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

MM 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 MM 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 phosphatidylinositol 3-kinase (PI3K)—protein kinase B (PKB/AKT) signaling in myeloma cells in the context of BM and cytokines led to the prediction that mammalian target of rapamycin (mTOR), a key protein of the PI3K/Akt pathway, may be a useful target in MM. 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. T^{2446} is phosphorylated by adenosine monophosphate kinase and is regulated by nutrient availability. S^{2448} is phosphorylated by P70S6K, reflecting the amino acid and nutrient status. S^{2481} is the autophosphorylation site, which directly represents intrinsic mTOR specific catalytic activity(5).

Initial studies targeting mTOR in MM 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). Based on recent preclinical testing of mTOR kinase in MM(10), it was predicted that reduction in phosphorylated -4E-BP1 and –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 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, IGF-1R, insulin receptor, and FGFR1-3) leading to subsequent PI3K activation and an mTOR-independent re-phosphorylation of AKT at T^308 is sufficient to reactivate AKT signaling(12). Therefore, the levels of p-Akt S^473 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. While 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 S^2481 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 Ser^473 phosphorylation (13, 14).

We have now tested these assertions in MM using the novel selective mTOR kinase inhibitor AZD8055(15). Initially, we evaluated p-mTOR S^2481 as the readout for mTORC2/Akt activity in MM cells alone and in the context of mTOR
inhibition via AZD8055, or rapamycin. We found that AZD8055 treatment induced upregulation of IGF-1R in MM cell lines with constitutive Akt activation, and that IGF-1/IGF-1R signaling mediated Akt upregulation despite TORC2 blockage. Our findings suggest that IGF-1/IGF-1R/Akt signaling may bypass mTORC2 when mediating Akt activation, which may reduce apoptosis induced by mTOR kinase inhibition. We believe the mTOR/Akt and IGF-1R/Akt pathways cross regulation might be of particular interest in MM tumors with IGF-1-enabled Akt activation. A better understanding of the feedback mechanisms responsible for Akt activation in MM with or without stimulatory signals will complement the design of clinical trials for mTOR inhibitors either alone or in combination for MM patients.

MATERIALS AND METHODS

Cell Culture and Reagents

*Human myeloma cell lines (HMCLs)*

Dex-sensitive MM.1S and -resistant MM.1R cells were provided by Dr. Steven Rosen (Northwestern University). U266 and RPMI8226 cell lines were obtained from American Type Culture Collection. Doxorubicin-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). IL-6 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 Microorganisms 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% fetal bovine serum (FBS, Sigma Chemical Co.), 2μM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin (GIBCO), with 2.5 and 5 ng/ml of recombinant interleukin-
6 (IL-6) only in INA-6 and ANBL-6 cells, respectively. Patient MM primary tumor cells were obtained from bone marrow (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-Hipaque density sedimentation, and plasma cells were purified by positive selection with anti-CD138 magnetic activated cell separation microbeads (Miltenyi Biotec, San Diego, CA). BM stroma cells (BMSCs) were generated from BM specimens from MM patients as described previously(16). BMSCs (10³ cells/well) were incubated in 96 well culture plates for 24 hours, MM cells were then added to the wells (2 x 10⁴ 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 (PBMCs).

AZD8055
Selective and orally bioavailable ATP-competitive mTOR kinase inhibitor was obtained from AstraZeneca (UK). For in vitro studies, AZD8055 was prepared as 10mmol/L stock solution in DMSO and stored at -20°C and diluted in culture medium (1-200 nM) immediately before use. For studies in mice AZD8055 was prepared as a suspension in sterile (autoclaved) vehicle 0.5% HPMC (hydroxpropylmethylcellulose [ref. 56336, Fluka]) + 0.1% polysorbate 80 [ref. 93780, Tween 80; Fluka]), sterilised by autoclaving at 121°C for 20mins @ 30psi (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 4h, and addition of 100μL isopropanol with 0.04 HCl. Absorbance readings at a wavelength of 570nm (with correction using readings at 630nm) 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 × 10,000 per well) were incubated in 96-well culture plates alone or in culture with BMSCs, IL-6 (10ng/mL), or IGF-I (50ng/mL) in the presence of media or varying concentrations of AZD8055 or rapamycin for 48 h at 37°C.

Immunoblotting

MM cells were cultured in media or with indicated concentrations of AZD8055, or rapamycin, harvested, and whole cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), as described previously(8). Immunoblotting was carried using specific antibodies: anti-phosphorylated (p)-mTOR S2481, -mTOR S2448, -mTOR, -p-P70S6K T389, -p-P70S6K T421/S424, -P70S6K, -p-4E-BP1 T37/46, -p-4E-BP1 S65, -p-Akt S473, -Akt, -p-NDRG1 S330, -LC3B, -cleaved caspase-3, -7, -8, -9, -PARP, -p-IGF-1R T1135/1136, and -IGF-1R β (Cell Signaling Technology, Beverly, MA). Same lysates have been used for blotting total proteins and their phosphorylated forms. Blots were re-probed with anti-GAPDH antibody (Cell Signaling Technology, Beverly, MA) for equal protein control when there was unequal expression of total protein.
Antigen–antibody complexes were detected by using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

**Protein Synthesis Assay**

Protein synthesis rate was measured using The CytoX-Red™ Cell Proliferation/Cytotoxicity Assay Kit (Epigentek Group Inc., Farmingdale, NY) according to the manufacturer’s protocol. MM.1S cells (2 × 10,000 per well) were incubated in 96-well culture plates alone or with AZD8055 (50, 100, 200 nM), or rapamycin (100 nM). After 24 or 48 h incubation the growth media was removed, cells washed with PBS and 50 uL of indicator solution added to each well for 10-15 min at RT. The plate was washed, 100 uL of dissolving solution added to each well, and O.D. measured at 570 nM.

**Detection of Apoptosis by Annexin V/PI Staining**

Detection of early apoptotic cells was performed with the annexin V-PI (Annexin/PI) detection kit (Immunotech/Beckman Coulter). Briefly, MM.1S cells (1 × 10^6) 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+ positive apoptotic cells were enumerated using the Epics flow cytometer.

**MM Xenograft Murine Model**

The *in vivo* anti-MM activity of AZD8055 was evaluated in a previously established MM xenograft model(8). CB-17 severe combined immunodeficient (SCID) mice obtained from Charles River Laboratories (Wilmington, MA) were subjected to animal studies according to the protocols approved by the Animal Ethics Committee. Forty 5-6 week old male mice were irradiated (2 Gy [200 rad]) using cesium 137 (137Cs)-irradiator source; 24 hours after irradiation 2.5 × 10^6
MM.1S cells suspended in 100μL of RPMI medium were inoculated subcutaneously in the upper back. When tumors were measurable with a tumor volume of approximately 150-200 mm³, mice were randomly assigned into three cohorts receiving daily gavages for 5 days/week for 4 consecutive weeks with either AZD8055 5mg/kg (10 mice), 10mg/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 > 2cm 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 were determined using Students t test, with minimal level of significance \( p<0.05 \). In vivo statistical tests were performed using 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 MM cells: p-mTOR S^{2481} as a Marker for mTORC2-Phosphorylatable Akt S^{473}.*
We first examined whether autophosphorylation of mTOR on S\(^{2481}\) can be used as readout for mTORC2/Akt signaling in MM cells. We evaluated the baseline phosphorylation of mTOR on S\(^{2481}\) in MM cell lines (Fig.1A). Normally, mTORC1 contains mainly mTOR phosphorylated at S\(^{2448}\), whereas mTORC2 contains predominantly mTOR phosphorylated at S\(^{2481}\)\(^{(13)}\). Through western blot analysis we found p-mTOR S\(^{2481}\) was present only in MM.1S, MM.1R, OPM1, OPM2 and U266 cells, whereas p-mTOR S\(^{2448}\) was expressed in all MM cell lines. We next compared the expression of mTOR phosphorylation on S\(^{2448}\) and S\(^{2481}\) 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 shows baseline expression of p-mTOR S\(^{2448}\) correlated with TORC1-phosphorylatable P70S6K and 4E-BP; expression of p-mTOR S\(^{2481}\) correlated with TORC2-phosphorylatable Akt (S\(^{473}\)) across all MM cell lines, except U266 in which p-mTOR S\(^{2481}\) is highly expressed while p-AKT S\(^{473}\) is not significantly expressed.

Since serum starvation leads to a uniform response in the mTOR pathway\(^{(17)}\), we next compared the kinetics of S\(^{2448}/S^{2481}\) phosphorylation of mTOR with p-Akt S\(^{473}\) levels in MM cell lines with relatively high (MM.1S and OPM1 cells) versus low (RPMI cells) constitutive expression of p-Akt S\(^{473}\) (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 h) were subjected to western blotting. In RPMI cells, P-mTOR S\(^{2481}\) and p-Akt S\(^{473}\) followed similar kinetics of expression during serum withdrawal and re-addition. In MM.1S cells, the initial induction of p-Akt S\(^{473}\) occurred before p-mTOR S\(^{2481}\) induction, suggesting that either low levels (not easily seen by WB) of p-mTOR S\(^{2481}\) are sufficient to induce p-AKT S\(^{473}\), or that TORC2 is not the only candidate to phosphorylate Akt at S\(^{473}\) in this cell line. In OPM1 cells p-mTOR S\(^{2481}\) and p-Akt S\(^{473}\) co-expression was present even in serum-starved cells, consistent with PI3K/Akt pathway constitutive activation due to PTEN deletion.
Since Akt plays a role in the regulation of mTOR, we next examined whether p-mTOR S\textsuperscript{2481} is dependent on the Akt activation status (Fig.1C). We found that reduced expression of p-Akt S\textsuperscript{473} via treatment with the Akt inhibitor Akti-1/2 resulted in reduced mTOR phosphorylation at S\textsuperscript{2481} in MM.1S and RPMI, but not in OPM1 cells.

Finally, we tested p-mTOR S\textsuperscript{2481} and p-Akt S\textsuperscript{473} baseline expression in CD138+ cells obtained from three MM patients; and in PBMCs from five healthy donors (Fig.1D). We found that p-mTOR S\textsuperscript{2481} expression correlated with p-Akt S\textsuperscript{473} in both CD138+ and healthy donor mononuclear cells.

**AZD8055, an mTOR Kinase Inhibitor, Inhibits MM Cell Growth in Vitro and in Vivo**

We then evaluated the effect of targeting TORC1 and TORC2 in multiple myeloma by using a novel selective mTOR kinase inhibitor, AZD8055 (15). We first assessed the effects of AZD8055 (25-200nM) treatment on cell number and metabolic activity of MM cell lines by MTT assay at 72h. We noted dose-dependent differences in growth among cells with high versus low p-mTOR S\textsuperscript{2481}/p-Akt S\textsuperscript{473} 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 MM patients. As evidenced by the MTT assays, AZD8055 (100-200 nM) treatment (72h) inhibited the proliferation of three out of eight tested samples (Fig.2B). Increased dosing of AZD8055 (0-800 nM) did not trigger significant cytotoxicity in healthy donors PBMCs (Fig.2C). We next validated AZD8055’s anti-MM activity in vivo in the MM.1S cell xenograft SCID mouse model (Fig.2D). Treatment with AZD8055 significantly suppressed MM tumor growth, with maximum TGI (%) noted at days 19 and 24 following start of treatment at 65% and 59% in the 10 mg/kg and 5 mg/kg treated cohorts, respectively (control vs treated p<0.05). AZD8055 treatment was also associated with improved overall survival (control vs treated...
p<0.05) (Fig.2D left panel). No toxic deaths occurred during this study: maximum percentage of body weight (BW) loss was observed on day 5 (1.3%) at 5mg/kg dosage schedule, and on day 8 (6%) after 10 mg/kg dosing, with weight recovery in the following two weeks with continued dosing (Fig.2D).

**AZD8055 Blocks p-mTOR S\(^{2481}\)/p-Akt S\(^{473}\) Signaling and Displays Higher Anti-Myeloma Effect Compared to Rapamycin**

We next examined how p-mTOR S\(^{2481}\)/p-Akt S\(^{473}\) expression correlates with response to mTOR-targeted treatment in MM cells. We compared p-mTOR S\(^{2481}\)/p-Akt S\(^{473}\) response to AZD8055 and to rapamycin treatment in MM.1S cells. Time-course western blot analysis of MM.1S cells exposed to AZD8055 (100nM) demonstrated inhibition of phosphorylation of both TORC1 and TORC2 substrates: P70S6K; 4E-BP1 including the rapamycin-resistant T\(^{37/46}\) – 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 S\(^{2481}\) along with p-Akt S\(^{473}\), 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 MM cells were exposed to the same concentration of AZD8055 and rapamycin (AZD8055 vs rapamycin MM1S \(p=0.0011\), MM1R \(p=0.0001\), OPM1 \(p=0.0001\), OPM2 \(p<0.0001\), RPMI \(p=0.0016\), DOX-40 \(p=0.0016\), LR5 \(p=0.0005\), 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 h) analysis of LC3 I, and LC3 II expression showed that AZD8055 (100nM) induced a clear cleavage of LC3BI into LC3BII. Such a clear
effect was not observed after rapamycin (100nM) treatment. While prolonged treatment with rapamycin increased the expression of both LC3 I, and LC3 II bands, prolonged treatment with AZD8055 reduced LC3 I at 3h and triggered the disappearance of both bands by 24h (Fig.3D left panel). Both AZD8055 and rapamycin 24 and 48 h treatments resulted in up to 80% inhibition of protein synthesis (AZD8055 50nM vs rapamycin 100 nM 48 h \( p=0.5 \), AZD8055 100nM vs rapamycin 100 nM 48 h \( p=0.16 \), AZD8055 200 nM vs rapamycin 100 nM 48 h \( p=0.11 \)) (Fig.3D right panel).

Because mTORC2 is a positive regulator of cell survival via Akt-mediated inhibition of apoptosis, we examined whether AZD8055 could trigger apoptosis in MM 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 panel); confirmed by increased population of apoptotic cells at 72h culture as detected by Annexin V/PI staining (Fig.3E right panel).

**IGF-1-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 MM cells, we next examined AZD8055’s efficacy in MM cells in culture with BMSCs or growth factors such as IL-6 and IGF-1. 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 \([^{3}H]\)-thymidine uptake at 48h. AZD8055 resulted in greater antiproliferative effects as compared to rapamycin. AZD8055 treatment however could not completely overcome BMSCs-mediated growth of MM.1S cells at 100nM dose (Fig.4A). Because of the major role played by IL-6 and IGF-1 in MM cell survival triggered by BM microenvironment, we next examined AZD8055 and
rapamycin treatments of MM.1S cells cultured with or without IL-6 (10ng/ml), or IGF-1 (50ng/ml). [3H]-thymidine uptake at 48h demonstrated that AZD8055 treatment completely abrogated IL-6-mediated proliferation; meanwhile, IGF-1 conferred relative resistance to AZD8055 in a similar manner to BMSCs (Fig.4B).

Because recent evidence suggests that mTOR and Akt negatively regulate IGF-1R expression, and that IGF-1R activity is re-activated by mTOR kinase inhibitors (12, 18, 19), we sought to determine whether AZD8055 triggered IGF-1R feedback activation in MM cells. We tested our hypothesis in two MM cell lines with relatively high baseline Akt activity (MM.1S and OPM1). MM cells were cultured for 6 hours in control media, AZD8055 (100nM), or Rapamycin (100nM), then stimulated with IGF-1 (50ng/ml) for 15 min and whole cell lysates subjected to western blot analysis. We found that AZD8055 alone induced the phosphorylation of tyrosine sites (Y1135/1136) in the activation loop of the IGF-1R kinase domain. IGF-1 clearly upregulated Akt phosphorylation in MM cells; however, it had no effect on mTOR Ser2481 phosphorylation. Moreover, AZD8055-treated cells exposed to IGF-1 sustained p-Akt Ser473 expression, while p-mTOR Ser2481 remained inhibited (Fig.4C).

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

**Blockage of IGF-1R Restores AZD8055-Mediated MM Cytotoxicity.**

We next determined whether blocking IGF-1R would 1) prevent IGF-1-mediated Akt re-activation in AZD8055-treated MM cells, and 2) re-institute AZD8055’s anti-MM activity compromised by IGF-1. We performed western blot analysis of MM.1S cells treated with AZD8055 (100nM), or anti-IGF-1R Ab (1ug/ml) or the
combination, with or without the addition of 50ng/ml IGF-1 for 6h. Blockade of IGF-1R signaling with the anti-IGF-1R Ab alone resulted in partial reduction of Akt S\textsuperscript{473} phosphorylation. We confirmed the anti-IGF-1R Ab-mediated downregulation of Akt activity by reduced p-FoxO1 T\textsuperscript{24}/FoxO3a T\textsuperscript{32}. Next, 50ng/ml IGF-1 partially rescued the phosphorylation of Akt S\textsuperscript{473}/FoxO1 T\textsuperscript{24}/FoxO3a T\textsuperscript{32} signaling in both AZD8055- and anti-IGF-1R-treated cells, but not in the combination treatment. Notably, mTOR S\textsuperscript{2481} phosphorylation remained unchanged either with IGF-1 stimulation, or with anti-IGF-1R Ab treatment (Fig.5A).

We hypothesized that IGF-1/IGF-1R-mediated signaling could rescue MM.1S cells from AZD8055-mediated apoptosis. Through western blot analysis of MM.1S cells cultured for 24h with 100nM AZD8055, or 1ug/ml anti-IGF-1R Ab, or the combination with or without 50ng/ml IGF-1, we found that IGF-1 significantly reduced caspase-3 cleavage induced by AZD8055 treatment in MM.1S. In contrast, the combination of AZD8055 with the anti-IGF-1R Ab increased the activation of caspases-9 and -3 (Fig.5B).

We therefore used Annexin/PI flowcytometry analysis to determine the rate of apoptosis in MM.1S cells subjected to longer treatment (72h) with AZD8055 (100nM) with or without anti-IGF-1R Ab pre-treatment, with or without the addition of IGF-1 (50ng/ml). AZD8055 alone induced 56.5% apoptotic cells; the addition of anti-IGF-1R Ab increased the apoptotic population to 77.9%. As hypothesized, exposure to IGF-1 reduced AZD8055-induced apoptosis from 56.5% to 15.1%; whereas blockage of IGF-1R via anti-IGF-1R Ab partially rescued AZD8055-mediated apoptotic effect (Fig.5C).

To confirm our observation, we evaluated AZD8055 and the anti-IGF-1R Ab combined treatment in two other MM cell lines, MM.1R and ANBL-6. MM.1R and ANBL-6 cells were pre-incubated for 1h with 1ug/ml Iso-IgG, or 1ug/ml anti-IGF-1R Ab and then cultured with AZD8055 (0-100nM) or regular media, with or
without 50ng/ml IGF-1. After 72h treatment anti-IGF-1R Ab alone reduced the cell numbers by 73% and 33% in MM.1R and ANBL-6 cells, respectively; next, anti-IGF-1R Ab enhanced AZD8055 activity (85% cell number decrease in MM1R treated with AZD8055 vs 94% cell number decrease in MM1R treated with AZD8055 and anti-IGF-1R Ab, 46% cell number decrease in ANBL-6 treated with AZD8055 vs 66% cell number decrease in ANBL-6 treated with AZD8055 and anti-IGF-1R Ab p<0.05) and reversed IGF-1-mediated partial rescue of MM 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 to scramble control and the combination of IGF1R knock out and AZD8055 100 nM showed a 78% decrease in viability compared to scramble control (supplementary figure S1).

DISCUSSION

The growing complexity of the mTOR pathway, as well as emerging discoveries regarding its autoregulatory mechanisms and activating feedback loops(4, 9) highlights the need for ongoing research of the mTOR pathway in MM. Our data aligned with previous findings in MM(10) suggest that targeting mTOR kinase produces more effective mTOR pathway inhibition than rapamycin with a more detrimental impact on MM cells. Unlike rapamycin, AZD8055 inhibition of mTORC1 and mTORC2 functions resulted in apoptosis in MM cells. However, we also found that mTOR kinase inhibition via AZD8055 induced phosphorylation of IGF-1R. Furthermore, exposure of AZD8055-treated cells to IGF-1 recovered p-Akt S473 and reduced apoptosis of MM cells despite mTOR kinase inhibition. In contrast, the addition of blocking IGF-1R antibody rescued AZD8055-induced apoptosis from IGF-1-mediated MM cell survival.

Taken together our findings indicate p-Akt S473 is not an accurate marker for mTORC2 inhibition in MM 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 S\textsuperscript{2481} as the more precise marker to monitor response to mTOR kinase inhibition in MM cells, specifically when it involves evaluating mTORC2/Akt function.

Reportedly, mTOR is phosphorylated differentially when associated with mTORC1 and mTORC2. Specifically, mTOR phosphorylated on S\textsuperscript{2448} with Raptor, and mTOR phosphorylated on S\textsuperscript{2481} with Rictor. Therefore it was believed that mTORC1 contains mTOR phosphorylated predominantly on S\textsuperscript{2448}, whereas mTORC2 contains mTOR phosphorylated predominantly on S\textsuperscript{2481}. Moreover, intact mTORC2 is obligatory for mTOR S\textsuperscript{2481} phosphorylation(13), thus p-mTOR S\textsuperscript{2481} is a marker of TORC2 activity(13, 14). In agreement with these data, our study in MM cells found that baseline expression of p-mTOR S\textsuperscript{2481} correlated with TORC2-phosphorylatable Akt (S\textsuperscript{473}). AZD8055, unlike rapamycin, consistently inhibited mTOR phosphorylation at S\textsuperscript{2481} along with p-Akt S\textsuperscript{473}. Importantly, p-mTOR S\textsuperscript{2481}/p-Akt S\textsuperscript{473} co-expression in MM cell lines correlated with sensitivity to mTOR kinase inhibition. Our data therefore suggest that phosphorylation of S\textsuperscript{2481} in mTOR may serve as a potential marker for intact mTORC2 activity and sensitivity to mTOR kinase inhibitors in MM. However, further studies are warranted to confirm that p-mTOR S\textsuperscript{2481} is a fair readout of mTORC2 activation in MM.

Until recently it was believed that TORC1/P70S6K suppression in the context of rapamycin treatment was responsible for triggering disinhibition of IGF-1R 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 MM cells by upstream IGF-1R/PI3K activation through interaction with growth factors and the bone marrow microenvironment. In our study, AZD8055 inhibition of mTOR kinase in MM cells associated with upregulation of the tyrosine phosphorylation sites (Y1135/1136) in the activation loop of the IGF-1R kinase domain. Furthermore, AZD8055-treated cells exposed to IGF-1 sustained p-Akt S473 expression, while TORC2 function remained inhibited, as suggested by inhibition of p-mTOR S2481. 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 disinhibition of RTK/PI3K feedback signaling stimulated by growth factors(12, 19). Given that, it is possible that AZD8055-triggered IGF-1R activation is in part due to mTORC1 inhibition and in part secondary to TORC2/Akt suppression.

In MM, activation of IGF-1R induces sustained activation of PI3K/Akt and NF-kB, phosphorylation of FKHR transcription factor and upregulation of a series of intracellular anti-apoptotic proteins including FLIP, survivin, cIAP-2, and XIAP thereby decreasing drug sensitivity of MM cells (21, 22). Moreover, IGF-1R inhibitors have been shown to trigger significant MM cell toxicity (22, 23). A phase I study in MM demonstrated the safe profile of fugitumumab, the human monoclonal antibody directed against IGF1R, and some responses were reported in combination with dexamethasone (24). In our study, we observed IGF-1/IGF-1R-mediated re-activation of Akt and rescue of MM cells from AZD8055-triggered apoptosis. Meanwhile, treatment with anti-IGF-1R Ab enhanced AZD8055 activity and reversed IGF-1-mediated rescue of MM cells from AZD8055-induced cytotoxicity, suggesting that treatments targeting IGF-1/IGF-1R signaling are promising strategies and complement mTOR kinase inhibition in MM.

Our assertion that relief of feedback inhibition of IGF-1R in MM tumors treated with mTOR kinase inhibitors decreases the efficacy of PI3K pathway inhibition
has yet to be addressed in patients. We believe our preclinical findings will inform and complement the ongoing clinical evaluation of mTOR kinase inhibition as targeted therapy and forms the basis for testing them in combination with inhibitors of the IGF-1R pathway.

**BIBLIOGRAPHY**

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FIGURE LEGENDS

Figure 1: mTOR/Akt signaling in MM cells: p-mTOR S\textsuperscript{2481} as a marker for mTORC2-phosphorylatable Akt S\textsuperscript{473}.

(A) Baseline mTOR profile in MM cell lines. MM cells grown in culture media alone were lysed and mTOR/TORC1/TORC2-related protein expression analyzed by western blotting.

(B) Effect of serum starvation/re-addition on mTOR activity in MM cells. MM.1S, OPM1 and RPMI MM cells were cultured in regular (10\%FBS) or serum starved media for 72 hours; next, regular media was 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 S\textsuperscript{2481}/S\textsuperscript{2448}, mTOR, p-P70S6K T\textsuperscript{389}, P70S6K, p-Akt S\textsuperscript{473}, and Akt expression. GAPDH was used for loading control.

(C) Akt regulates p-mTOR S\textsuperscript{2481}. MM.1S, OPM1 and RPMI cells exposed to media alone or with Akti-1/2 (5uM) for 30 min, 1h, 3h, or 6h were analyzed by western blotting for the expression of p-Akt S\textsuperscript{473}, Akt, p-mTOR S\textsuperscript{2481} and mTOR.

(D) P-mTOR S\textsuperscript{2481} correlates with p-Akt S\textsuperscript{473} expression in CD138+ MM primary tumor cells. CD138+ primary tumor cells from MM patients and PBMCs from healthy donors were subjected to western blotting and p-
mTOR S\(^{2481}\), mTOR, p-Akt S\(^{473}\), Akt and GAPDH expression detected using specific antibodies.

Figure 2: AZD8055, an mTOR kinase inhibitor, shows in vitro and in vivo anti myeloma effect

A) AZD8055 decreases MM viability. MM cell lines were cultured in culture media or at varying concentrations of AZD8055 (0-200 nM), and cytotoxicity evaluated by MTT assay at 72h; data represent means (+/-SD) of triplicate culture.

B) Patient MM cells exhibited differential sensitivity to AZD8055. MM patient cells isolated from BM by CD138 positive selection were cultured with or without AZD8055 (100, or 200 nM) for 72h and viability assessed via MTT assay. Data represent means (+/-SD) of triplicate culture.

C) AZD8055 was not toxic to PBMCs. Healthy donors PBMCs were incubated with or without increasing concentrations of AZD8055 (0-800 nM) and viability assessed at 72h by MTT assay; data represent means (+/-SD) of triplicate culture.

D) AZD8055 anti-MM activity in vivo: tumor growth, host weight, and Kaplan–Meier survival curves in xenografted with MM.1S cells mice. Immunodeficient SCID-CB17 mice were inoculated subcutaneously MM.1S cells. Oral AZD8055 was administered daily 5 days per week for 4 consecutive weeks either 5 mg/kg (10 mice), or 10 mg/kg (10 mice), or control vehicle (10 mice) starting after the development of measurable tumor with a tumor volume of approximately 150-200 cm\(^3\). AZD8055 treatment resulted in tumor growth inhibition and prolonged survival.
Figure 3: AZD8055 inhibits cell growth by blocking p-mTOR S^{2481}/p-AKT S^{473} signaling

(A) AZD8055, an mTOR kinase inhibitor induces dual inhibition of TORC1/TORC2 signaling in MM cells. MM.1S cells were incubated with AZD8055 (100 nM) or rapamycin (100 nM) for the specified time points, subjected to western blot and mTORC1/mTORC2 downstream targets expression was detected using specific antibodies.

(B) p-mTOR S^{2481} is a marker of mTOR kinase inhibition in MM cells. MM.1S, OPM1, and RPMI cells were incubated with AZD8055 (100 nM) or Rapamycin (100 nM) for short time points (15 min – 6 hours). Cells were pelleted and subjected to western blotting. p-mTOR S^{2481}, mTOR, p-Akt S^{473} and Akt expression was determined using specific antibodies.

(C)p-mTOR S^{2481}/p-Akt S^{473} co-expression correlates with sensitivity to mTOR kinase inhibition in MM cell lines. MM cell lines were cultured in culture media or at AZD8055 (100 nM), or rapamycin (100 nM) and cytotoxicity evaluated by MTT assay at 72h; data represent means (+/-SD) of triplicate culture.

(D) Left panel: AZD8055 induced autophagy, consistent with TORC1 inhibition. MM.1S cells were incubated with media, AZD8055 (100 nM), or Rapamycin (100 nM) for 3, 6, 12, 24, or 48 h and cells analyzed for LC3B expression using western blot. GAPDH expression served as a loading control. Right panel AZD8055 blocked protein synthesis, a TORC1-related function. Protein synthesis was assayed in MM.1S cells cultured for 24 and 48 h either in regular media or with increasing concentrations of AZD8055 (50-200 nM), or rapamycin (100nM).
(E) Left panel: AZD8055 induced caspases activation, consistent with TORC2/Akt inhibition. MM.1S cells were incubated with or without AZD8055 (100 nM), or Rapamycin (100 nM) for 24 and 48 h. Cleaved caspase-7, 8, 9, 3 and PARP were detected via WB using specific antibodies. Right panel AZD8055 induced apoptosis. MM.1S cells were treated with AZD8055 (100 nM), or Rapamycin (100 nM) for 48 and 72 hours. The rate of apoptosis was assessed by Annexin/PI flow cytometry analysis.

Figure 4: IGF-1-induced Akt activation confers relative resistance to AZD8055-mediated cytotoxicity.

(A) BMSCs-stimulated MM.1S partially escape AZD8055-mediated growth inhibition. MM.1S cells were cultured for 48h in control media, AZD8055 (25-200 nM), or rapamycin (10-100 nM) in the presence or absence of BMSCs. Cell proliferation was assessed by [3H]TdR assay; data represent means (+/-SD) of triplicate cultures.

(B) AZD8055-inhibited proliferation of MM.1S is partially rescued by IGF-1. MM.1S cells were cultured for 48h in control media, AZD8055 (25-200 nM), or rapamycin (10-100 nM) in the presence or absence of IL-6 (10 ng/ml) or IGF-1 (25 ng/ml). Cell proliferation was assessed by [3H]TdR assay; data represent means (+/-SD) of triplicate cultures.

(C) AZD8055 treatment triggered upregulation of the tyrosine phosphorylation sites in the activation loop of the IGF-1R kinase domain T1135/1136, which associated with activated p-Akt S473 in MM cells despite AZD8055 exposure. MM.1S and OPM1 cells were cultured for 6h in regular media, AZD8055 100 nM, or Rapamycin 100
nM. Cells were then pelleted down and re-suspended in PBS with or without IL-6 (10 ng/ml), or IGF-1 (50 ng/ml) for 15 min. Expression of p-IGF-1 T1135/1136, IGF-1Rβ, p-mTOR S2481, p-mTOR S2448, mTOR, p-Akt S437, and Akt was examined via western blot using GAPDH for loading control.

(D) IGF-1 confers relative resistance to AZD8055-induced cytotoxicity of MM. MM.1S and OPM1 MM cells incubated for 48h in media alone or AZD8055 (50, 100 nM), with or without 50 ng/ml IGF-1 were assessed for viability via MTT assay; data represent means (+/- SD) of triplicate cultures.

Figure 5: Blockage of IGF-1R restores AZD8055-mediated MM cytotoxicity.

(A) IGF-1R antibody prevents IGF-1-mediated Akt re-activation in AZD8055-treated MM.1S cells. MM.1S cells were pre-incubated for 1h with Iso-IgG Ab (1ug/ml) or anti-IGF-1R Ab (1ug/ml) and then exposed to 100nM AZD8055 with or without 50ng/ml IGF-1 for 6h. Expression of p-mTOR S2481, mTOR, p-Akt S437, 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-IGF-1R Ab restores AZD8055-mediated caspase cleavage impaired by IGF-1. MM.1S cells cultured for 24h with 100nM AZD8055, or 1ug/ml anti-IGF-1R Ab, or their combination, with or without 50ng/ml IGF-1 were subjected to western blot analysis for caspase-9 and -3 expression.

(C) Blockage of IGF-1R via anti-IGF-1R Ab prevents IGF-1-mediated reduction in apoptosis produced by AZD8055 treatment.
Annexin/PI flowcytometry analysis was performed to determine the rate of apoptosis in MM.1S cells subjected to longer treatment (72h) with AZD8055 (100nM) with or without anti-IGF-1R Ab pre-treatment, with or without IGF-1 (50ng/ml).

(D) IGF-1R blockage via IGF-1R Ab potentiates AZD8055 activity in MM.1R and ANBL-6 cells. MM.1R and ANBL-6 cells pre-incubated for 1h with Iso-IgG Ab (1ug/ml) or anti-IGF-1R Ab (1ug/ml) were exposed to AZD8055 (100nM) with or without IGF-1 (50ng/ml) for 72h and viability assessed via MTT assay; data represent means (+/-SD) of triplicate cultures.
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A

B

C

D

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Figure 2: AZD8055, an mTOR kinase inhibitor, shows in vitro and in vivo anti MM effect.
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A

B

C

D

E

Protein Synthesis Assay in MM.1S

AZD8055 (nM) Rapamycin (nM)
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