Targeting the Pro-Survival Protein MET with Tivantinib (ARQ 197) Inhibits Growth of Multiple Myeloma Cells¹,²

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Abstract
The hepatocyte growth factor (HGF)/MNNG HOS transforming gene (MET) pathway regulates cell growth, survival, and migration. MET is mutated or amplified in several malignancies. In myeloma, MET is not mutated, but patients have high plasma concentrations of HGF, high levels of MET expression, and gene copy number, which are associated with poor prognosis and advanced disease. Our previous studies demonstrated that MET is critical for myeloma cell survival and its knockdown induces apoptosis. In our current study, we tested tivantinib (ARQ 197), a small-molecule pharmacological MET inhibitor. At clinically achievable concentrations, tivantinib induced apoptosis by >50% in all 12 human myeloma cell lines tested. This biologic response was associated with down-regulation of MET signaling and inhibition of the mitogen-activated protein kinase and phosphoinositide 3-kinase pathways, which are downstream of the HGF/MET axis. Tivantinib was equally effective in inducing apoptosis in myeloma cell lines resistant to standard chemotherapy (melphalan, dexamethasone, bortezomib, and lenalidomide) as well as in cells that were co-cultured with a protective bone marrow microenvironment or with exogenous cytokines. Tivantinib induced apoptosis in CD138+ plasma cells from patients and demonstrated efficacy in a myeloma xenograft mouse model. On the basis of these data, we initiated a clinical trial for relapsed/refractory multiple myeloma (MM). In conclusion, MET inhibitors may be an attractive target-based strategy for the treatment of MM.

Neoplasia (2015) 17, 289–300

Introduction
Multiple myeloma (MM) is an incurable B-cell disorder characterized by the accumulation of mature plasma cells producing a monoclonal protein in the bone marrow [1]. The incidence of myeloma in the United States is four to five per 100,000 individuals [2]. Even though new therapeutic drugs have been introduced for myeloma, resistance
to these agents is common and patients have a median survival of only 7 to 8 years [1]. Thus, new targets for drug development are needed for the treatment of MM.

One target may be hepatocyte growth factor (HGF). In MM, patients have high serum concentrations of HGF as compared to healthy subjects [3]. High HGF level in MM patients has been correlated with poor prognosis [4–6]. Therefore, investigators have targeted HGF with anti-HGF antibodies or HGF antagonists to treat this disease [7,8]. Additionally, compared to CD138-negative cells, increased copy number of the MNNG HOS transforming gene (MET) gene as well as an increase in MET mRNA levels, which encodes for the HGF receptor, has been reported in myeloma patients [9]. Furthermore, higher MET levels were also associated with poor response and survival of myeloma patients treated with bortezomib-based induction therapy.

The MET receptor tyrosine kinase is a proto-oncogene that regulates cell growth, survival, and migration [10,11]. When HGF binds to MET, it leads to dimerization of MET and phosphorylation of tyrosine residues in the kinase domain (Y1230, Y1234, and Y1235). This triggers autophosphorylation of tyrosine residues (Y1349 and Y1356) in the carboxyl-terminal substrate binding site, resulting in the binding of effector molecules such as growth factor receptor-bound protein 2, GRB2-associated-binding protein 1, phospholipase C, and cellular SRC kinase. The effector molecules activate a signaling cascade that includes the phosphoinositide 3-kinase/AKT and mitogen-activated protein kinase. The effector molecules activate a signaling cascade that includes the phosphoinositide 3-kinase/AKT and mitogen-activated protein kinase (MAPK) pathways, which leads to stimulation of cell proliferation, survival, and migration [11].

MET knockdown in MM cells by ribozyme or shRNA has demonstrated that MET is required for cell survival, and its knockdown inhibited the growth of myeloma cells and induced apoptosis in these cells [12,13]. In addition, proof of principle studies targeting MET with small-molecule inhibitors such as PHA-665752, SU11274, and amuvatinib showed efficacy in myeloma cells [14–16]. These studies suggested that targeting MET could be an effective strategy for treating MM patients. While shRNA and ribozyme strategies are not clinically practical and the MET inhibitors, PHA-665752, SU11274, and amuvatinib, are not clinically viable choices, new small-molecule inhibitors of MET are being designed and developed.

ARQ 197 (tivantinib) is a small-molecule, non–ATP-competitive inhibitor of MET. In an in vitro kinase assay, in which ARQ 197 was tested against a panel of 230 human kinases, it inhibited MET with high specificity ($K_i = 355$ nM) [17]. ARQ 197 inhibits MET by binding to the inactive conformation of MET to stabilize this conformation [18]. This prevents MET phosphorylation and dampens downstream signaling, thereby resulting in growth inhibition and induction of apoptosis in solid tumors [17,19].

Phase I clinical trials of tivantinib have demonstrated activity in primary and metastatic solid tumors [7,20]. The maximum tolerated oral dose was 360 mg twice daily. At this dose, a steady-state plasma concentration of 6 to 7 μM was achieved [20]. Phase II investigations with single-agent tivantinib demonstrated efficacy in solid tumors [21,22]. The combination of tivantinib and erlotinib has been tested in phase I and II investigations, and phase III trials are ongoing [23–25]. Both as a single agent and in combination, tivantinib has been well tolerated. Furthermore, biomarker studies have demonstrated the effectiveness of tivantinib in inhibiting MET and inducing apoptosis of the target tumor cells [20].

On the basis of the importance of MET in myeloma and the results of clinical trials with tivantinib in solid tumors, we hypothesized that this molecule will affect the HGF/MET axis in myeloma cells and induce cytotoxicity. We used several myeloma model systems and found that ARQ 197 was highly efficacious in inhibiting growth and inducing apoptosis even under conditions associated with resistance to standard myeloma therapies. Additionally, this drug blocked HGF/MET signaling in these cells and was cytotoxic to primary myeloma cells. On the basis of these encouraging data, we are testing tivantinib in a National Cancer Institute Cancer Therapy Evaluation Program–sponsored phase II clinical trial for patients with relapsed/refractory MM (NCT01447914).

**Materials and Methods**

**Cell Cultures and Reagents**

Twelve human myeloma cell lines were used for the current investigation (Table 1) [26–29]. These were maintained at 37°C in a humidified incubator with 5% CO2 and were authenticated and tested for Mycoplasma infection by The University of Texas (UT) MD Anderson Cancer Center Characterized Cell Line Core. Resistant cell lines were maintained as described before [26,27,29,30]. NKtert human marrow stromal cells (NKtert; RIKEN Cell Bank, Koyadai, Japan [31]) were maintained as described previously [32]. Tivantinib (ARQ 197) was obtained from Active Biochem (Maplewood, NJ) and ArQule (Woburn, MA).

**Flow cytometry analysis of annexin V/propidium iodide (PI) staining as a measure of cell survival was performed as described before [12]. The effect of ARQ 197 treatment on cell growth was measured in exponentially growing cells by determining the cell number using a Coulter counter (Beckman Coulter, Fullerton, CA).**

**Table 1. List of Human Myeloma Cell Lines**

| Cell Line | Origin | Medium + Supplements | Characteristics |
|-----------|--------|----------------------|----------------|
| U266      |        | RPMI-1640 + 10% FBS  |                |
| OPM-2     |        | RPMI-1640 + 10% FBS  |                |
| MM.1S     | Dr Rosen, Lurie Comprehensive Cancer Center (Chicago, IL) [36] | RPMI-1640 + 10% FBS | Glucocorticoid sensitive |
| MM.1R     | Dr Rosen, Lurie Comprehensive Cancer Center (Chicago, IL) [36] | RPMI-1640 + 10% FBS | Glucocorticoid sensitive |
| RPMI-8226 | Dr Orloff, UT MD Anderson Cancer Center (Houston, TX) [28,29] | RPMI-1640 + 10% FBS | Bortezomib sensitive |
| ANBL-6    | Dr Orloff, UT MD Anderson Cancer Center (Houston, TX) [28,29] | RPMI-1640 + 10% FBS | Bortezomib sensitive |
| ANLB-6    | Dr Orloff, UT MD Anderson Cancer Center (Houston, TX) [28,29] | RPMI-1640 + 10% FBS | Bortezomib sensitive |
| V10R      | Dr Orloff, UT MD Anderson Cancer Center (Houston, TX) [28,29] | RPMI-1640 + 10% FBS | Bortezomib sensitive |
| KAS-6/1   | Dr Orloff, UT MD Anderson Cancer Center (Houston, TX) [28,29] | RPMI-1640 + 10% FBS | Bortezomib and lenalidomide sensitive |
| KAS-6/2   | Dr Orloff, UT MD Anderson Cancer Center (Houston, TX) [28,29] | RPMI-1640 + 10% FBS | Bortezomib resistant |
| V10R      | Dr Orloff, UT MD Anderson Cancer Center (Houston, TX) [28,29] | RPMI-1640 + 10% FBS | Bortezomib resistant |
| KAS-6/3   | Dr Orloff, UT MD Anderson Cancer Center (Houston, TX) [28,29] | RPMI-1640 + 10% FBS | Bortezomib resistant |
| 8226/S    | Dr Dalton, Moffitt Cancer Center (Tampa, FL) [25] | RPMI-1640 + 10% FBS | Melphalan sensitive |
| 8226/LR-5 | Dr Dalton, Moffitt Cancer Center (Tampa, FL) [25] | RPMI-1640 + 10% FBS | Melphalan resistant |
**Thymidine Incorporation Assay.** To measure DNA synthetic capacity of cells, myeloma cell lines (U266 and OPM-2) were treated with interleukin 6 (IL-6) or HGF or were placed on stroma. One hour before harvesting, cells were incubated with 2 μCi [³H]thymidine at 37°C. Cell concentration was measured using a Coulter counter. DNA synthesis was measured as described before [33].

**Western Blot Analysis and Antibodies**

Whole-cell lysates were prepared as described before [34]. Immunoblots were scanned and quantitated on an Odyssey imaging system (LI-COR Biosciences, Lincoln, NE). Antibody information for all proteins is provided in Supplementary Table 1.

**Co-Culturing U266 or OPM-2 Cells with NKtert Cells**

To co-culture myeloma (U266, OPM-2) cells with bone marrow stromal cells, NKtert cells were seeded in 12-well plates at 2.5 × 10⁴ cells/ml. After 16 to 18 hours, myeloma cells were seeded at 5 × 10⁵ cells/ml in RPMI-1640 medium supplemented with 10% FBS. Cells were co-cultured for 2 hours before treatment was initiated. Forty-eight hours later, myeloma cells, which are suspension cells, were removed for analysis without disturbing the adherent NKtert cells. Attached stromal cells were harvested by detaching with Accutase Cell Detachment Solutions (Innovative Cell Technologies, San Diego, CA) and similarly assessed.

**Bone Marrow Samples and Isolation of CD138+ Cells**

Bone marrow samples were collected from 15 patients with myeloma through a protocol approved by the MD Anderson Cancer Center Institutional Review Board and in accordance with the Declaration of Helsinki. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO₂ for 72 hours with or without ARQ 197. CD138+ fraction was isolated from bone marrow for 10 patient samples at the MD Anderson Myeloma Core as described before [27]. For the other five patients, cells were obtained unsorted. These samples were stained with 10 μl of CD138-APC (Miltenyi Biotec, Auburn, CA) for 10 minutes just before processing for annexin V/PI staining for flow cytometry analysis.

**Flow Cytometry Analysis of MET and Phospho-MET**

CD138+ plasma cells were isolated from bone marrow of MM patients. Cells were fixed and permeabilized with BD cytofix/cytoperm fixation and permeabilization solution kit (BD Biosciences, San Jose, CA). For detection of phospho-MET, cells were incubated with phosho-MET(Y1234/1235) for 30 minutes at room temperature followed by a wash with 1× BD Perm/Wash Buffer. Control cells were incubated with normal rabbit IgG. Next, cells were incubated with secondary antibody (goat anti-rabbit IgG-phycocerythrin) for 30 minutes at 4°C. Cells were washed with 1× BD Perm/Wash Buffer and analyzed by flow cytometry. Antibody information is provided in Supplementary Table 1.

**Murine Xenograft Model of Myeloma**

All mice procedures were conducted through a protocol approved by the UT MD Anderson Cancer Center Institutional Animal Care and Use Committee. Six- to eight-week-old severe combined immunodeficient mice (BALB/c background; National Cancer Institute Frederick Laboratory for Cancer Research, Frederick, MD) were subcutaneously injected once into the right flank with 2 × 10⁶ MM.1S cells. When a tumor reached 0.5 mm in the longest dimension, the mouse was randomly placed in the vehicle (control) group or the ARQ 197–treated group. Mice were treated as described before [17]. Tumor volume was calculated with the formula: (width² × length × π)/6.

**Statistical Analysis**

Data were analyzed and graphed using GraphPad Prism (GraphPad software version 6, San Diego, CA). The statistical analyses used are indicated in the respective sections.

**Results**

**ARQ 197 Inhibited Cell Growth and Induced Cell Death in Myeloma Cell Lines**

To investigate the effect of ARQ 197 on the growth of myeloma cells, we treated U266, MM.1S, OPM-2, and RPMI-8226 cells with increasing doses of ARQ 197. Compared to control treatment, dose-dependent inhibition of cell growth increased notably at 1 μM ARQ 197 in all four cell lines (46%, 33%, 29%, and 48%, respectively; Figure 1A). At 3 μM ARQ 197, cell growth was inhibited even further for all cell lines. There was a corresponding increase in cell death as measured by an increase in annexin V/PI staining and detection of procaspase-3 cleavage. At 1 μM ARQ 197, the percentage of dead cells increased by 54% in U266, 25% in MM.1S, 18% in OPM-2, and 58% in RPMI-8226 cell lines compared with the DMSO-treated cells (Figure 1B). In all the cell lines, 3 μM ARQ 197 induced >70% cell death. Consistent with the cell death data, ARQ 197 treatment increased procaspase-3 cleavage in a dose-dependent manner (Figure 1C). The half maximal inhibitory concentration (IC₅₀) for cell death for these cell lines ranged from 0.9 to 1.8 μM (RPMI-8226 = 0.9 μM, U266 = 1.1 μM, MM.1S = 1.6 μM, OPM-2 = 1.8 μM). This IC₅₀ range was well below the steady-state plasma concentration of 6 to 7 μM that is achievable in the clinic, suggesting that ARQ 197 can effectively target myeloma cells in patients [20]. In addition, a time-dependent increase in annexin V/PI positive cells was observed in U266 and MM.1S cells with both 1 and 3 μM ARQ 197 (Figure 1, D and E). These data demonstrated that ARQ 197 induced cell death in myeloma cell lines in a dose- and time-dependent fashion.

**ARQ 197 Overcame Microenvironment-Mediated Drug Resistance and Induced Cell Death in Myeloma Cell Lines**

The bone marrow microenvironment is a major source of drug resistance for myeloma cells. It produces cytokines such as HGF and IL-6, which stimulate the growth of myeloma cells and contribute to their resistance to treatment [35]. HGF and, to some extent, IL-6 stimulated DNA synthesis in U266 and OPM-2 cells (Supplementary Figure 1, A and B). To determine whether ARQ 197 overcomes the protection conferred by the bone marrow microenvironment, we treated U266 and OPM-2 cells with 3 μM ARQ 197 in the presence of high concentrations of exogenous IL-6 or HGF for 48 hours. ARQ 197 inhibited cell growth in the presence of IL-6 or HGF to a similar extent as treatment with ARQ 197 alone (Figure 2, A and C). In U266 cells, compared with DMSO-treated cells, 3 μM ARQ 197 significantly inhibited growth in the presence of 50 ng/ml IL-6 (60% inhibition, P < .0001) or 50 ng/ml HGF (64% inhibition, P < .0001). Similarly, in OPM-2 cells, 3 μM ARQ 197 significantly inhibited growth compared...
to DMSO-treated cells in the presence of 50 ng/ml IL-6 (62.5%, \( P < .0001 \)) or 50 ng/ml HGF (61% inhibition, \( P < .0001 \)). Consistent with the growth inhibition results, incubation with ARQ 197 resulted in inhibition of cell survival in both U266 and OPM-2 cells in the presence of these growth factors (Figure 2, B and D).

To further investigate the impact of the microenvironment on drug resistance, we co-cultured U266 or OPM-2 cells with human NKtert stromal cells, which provide conditions similar to the bone marrow microenvironment. Myeloma cells were seeded on NKtert at a concentration 20× that of NKtert cells. Under these conditions, NKtert cells stimulated DNA synthesis in U266 cells and, to some extent, in OPM-2 cells (Supplementary Figure 1C). Forty-eight hours of treatment with 3 \( \mu M \) ARQ 197 resulted in cell death of 54% in U266 cells (Figure 2E) and 69% in OPM-2 cells (Figure 2F) when co-cultured with NKtert cells; these values were similar to that of U266 cells cultured alone (55% annexin V+/PI+ cells; Figure 2E) and OPM-2 cells cultured alone (82% annexin V+/PI+ cells; Figure 2F). Importantly, ARQ 197 did not induce a notable increase in NKtert cell death (22% at 3 \( \mu M \)), suggesting that ARQ 197 spares normal stromal cells.

**Activity of ARQ 197 in Melphalan- and Dexamethasone-Resistant Cell Lines**

Melphalan is an alkylating agent that was one of the earliest treatments introduced for myeloma. We tested ARQ 197 on paired cell lines that were melphalan resistant (8226/LR-5) and melphalan sensitive (8226/S). ARQ 197 inhibited cell growth by 54% at 3 \( \mu M \) in 8226/S cells (Figure 3A). This was associated with 77% cell death (Figure 3B). Despite the melphalan-resistant 8226/LR-5 cells having a high percentage of endogenous cell death (44%), 3 \( \mu M \) ARQ 197 was still able to inhibit cell growth by 54% and induced 98% cell death (Figure 3, A and B). The correlation in cell death was high between 8226/S and 8226/LR-5 cells in their response to ARQ 197 (Pearson correlation 0.8718, \( P = .0105 \)).

Dexamethasone is another approved agent for myeloma treatment. Hence, we tested both MM.1R cells, which are resistant to the synthetic steroid dexamethasone, and MM.1S cells, which are sensitive, to determine whether ARQ 197 can induce cell death in these cell lines. At 1 and 3 \( \mu M \) ARQ 197, cell growth was inhibited by 33% and 49%, respectively, in sensitive cells and by 28% and 44%,
respectively, in resistant cells (Figure 3C). Similar to the growth inhibition data, an increase in cell death was detectable with 3 μM ARQ 197 and was similar in both cell lines (Figure 3D). The correlation in cell death was high between MM.1R and MM.1S cells in their response to ARQ 197 (Pearson correlation 0.9741, \(P = .0010\)).

Activity of ARQ 197 in Cells Resistant to Bortezomib or Lenalidomide

Newly approved drugs for myeloma include proteasome inhibitors such as bortezomib and immunomodulatory drugs such as thalidomide or lenalidomide. However, patients acquire resistance to these drugs. We examined two pairs of cell lines that were either sensitive or resistant to bortezomib (ANBL-6 vs ANBL-6/V10R and KAS-6 vs KAS-6/V10R). In these cell lines, growth inhibition was observed starting with 0.3 μM, and by 3 and 10 μM, 50% inhibition was achieved in both sensitive and resistant cell lines (Figure 4, A and C). This finding was consistent with that of induction of cell death (Figure 4, B and D), which was also similar in sensitive and resistant cell lines. Both paired cell lines exhibited high correlation in their response to ARQ 197 (Pearson correlation 0.9844, \(P < .0001\) for both ANBL-6 vs ANBL-6/V10R and KAS-6 vs KAS-6/V10R).

Finally, a cell line that was resistant to lenalidomide (KAS-6/R10R) was tested for sensitivity to ARQ 197. Cell growth was inhibited and cell death was induced by more than 50% at 1 μM ARQ 197 in this cell line (Figure 4, E and F). ARQ 197–induced cell death was similar between KAS-6 and KAS-6/R10R cells (Pearson correlation 0.9584, \(P = .0007\)). These results suggested that ARQ 197 is able to
overcome the resistance that these cell lines developed to bortezomib or lenalidomide and can inhibit the growth of myeloma cells.

**ARQ 197 Inhibited the MET Signaling Pathway**

To elucidate the mechanism of ARQ 197–mediated cytotoxicity, we examined the MET signaling pathway in OPM-2, KAS-6/1, and KAS-6/R10R cells. For OPM-2, the cells were serum starved for 24 hours in 0.1% FBS and then treated with ARQ 197 for 6 hours (Figure 5A and Supplementary Figure 2A) or serum starved in 0.1% FBS and treated with ARQ 197 for 24 hours (Figure 5B and Supplementary Figure 2B). MET signaling was examined after stimulation with HGF for 15 minutes. As shown in Figure 5, A and B, although these cells showed an HGF-dependent increase in phospho-MET, ARQ 197 treatment decreased levels of this phosphorylation in a dose-dependent manner. In addition, total MET levels decreased with ARQ 197 treatment even though glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels did not decrease dramatically. These findings are consistent with an earlier study of ARQ 197 that demonstrated drug-enhanced MET degradation [36]. We also observed that decreases in phospho-MET and total MET were associated with decreases in phospho-AKT (S473). At high concentrations of ARQ 197 (≥ 1 μM ARQ 197), total AKT levels also decreased, as has been observed previously with other MET inhibitors [15]. MET inhibition also decreased phospho-glycogen synthase kinase-3 (GSK-3β)(S9) levels, thus inhibition of the MET downstream signaling pathway. These molecular events were associated with an increase in the levels of cleaved poly(ADP-ribose) polymerase (PARP), a target of executioner caspase-3, indicating the induction of apoptosis. ARQ 197–mediated decrease in MET signaling was also observed in OPM-2 cells treated with ARQ 197 for 24 hours (Figure 5B and Supplementary Figure 2B).

**Immunoblot analysis of KAS-6/1 cells treated with ARQ 197 in serum-starved media (0.1% FBS) produced similar results as in OPM-2 cells. Treatment of cells with HGF increased the levels of phospho-MET(Y1234/1235) (Figure 5C and Supplementary Figure 2C). This increase in phospho-protein level did not occur when cells were incubated with ARQ 197 before HGF stimulation. The decrease in phospho-MET(Y1234/1235) was associated with the decrease in downstream MET signaling targets such as phospho-AKT(S473), phospho-GSK3β(S9), and phospho-extracellular-signal-regulated kinases 1/2(T202/204).

To compare the effects of ARQ 197 on both HGF-dependent MET signaling and signaling in the presence of full serum, lenalidomide-resistant KAS-6/R10R cells were treated with ARQ 197 for 16 or 24 hours, in the absence or presence of serum, respectively. Similar to the OPM-2 cells, ARQ 197 treatment inhibited HGF-dependent phospho-MET (Y1234) with a corresponding decrease in the downstream MET signaling targets and an
increase in apoptosis, as indicated by an increase in cleaved PARP (Figure 5D and Supplementary Figure 2D). As expected, in the presence of full serum, ARQ 197 inhibited phospho-MET(Y1349) levels. In addition, as expected, the steady-state phosphorylation (thus, signaling) levels of the various downstream signaling molecules in the untreated cells in the presence of full serum was lower than in the HGF-stimulated cells. Interestingly, despite the presence of other growth factors in the full serum to induce signaling, ARQ 197 treatment was able to further reduce AKT, ERK1/2, and GSK3β phosphorylation. This may be indicating that a significant amount of cellular signaling through AKT and MAPK pathways in MM cells is induced by MET activity. Overall, the results demonstrate that the inhibition of MET activity with ARQ 197 inhibited the MET signaling pathway, leading to inhibition of cell growth and induction of cell death.

ARQ 197 in CD138+ Plasma Cells from Myeloma Patients and in Murine Xenograft Model of Myeloma

To extend the cell line data to primary myeloma plasma cells, CD138+ cells obtained from 15 patients with myeloma were treated with 10 μM ARQ 197 for 72 hours and annexin V/PI stained cells were analyzed by flow cytometry. CD138+ cells treated with 10 μM ARQ 197 demonstrated statistically significant increase in annexin V/PI positivity compared to control cells (P < .05 by unpaired t test; Figure 6A). These results indicated that ARQ 197 reduced viability of primary myeloma samples. Treatment of CD138+ plasma cells with ARQ 197 was associated with decreased levels of phospho-MET (Y1234/1235) (Figure 6B). The geometric mean decreased from 32.3 to 28.9 for patient 44, 32.2 to 25.7 for patient 45, and 54.1 to 41.1 for patient 46 upon treatment with 10 μM ARQ 197. Total MET levels (Figure 6C) also decreased upon ARQ 197 treatment (9.04 to 6.81 in patient 44, 8.95 to 7.84 in patient 45, and 6.30 to 5.25 in patient 46). These data demonstrate that the decrease in cell viability was correlated with decreased MET signaling.

ARQ 197 was also tested in a mouse xenograft model of myeloma. Immunodeficient mice were injected subcutaneously with MM.1S myeloma cells to generate xenograft tumors. These mice were treated with ARQ 197 or vehicle using an established schedule that was approved by UT MD Anderson Cancer Center Institutional Animal Care and Use Committee [17]. The mice were randomly placed in either the vehicle treatment group or the ARQ 197 treatment group (200 mg/kg per day × 5 days/week for 4 weeks). At the end of the treatment cycle, tumors were excised and their volume was determined. Compared with the vehicle-treated group (12 mice), the ARQ 197–treated group (15 mice) showed median lower tumor volume (Supplementary Figure 3).

Discussion

Myeloma was previously treated with steroids or with the DNA-damaging agent melphalan. During the last decade, several novel agents that
impact the proteasomal degradation pathway such as bortezomib or carfilzomib were approved as well as immunomodulatory agents such as thalidomide or lenalidomide. These new agents, either as a single agent or in combination, have now become the standard of care for MM.

While the therapeutic landscape has changed for this difficult disease, none of the agents are targeted therapies.

Overproduction and expression of HGF have been established in myeloma. This growth factor binds to the MET receptor tyrosine kinase and this cassette propagates survival, migration, and proliferation signals. High levels of HGF have been found in myeloma patients’ plasma [3–6]. Studies from our group have identified that HGF is overexpressed in primary myeloma plasma cells [15]. Furthermore, genetic knockdown of this pathway or pharmacological inhibition impacted myeloma cell survival and proliferation. However, genetic manipulation is not practical and previous small molecule inhibitors are not clinically viable candidates.

ARQ 197 is not only a potent inhibitor of MET kinase but is also already in the clinics as an oral agent. Our present work focused on testing this agent in the preclinical setting to demonstrate utility of this compound for myeloma. Using a number of myeloma cell lines (U266, MM.1S, OPM-2, RPMI-8226, ANBL-6, and KAS-6/1), we demonstrated that ARQ 197 inhibited growth in a dose- and time-dependent manner. Growth inhibition was associated with an increase in apoptotic cells as observed by annexin/PI positivity and PARP cleavage. Consistent with the cell line data, ARQ 197 treatment decreased phospho-MET(Y1234/1235) and total MET levels in CD138 + plasma cells from patients with myeloma and showed ARQ 197–mediated apoptosis.

Analysis of the HGF/MET signaling pathways in myeloma cell lines demonstrated that ARQ 197 inhibited HGF-dependent induction of phospho-MET. In addition, we observed a decrease in total MET levels, which is consistent with earlier reports with cell

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**Figure 5.** ARQ 197 inhibited the MET signaling pathway. (A) Immunoblot analysis of OPM-2 cells serum starved (0.1% FBS) for 24 hours and treated with ARQ 197 for 6 hours, followed by stimulation with 50 ng/ml HGF for 15 minutes. (B) Immunoblot analysis of OPM-2 cells serum starved (0.1% FBS) and treated with ARQ 197 for 24 hours, followed by stimulation with 50 ng/ml HGF for 15 minutes. (C) KAS-6/1 cells were serum starved for 8 hours, followed by treatment with ARQ 197 for 16 hours. Cells were stimulated with 50 ng/ml HGF and examined by immunoblot analysis. A representative immunoblot from three biologic replicates is shown here. (D) KAS-6/R10R cells were either serum starved (0.1% FBS) for 24 hours and treated with ARQ 197 for 16 hours or they were grown in 10% FBS and treated with ARQ 197 for 24 hours. Serum-starved cells were further stimulated with 50 ng/ml HGF, and the cells were analyzed as in A. For each immunoblot of this figure (A–D), only one GAPDH was needed because all proteins were done from the same gel and membrane by cutting in several sections and using antibodies from different species. We use a fluorescence-based imaging system (LI-COR Odyssey System) to analyze our immunoblots. With this system, our secondary antibodies against primary antibodies derived from different species are tagged with different fluorescence colors.
lines and tumor tissues analyzed during clinical trial [17,20]. This decline might be due to ARQ 197–mediated ubiquitin-dependent degradation of MET [20,36]. The decreases in phospho-MET and total MET were associated with down-regulation of HGF/MET effector pathways such as the AKT and MAPK pathways, which control cell survival and proliferation.

Development of drug-induced cross-resistance is a common phenomenon for myeloma. Hence, we tested drug-resistant myeloma cell lines for biologic actions of ARQ 197. Melphalan and glucocorticoids were once the standard of care for myeloma and are still used in combination. Melphalan, an alkylating agent, has been the mainstay for myeloma treatment since the 1960s. Myeloma initially responds well to melphalan but quickly acquires resistance. A major mechanism of melphalan resistance was through the up-regulation of the Fanconi anemia/BRCA DNA damage repair pathway, which results in repair of DNA damage induced by melphalan [37]. We tested ARQ 197 against melphalan-sensitive (8226/S) and melphalan-resistant (8226/LR-5) cell lines. 8226/LR-5 cells have an IC₅₀ of 20 μM for melphalan, which translates to a seven-fold resistance to melphalan compared with the sensitive cell line [26]. However, these cell lines were equally sensitive to ARQ 197.

Myeloma patients are also treated with glucocorticoids such as dexamethasone, mostly in combination with other drugs. To examine whether ARQ 197 is effective in cells that are glucocorticoid resistant, we used MM.1R cells [28,38,39]. This glucocorticoid-resistant cell line has low expression as well as alternatively spliced isoforms of the receptor and therefore does not undergo cytolysis upon binding of glucocorticoids [38]. We tested ARQ 197 in this cell line and MM.1S, a glucocorticoid-sensitive cell line. In both cell lines, cytotoxicity was comparable, indicating that there was no cross-resistance.

During the last decade, immunomodulatory drugs such as thalidomide and lenalidomide and proteasome inhibitors such as bortezomib and carfilzomib have been introduced and approved for patients with myeloma. Bortezomib is a proteasome inhibitor that induces apoptosis in myeloma cells, in part by blocking the nuclear factor kappa-light-chain-enhancer of activated B cells pathway and stabilizing pro-apoptotic
proteins such as p53 and BAX and decreasing the levels of anti-apoptotic proteins such as BCL-2 [40]. It has been approved as a front-line single-agent therapy for myeloma. In combination with conventional drugs for myeloma, bortezomib exhibits increased activity against myeloma cells and has significantly improved patient survival. We found that bortezomib-resistant myeloma lines ANBL-6/V10R and KAS-6/R10R were sensitive to ARQ 197.

Lenalidomide is a second-generation agent of thalidomide that has been effective as a single agent in myeloma treatment since 2002 [41]. Lenalidomide has been shown to alter ubiquitination of Ikaros transcription factors [42,43], which leads to numerous cellular and immunomodulatory effects such as induction of apoptosis in myeloma cells; decreased attachment of myeloma cells to the bone marrow; decreased production of cytokines such as IL-6, vascular endothelial growth factor, and tumor necrosis factor-α in the bone marrow microenvironment; induction of caspase-8–dependent apoptosis; blockage of angiogenesis; and stimulation natural killer cells’ immunity against MM [41,44]. However, some MM patients eventually become resistant to lenalidomide by activation of the Wnt/β-catenin pathway [27] or overactivation of the HGF/MET pathway (our unpublished data). Yet, in our current study, KAS-6/1 and KAS-6/R10R cells were equally sensitive to ARQ 197. Collectively, these data suggest that ARQ 197 ignores diverse mechanisms of resistance induced by a variety of myeloma therapeutics.

In addition to developing drug-induced resistance mechanisms, myeloma can acquire chemoresistance due to cell adhesion–mediated drug resistance [35]. This phenomenon occurs due to interaction with the bone marrow microenvironment that produces cytokines such as IL-6, vascular endothelial growth factor, insulin-like growth factor-1, and HGF, stimulating myeloma cell survival [1] However, our investigations demonstrate that ARQ 197 overcomes the resistance conferred by IL-6, HGF, or the bone marrow microenvironment.

In our study, ARQ 197 induced cell death in myeloma cell lines and primary myeloma patient samples. Additionally, ARQ 197 treatment in a myeloma murine xenograft model showed median lower tumor volume. While the efficacy in the xenograft model was not dramatic as in the cell line models, the apparent difference could be due to the establishment of xenografts using human myeloma MM.1S cells. These cells are dependent on HGF stimulation for activation of the MET signaling pathway. However, mouse HGF do not activate human MET [45,46]. This would suggest that the xenografts that formed developed independent of MET signaling, and therefore, a MET kinase inhibitor would not be as effective in reducing the tumor volume. This is supported by the observation that there was heterogeneity in mice acquiring tumors. For both the vehicle- and ARQ 197–treated groups, there was a wide variation in the onset of tumors (from 4.4 to 15 weeks). Therefore, a heterogenic feature of mice in acquiring tumors could have translated into heterogeneity in their response rate.

In general, our current investigation support the notion that in myeloma cell lines, ARQ 197 effectively inhibits MET phosphorylation, downregulates the MET signaling pathway, and induces cytotoxicity. Our results are consistent with previous data where we demonstrated that partially decreasing MET levels in myeloma cells by stably transfecting a MET ribozyme construct increased the therapeutic sensitivity of agents that further depleted MET levels [12,13]. In concert, shRNA-mediated MET knockdown diminished MET protein levels and induced apoptosis. Moreover, in our current study, immunoblot analysis indicated that ARQ 197 inhibited HGF-stimulated induction of phospho-MET in OPM-2 and KAS-6/ R10R cell lines. We observed approximately 50% inhibition of phospho-MET at 300 nmol/l ARQ 197. This range of phospho-MET inhibition is similar to that observed in solid tumors, for which MET phosphorylation was inhibited by 100 to 300 nmol/l ARQ 197 [17]. Collectively, these data indicate that MET is a viable anti-cancer target for myeloma treatment and that a MET inhibitor that works in vivo will be effective in myeloma patients.

Our preclinical studies with ARQ 197 demonstrated that it induced apoptosis in ex vivo models of myeloma. However, cell apoptosis was induced in the presence of 10 μM concentration of the drug. We note that at this concentration, ARQ 197 can target other pathways. Recent studies have demonstrated that in addition to its impact on the MET pathway, ARQ 197 also inhibits tubulin in lung cancer cell lines [47,48]. The influence on tubulin resulted in cell arrest in the G2/M phase of the cell cycle. Tivantinib reduced tubulin polymerization in cells and in xenograft tumors by directly binding to the colchicine binding site of tubulin [49]. We are now focusing on the effect of ARQ 197 on tubulin and its biologic consequence in myeloma cell lines.

In conclusion, we have demonstrated that inhibiting MET with ARQ 197 induces apoptosis in myeloma cells. On the basis of these data, we have initiated a phase II clinical trial of ARQ 197 in relapsed/refractory myeloma (NCT01447914). This is a novel target-based strategy that could lead to another drug for myeloma treatment.

Acknowledgements
The authors thank Thomas Chan (ArQule), Chang-Rung Chen (ArQule), and Zenta Tsuchihashi (Daiichi Sankyo, Inc, Parsippany, NJ) for providing us ARQ 197 for animal use. We thank Ivory J. Ellis, Safia Maredia, Rebecca A. Murray, Kavita S. Chathaun, and Trang T. Nguyen from the MD Anderson Myeloma Core for processing bone marrow samples, Adam Stein and Catherine M. Claussen for coordinating the collection and distribution of myeloma samples, and Mary Ayres and Kumudha Balakrishnan for advice on co-culture experiments. We thank Elizabeth L. Hess from the Department of Scientific Publications at UT MD Anderson Cancer Center for editorial help in preparing this manuscript.

Appendix A. Supplementary Data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2015.01.006.

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