with weight recovery in the following 2 weeks with continued dosing (Fig. 2D).

AZD8055 blocks p-mTOR S2481/p-Akt S473 signaling and displays higher antimyeloma effect compared with rapamycin

We next examined how p-mTOR S2481/p-Akt S473 expression correlates with response to mTOR-targeted treatment in multiple myeloma cells. We compared p-mTOR S2481/p-Akt S473 response to AZD8055 and to rapamycin treatment in MM.1S cells. Time course Western blot analysis of MM.1S cells exposed to AZD8055 (100 nmol/L) demonstrated inhibition of phosphorylation of both TORC1 and TORC2 substrates: P70S6K and 4E-BP1 including the rapamycin-resistant T37/44e—downstream targets of TORC1, as well as Akt and NDRG1—effectors of TORC2 refractory to rapamycin (Fig. 3A). Unlike rapamycin, AZD8055 consistently inhibited mTOR phosphorylation at S2481 along with p-Akt S473, as evidenced by Western blot studies in MM.1S, OPM1, and RPMI cells (Fig. 3B). These results reflected in a different response in term of cell viability when the multiple myeloma cells were exposed to the same concentration of AZD8055 and rapamycin (AZD8055 vs. rapamycin MM.1S P = 0.0011, MM1R P = 0.0001, OPM1 P = 0.0001, OPM2 P < 0.0001, RPMI P = 0.0016, DOX-40 P = 0.0016, LR5 P = 0.0005, and INA-6 P = 0.0034), as shown by MTT assay (Fig. 3C). AZD8055 was more potent than rapamycin and resulted in a greater impact on cell viability.

We next evaluated the cellular effects of the AZD8055-mediated inhibition of mTORC1 and mTORC2 functions in MM.1S cells. mTORC1 is a positive regulator of protein synthesis through both inhibition of autophagy and induction of P70S6K/4E-BP1—mediated protein translation. Our Western blot time course (0, 3, 6, 12, 24, and 48 hours) analysis of LC3 I and LC3 II expression showed that AZD8055 (100 nmol/L) induced a clear cleavage of LC3BI into LC3BII. Such a clear effect was not observed after rapamycin (100 nmol/L) treatment. Although prolonged treatment with rapamycin increased the expression of both LC3 I and LC3 II bands, prolonged treatment with AZD8055 reduced LC3 I at 3 hours and triggered the disappearance of both bands by 24 hours (Fig. 3D, left). Both AZD8055 and rapamycin 24- and 48-hour treatments resulted in up to 80% inhibition of protein synthesis (AZD8055 50 nmol/L vs. rapamycin 100 nmol/L, 48 hours, P = 0.5; AZD8055 100 nmol/L vs. rapamycin 100 nmol/L, 48 hours, P = 0.16; AZD8055 200 nmol/L vs. rapamycin 100 nmol/L, 48 hours, P = 0.11; Fig. 3D, right).

Because mTORC2 is a positive regulator of cell survival via Akt-mediated inhibition of apoptosis, we examined whether AZD8055 could trigger apoptosis in multiple myeloma cells. Consistent with mTORC2 inhibition, AZD8055, unlike rapamycin, induced apoptosis, as evidenced by Western blot detection of caspase-7, -8, -9, -3, and PARP cleavage in a time-dependent fashion (Fig. 3E, left), and confirmed by increased population of apoptotic cells at 72-hour culture as detected by Annexin V/PI staining (Fig. 3E, right).

IGF1-induced Akt activation confers relative resistance to AZD8055-mediated cytotoxicity

Because we, and others, have reported on the role of the BM microenvironment in mTOR/Akt-mediated proliferation and survival in multiple myeloma cells, we next examined AZD8055’s efficacy in multiple myeloma cells in culture with BMSCs or growth factors such as IL6 and IGF1. We have previously shown that rapamycin alone was unable to overcome the protective effects of BMSCs and growth factors (7, 8). We therefore evaluated AZD8055’s activity next to rapamycin in MM.1S cultures with or without BMSCs via [3H]-thymidine uptake at 48 hours. AZD8055 resulted in greater antiproliferative effects as compared with rapamycin. AZD8055 treatment however could not completely overcome BMSCs-mediated growth of MM.1S cells at 100 nmol/L dose (Fig. 4A). Because of the major role played by IL6 and IGF1 in multiple myeloma cell survival triggered by BM microenvironment, we next examined AZD8055 and rapamycin treatments of MM.1S cells cultured with or without IL6 (10 ng/mL) or IGF1 (50 ng/mL). [3H]-thymidine uptake at 48 hours demonstrated that AZD8055 treatment completely abrogated IL6-mediated proliferation; meanwhile, IGF1 conferred relative resistance to AZD8055 in a manner similar to BMSCs (Fig. 4B).

Because recent evidence suggests that mTOR and Akt negatively regulate IGF1R expression, and that IGF1R activity is reactivated by mTOR kinase inhibitors (12, 18, 19), we sought to determine whether AZD8055 triggered IGF1R feedback activation in multiple myeloma cells. We tested our hypothesis in two multiple myeloma cell lines with relatively high baseline Akt activity (MM.1S and OPM1). Multiple myeloma cells were cultured for 6 hours in control media, AZD8055 (100 nmol/L) or rapamycin (100 nmol/L), then stimulated with IGF1 (50 ng/mL) for 15 minutes, and whole-cell lysates were subjected to Western blot analysis. We found that AZD8055 alone induced the phosphorylation of tyrosine sites (Y1135/1136) in the activation loop of the IGF1R kinase.
Figure 3. AZD8055 inhibits cell growth by blocking p-mTOR S2481/p-AKT S473 signaling. A, AZD8055, an mTOR kinase inhibitor, induces dual inhibition of TORC1/TORC2 signaling in multiple myeloma cells. MM.1S cells were incubated with AZD8055 (100 nmol/L) or rapamycin (100 nmol/L) for the specified time points, subjected to Western blot, and mTORC1/mTORC2 downstream targets expression was detected using specific antibodies. B, p-mTOR S2481 is a marker of mTOR kinase inhibition in multiple myeloma cells. MM.1S, OPM1, and RPMI cells were incubated with AZD8055 (100 nmol/L) or rapamycin (100 nmol/L) for short time points (15 minutes to 6 hours). Cells were pelleted and subjected to Western blotting. p-mTOR S2481, mTOR, p-Akt S473, and Akt expression was determined using specific antibodies. C, p-mTOR S2481/p-Akt S473 coexpression correlates with sensitivity to mTOR kinase inhibition in multiple myeloma cell lines. Multiple myeloma cell lines were cultured in culture media or with AZD8055 (100 nmol/L) or rapamycin (100 nmol/L), and cytotoxicity was evaluated by MTT assay at 72 hours; data, mean (±SD) of triplicate culture. D, left, AZD8055 induced autophagy, consistent with TORC1 inhibition. MM.1S cells were incubated with media, AZD8055 (100 nmol/L), or rapamycin (100 nmol/L) for 3, 6, 12, 24, or 48 hours, and cells analyzed for LC3B expression using Western blot. GAPDH expression served as a loading control. Right, AZD8055 blocked protein synthesis, a TORC1-related function. Protein synthesis was assayed in MM.1S cells cultured for 24 and 48 hours either in regular media or with increasing concentrations of AZD8055 (50–200 nmol/L) or rapamycin (100 nmol/L), and cytotoxicity was evaluated by MTT assay at 72 hours; data, mean (±SD) of triplicate culture. D, left, AZD8055 induced autophagy, consistent with TORC1 inhibition. MM.1S cells were incubated with media, AZD8055 (100 nmol/L), or rapamycin (100 nmol/L) for 3, 6, 12, 24, or 48 hours, and cells analyzed for LC3B expression using Western blot. GAPDH expression served as a loading control. Right, AZD8055 blocked protein synthesis, a TORC1-related function. Protein synthesis was assayed in MM.1S cells cultured for 24 and 48 hours either in regular media or with increasing concentrations of AZD8055 (50–200 nmol/L) or rapamycin (100 nmol/L), and cytotoxicity was evaluated by MTT assay at 72 hours; data, mean (±SD) of triplicate culture. D, left, AZD8055 induced autophagy, consistent with TORC1 inhibition. MM.1S cells were incubated with media, AZD8055 (100 nmol/L), or rapamycin (100 nmol/L) for 3, 6, 12, 24, or 48 hours, and cells analyzed for LC3B expression using Western blot. GAPDH expression served as a loading control. Right, AZD8055 blocked protein synthesis, a TORC1-related function. Protein synthesis was assayed in MM.1S cells cultured for 24 and 48 hours either in regular media or with increasing concentrations of AZD8055 (50–200 nmol/L) or rapamycin (100 nmol/L), and cytotoxicity was evaluated by MTT assay at 72 hours; data, mean (±SD) of triplicate culture. D, left, AZD8055 induced autophagy, consistent with TORC1 inhibition. MM.1S cells were incubated with media, AZD8055 (100 nmol/L), or rapamycin (100 nmol/L) for 3, 6, 12, 24, or 48 hours, and cells analyzed for LC3B expression using Western blot. GAPDH expression served as a loading control. Right, AZD8055 blocked protein synthesis, a TORC1-related function. Protein synthesis was assayed in MM.1S cells cultured for 24 and 48 hours either in regular media or with increasing concentrations of AZD8055 (50–200 nmol/L) or rapamycin (100 nmol/L), and cytotoxicity was evaluated by MTT assay at 72 hours; data, mean (±SD) of triplicate culture. D, left, AZD8055 induced autophagy, consistent with TORC1 inhibition. MM.1S cells were incubated with media, AZD8055 (100 nmol/L), or rapamycin (100 nmol/L) for 3, 6, 12, 24, or 48 hours, and cells analyzed for LC3B expression using Western blot. GAPDH expression served as a loading control. Right, AZD8055 blocked protein synthesis, a TORC1-related function. Protein synthesis was assayed in MM.1S cells cultured for 24 and 48 hours either in regular media or with increasing concentrations of AZD8055 (50–200 nmol/L) or rapamycin (100 nmol/L), and cytotoxicity was evaluated by MTT assay at 72 hours; data, mean (±SD) of triplicate culture.
Figure 4. IGF1-induced Akt activation confers relative resistance to AZD8055-mediated cytotoxicity. A, BMSC-stimulated MM.1S cells partially escape AZD8055-mediated growth inhibition. MM.1S cells were cultured for 48 hours in control media, AZD8055 (25–200 nmol/L), or rapamycin (10–100 nmol/L) in the presence or absence of BMSCs. Cell proliferation was assessed by [3H]Thymidine assay; data, mean (±SD) of triplicate cultures.

B, AZD8055-inhibited proliferation of MM.1S is partially rescued by IGF1. MM.1S cells were cultured for 48 hours in control media, AZD8055 (25–200 nmol/L), or rapamycin (10–100 nmol/L) in the presence or absence of IL6 (10 ng/mL) or IGF1 (25 ng/mL). Cell proliferation was assessed by [3H]Thymidine assay; data, mean (±SD) of triplicate cultures.

C, AZD8055 treatment triggered upregulation of the tyrosine phosphorylation sites in the activation loop of the IGF1R kinase domain T1135/1136, which associated with activated p-Akt S473 in multiple myeloma cells despite AZD8055 exposure. MM.1S and OPM1 cells were cultured for 6 hours in regular media, AZD8055 (100 nmol/L), or rapamycin (100 nmol/L). Cells were then pelleted down and resuspended in PBS with or without IL6 (10 ng/mL) or IGF1 (50 ng/mL) for 15 minutes. Expression of p-IGF1R T1135/1136, IGF1R, p-mTOR S2448, mTOR, p-Akt S473, and Akt was examined via Western blot using GAPDH for loading control.

D, IGF1 confers relative resistance to AZD8055-induced cytotoxicity of multiple myeloma. MM.1S and OPM1 multiple myeloma cells incubated for 48 hours in media alone or AZD8055 (50, 100 nmol/L), with or without 50 ng/mL IGF1, were assessed for viability via MTT assay; data, mean (±SD) of triplicate cultures.
domain. IGF1 clearly upregulated Akt phosphorylation in multiple myeloma cells; however, it had no effect on mTOR Ser2481 phosphorylation. Moreover, AZD8055-treated cells exposed to IGF1 sustained p-Akt Ser473 expression, whereas p-mTOR Ser2481 remained inhibited (Fig. 4C).

Reactivation of IGF1R signaling in multiple myeloma cells in the context of mTOR kinase inhibitors suggests that multiple myeloma may survive in an IGF1-dependent fashion. We therefore next treated MM.1S and OPM1 cells with AZD8055, in the presence or absence of IGF1 (50 ng/mL). As evidenced by the MTT assay at 48 hours, multiple myeloma cells partially escaped AZD8055 cytotoxicity (Fig. 4D).

### Blockage of IGF1R restores AZD8055-mediated multiple myeloma cytotoxicity

We next determined whether blocking IGF1R would (i) prevent IGF1-mediated Akt reactivation in AZD8055-treated multiple myeloma cells, and (ii) reinstitute AZD8055’s anti–multiple myeloma activity compromised by IGF1. We performed Western blot analysis of MM.1S cells treated with AZD8055 (100 nmol/L) or anti–IGF1R Ab (1 μg/mL), or the combination, with or without the addition of 50 ng/mL IGF1 for 6 hours. Blockade of IGF1R signaling with the anti–IGF1R Ab alone resulted in partial reduction of Akt S473 phosphorylation. We confirmed the anti–IGF1R Ab–mediated downregulation of Akt activity

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**Figure 5.** Blockage of IGF1R restores AZD8055-mediated multiple myeloma cytotoxicity. A, IGF1R antibody prevents IGF1-mediated Akt reactivation in AZD8055-treated MM.1S cells. MM.1S cells were preincubated for 1 hour with Iso-IgG Ab (1 μg/mL) or anti–IGF1R Ab (1 μg/mL) and then exposed to 100 nmol/L AZD8055 with or without 50 ng/mL IGF1 for 6 hours. Expression of p-mTOR S2481, mTOR, p-Akt S473, Akt, and p-FoxO1 T24/FoxO3a T32 was examined via Western blot using GAPDH for loading control. B, the combination of AZD8055 with the anti–IGF1R Ab restores AZD8055-mediated caspase cleavage impaired by IGF1. MM.1S cells cultured for 24 hours with 100 nmol/L AZD8055, 1 μg/mL anti–IGF1R Ab, or their combination, with or without 50 ng/mL IGF1, were subjected to Western blot analysis for caspase-9 and -3 expression. C, blockage of IGF1R via anti–IGF1R Ab prevents IGF1-mediated reduction in apoptosis produced by AZD8055 treatment. Annexin/PI flow cytometry analysis was performed to determine the rate of apoptosis in MM.1S cells subjected to longer treatment (72 hours) with AZD8055 (100 nmol/L) with or without anti–IGF1R Ab pretreatment, with or without IGF1 (50 ng/mL). D, IGF1R blockade via IGF1R Ab potentiates AZD8055 activity in MM.1R and ANBL-6 cells. MM.1R and ANBL-6 cells preincubated for 1 hour with Iso-IgG Ab (1 μg/mL) or anti–IGF1R Ab (1 μg/mL) were exposed to AZD8055 (100 nmol/L) with or without IGF1 (50 ng/mL) for 72 hours and viability assessed via MTT assay; data, mean (±SD) of triplicate cultures.
by reduced p-FoxO1 T24/FoxO3a T32. Next, 50 ng/mL IGF1 partially rescued the phosphorylation of Akt S473/ FoxO1 T24/FoxO3a T32 signaling in both AZD8055- and anti-IGF1R-treated cells, but not in the combination treatment. Notably, mTOR S2481 phosphorylation remained unchanged either with IGF1 stimulation or with anti-IGF1R Ab treatment (Fig. 5A).

We hypothesized that IGF1/IGF1R-mediated signaling could rescue MM.1S cells from AZD8055-mediated apoptosis. Through Western blot analysis of MM.1S cells cultured for 24 hours with 100 nmol/L AZD8055 or 1 µg/mL anti–IGF1R Ab; or the combination with or without 50 ng/mL IGF1, we found that IGF1 significantly reduced caspase-3 cleavage induced by AZD8055 treatment in MM.1S. In contrast, the combination of AZD8055 with the anti–IGF1R Ab increased the activation of caspases-9 and -3 (Fig. 5B).

We therefore used Annexin/PI flow cytometry analysis to determine the rate of apoptosis in MM.1S cells subjected to longer treatment (72 h) with AZD8055 (100 nmol/L) with or without anti–IGF1R Ab pretreatment, with or without the addition of IGF1 (50 ng/mL). AZD8055 alone induced 56.5% apoptotic cells; the addition of anti–IGF1R Ab increased the apoptotic population to 77.9%. As hypothesized, exposure to IGF1 reduced AZD8055-induced apoptosis from 56.5% to 15.1%, whereas blockage of IGF1 partially rescued AZD8055-mediated apoptosis from IGF1R via anti–IGF1R Ab (Fig. 5C).

To confirm our observation, we evaluated AZD8055 and the anti–IGF1R Ab combined treatment in two other multiple myeloma cell lines, MM.1R and ANBL-6. MM.1R and ANBL-6 cells were preincubated for 1 hour with 1 µg/mL Iso-IGF or 1 µg/mL anti–IGF1R Ab and then cultured with AZD8055 (0–100 nmol/L) or regular media, with or without 50 ng/mL IGF1. After 72-hour treatment, anti–IGF1R Ab alone reduced the cell numbers by 73% and 33% in MM.1R and ANBL-6 cells, respectively; next, anti–IGF1R Ab enhanced AZD8055 activity (85%) cell number decrease in MM.1R treated with AZD8055 vs. 94% cell number decrease in MMIR treated with AZD8055 and anti–IGF1R Ab, 46% cell number decrease in ANBL-6 treated with AZD8055 vs. 66% cell number decrease in ANBL-6 treated with AZD8055 and anti–IGF1R Ab. P < 0.05 and reversed IGF1-mediated partial rescue of multiple myeloma cells from AZD8055-induced cytotoxicity (Fig. 5D). To confirm these findings, we suppressed the expression of IGF1R using siRNA in U266 cell lines. Cells with IGF1R knockout showed 39% decrease in viability compared with scramble control, and the combination of IGF1R knock out and AZD8055 100 nmol/L showed a 78% decrease in viability compared with scramble control (Supplementary Fig. S1).

Discussion

The growing complexity of the mTOR pathway, as well as emerging discoveries about its autoregulatory mechanisms and activating feedback loops (4, 9), highlights the need for ongoing research of the mTOR pathway in multiple myeloma. Our data aligned with previous findings in multiple myeloma (10) suggest that targeting mTOR kinase produces more effective mTOR pathway inhibition than rapamycin with a more detrimental impact on multiple myeloma cells. Unlike rapamycin, AZD8055 inhibition of mTORC1 and mTORC2 functions resulted in apoptosis in multiple myeloma cells. However, we also found that mTOR kinase inhibition via AZD8055-induced phosphorylation of IGF1R. Furthermore, exposure of AZD8055-treated cells to IGF1 recovered p-Akt S473 and reduced apoptosis of multiple myeloma cells despite mTOR kinase inhibition. In contrast, the addition of blocking IGF1R antibody rescued AZD8055-induced apoptosis from IGF1-mediated multiple myeloma cell survival.

Taken together, our findings indicate that p-Akt S473 is not an accurate marker for mTORC2 inhibition in multiple myeloma cells. So far, several biomarkers have been developed to monitor the effects of mTOR kinase inhibitors. These include measurements by Western blot or immunohistochemistry of P70S6K, 4E-BP1, and Akt phosphorylation. Because these markers may lack the required selectivity and sensitivity, there is a clear need for the identification and validation of additional biomarkers allowing us to predict and monitor responses to mTOR inhibitors. We propose p-mTOR S2481 as the more precise marker to monitor response to mTOR kinase inhibition in multiple myeloma cells, specifically when it involves evaluating mTORC2/Akt function.

Reportedly, mTOR is phosphorylated differentially when associated with mTORC1 and mTORC2. Specifically, mTOR phosphorylated on S2448 with Raptor, and mTOR phosphorylated on S2481 with Rictor. Therefore, it was believed that mTORC1 contains mTOR phosphorylated predominantly on S2448, whereas mTORC2 contains mTOR phosphorylated predominantly on S2481. Moreover, intact mTORC2 is obligatory for mTOR S2481 phosphorylation (13, 14). In agreement with these data, our study in multiple myeloma cells found that baseline expression of p-mTOR S2481 correlated with TORC2-phosphorylatable Akt (S473). AZD8055, unlike rapamycin, consistently inhibited mTOR phosphorylation at S2481 along with p-Akt S473. Importantly, p-mTOR S2481/p-Akt S473 coexpression in multiple myeloma cell lines correlated with sensitivity to mTOR kinase inhibition. Our data therefore suggest that phosphorylation of S2481 in mTOR may serve as a potential marker for intact mTORC2 activity and sensitivity to mTOR kinase inhibitors in multiple myeloma. However, further studies are warranted to confirm that p-mTOR S2481 is a good readout of mTORC2 activation in multiple myeloma.

Until recently it was believed that TORC1/P70S6K suppression in the context of rapamycin treatment was responsible for triggering disinhibition of IGF1R signaling and subsequent Akt activation (20). In this regard, identification and characterization of mTORC2/Akt signaling and the discovery of mTOR kinase inhibitors
demonstrating dual inhibition of TORC1 and TORC2 pathways predicted the blockage of Akt signaling induced in multiple myeloma cells by upstream IGF1R/Pi3K activation through interaction with growth factors and the BM microenvironment. In our study, AZD8055 inhibition of mTOR kinase in multiple myeloma cells associated with upregulation of the tyrosine phosphorylation sites (Y1135/1136) in the activation loop of the IGFIR kinase domain. Furthermore, AZD8055-treated cells exposed to IGF1-sustained p-Akt S473 expression, while TORC2 function remained inhibited, as suggested by inhibition of p-mTOR S2441. Our observation aligned with recent findings in other tumor types that Akt activation may occur even in the context of mTOR kinase inhibition because of blockage of TORC2/Akt and following dis-inhibition of RTK/Pi3K feedback signaling stimulated by growth factors (12, 19). Given that, it is possible that AZD8055-triggered IGF1R activation is in part due to mTORC1 inhibition and in part secondary to TORC2/Akt suppression.

In multiple myeloma, activation of IGF1R induces sustained activation of Pi3K/Akt and NF-κB, phosphorylation of FKHR transcription factor, and upregulation of a series of intracellular antiapoptotic proteins, including FLIP, survivin, cIAP-2, and X-linked inhibitor of apoptosis protein (XIAP), thereby decreasing drug sensitivity of multiple myeloma cells (21, 22). Moreover, IGF1R inhibitors have been shown to trigger significant multiple myeloma cell toxicity (22, 23). A phase I study in multiple myeloma demonstrated the safe profile of fugitumumab, targeting data, constructing databases:

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Delineating the mTOR Kinase Pathway Using a Dual TORC1/2 Inhibitor, AZD8055, in Multiple Myeloma

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