Targeting MEK induces myeloma-cell cytotoxicity and inhibits osteoclastogenesis

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Activation of the extracellular signal-regulated kinase1/2 (ERK1/2) signaling cascade mediates human multiple myeloma (MM) growth and survival triggered by cytokines and adhesion to bone marrow stromal cells (BMSCs). Here, we examined the effect of AZD6244 (ARRY-142886), a novel and specific MEK1/2 inhibitor, on human MM cell growth in the bone marrow (BM) milieu. AZD6244 blocks constitutive and cytokine-stimulated ERK1/2 phosphorylation and inhibits proliferation and survival of human MM cell lines and patient MM cells, regardless of sensitivity to conventional chemotherapy. Importantly, AZD6244 (200 nM) induces apoptosis in patient MM cells, even in the presence of exogenous interleukin-6 or BMSCs associated with triggering of caspase 3 activity. AZD6244 sensitizes MM cells to both conventional (dexamethasone) and novel (perifosine, lenalidomide, and bortezomib) therapies. AZD6244 down-regulates the expression/secretion of osteoclast (OC)–activating factors from MM cells and inhibits in vitro differentiation of MM patient PBMCs to OCs induced by ligand for receptor activator of NF-κB (RANKL) and macrophage-colony stimulating factor (M-CSF). Finally, AZD6244 inhibits tumor growth and prolongs survival in vivo in a human plasmacytoma xenograft model. Taken together, these results show that AZD6244 targets both MM cells and OCs in the BM microenvironment, providing the preclinical framework for clinical trials to improve patient outcome in MM. (Blood. 2007;110: 1656-1663)

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Introduction

Multiple myeloma (MM) is characterized by the accumulation of clonal malignant plasma cells in the bone marrow (BM) and monoclonal protein in blood and/or urine.[1] Clinical features include increased risk for infection, pancytopenia, renal disease, hypercalcemia, and bone disease. Although conventional treatments achieve high response rates, disease relapse occurs as a result of acquired drug resistance. Novel agents including thalidomide, lenalidomide, and bortezomib can achieve responses in patients with relapsed and/or refractory MM,[2-5] but resistance develops to these agents. Thus, there is an urgent need for novel biologically based treatment strategies in MM.

Accumulating evidence implicates the RAS/MEK/ERK signaling pathway in pathogenesis of MM. First, specific mutations of *NRAS* (Q61R) or *RAF* (V600E) resulting in the highly activated MEK/ERK pathway are associated with enhanced and selective sensitivity to MEK inhibition.[6] Second, MM cells in the BM microenvironment may also be more susceptible to ERK inhibition due to adhesion-induced ERK activation. ERK1/2 activation is induced in MM cells both by binding to bone marrow stromal cells (BMSCs) and associated cytokine secretion.[7,8] Interleukin-6 (IL-6) and insulin growth factor-1 (IGF-1), 2 major growth, survival, and drug-resistance factors for MM cells, stimulate cell proliferation and block apoptosis by activating this pathway.[1,9-13] MEK and ERK activity is also induced by vascular endothelial growth factor (VEGF) and B-cell activating factor (BAFF) through autocrine and paracrine mechanisms; conversely, pharmacologic inhibition of MEK/ERK signaling blocks MM-cell proliferation and migration induced by these cytokines.[14,15] Dexamethasone (Dex) is a major therapy for MM, and MEK inhibitors synergize with Dex.[16-18] Specifically, resistance to Dex in MM cells is conferred by IL-6,[9,19] IGF-1,[13,20] and BAFF,[15,21] whereas ERK inhibition overcomes Dex resistance by down-regulating these cytokines. Therefore, the MEK/ERK pathway mediates MM-cell growth and survival induced by BM cytokines/growth factors and adhesion to BMSCs, thereby conferring growth and resistance to apoptosis in the BM milieu. Finally, increased angiogenesis in BM of patients is associated with active MM[22]; conversely, ERK inhibition decreases VEGF secretion from MM cells and the BM microenvironment, thereby decreasing in vivo vessel formation induced by MM cells.[23,24] This antiangiogenic effect of MEK/ERK pathway inhibition therefore represents additional potential mechanism of its anti-MM activity.

AZD6244, a novel oral and highly specific MEK1/2 inhibitor,[25,26] induces sustained inhibition of ERK1/2 phosphorylation and tumor cell growth in human solid-tumor xenograft models. It has now entered phase 1 clinical trials in patients with melanoma and non–small-cell lung cancer.[27,28] In the present study, we investigated the impact of AZD6244 on MM cells and osteoclasts (OCs) within the BM microenvironment. Our in vitro and in vivo studies show that AZD6244 targets both MM cells and OCs in the BM milieu and enhances cytotoxicity of both conventional and novel anti-MM agents. These preclinical in vitro and in vivo studies provide the framework for derived clinical trials to improve patient outcome in MM.

Patients, materials, and methods

Cell culture

The CD138^{+} human MM-derived cell lines were maintained as described.[15,29] All MM lines express CD138 and CD38 (> 95% of cells), as evidenced by flow-cytometric analysis. Dex-sensitive MM1S and Dex-resistant MM1R cells were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL). MC/CAR and RPMI8226 lines were obtained from ATCC (Manassas, VA). Doxorubicin-resistant (DOX40) and melphalan-resistant (LR5) RPMI8226 MM lines were provided by Dr William Dalton (Mofit Cancer Center, Tampa, FL). The 28BM and 28BM MM cell lines were provided by Dr Otsuki (Kawasaki Medical School, Okayama, Japan). The IL-6–dependent INA-6 cell line was kindly provided by Dr Renate Burger (University of Kiel, Kiel, Germany). Freshly isolated MM cells (CD138^{+}) were prepared by positive selection using CD138 microbeads (Miltenyi Biotech, Auburn, CA), according to the manufacturer's protocol.[15] CD138^{−} bone marrow mononuclear cells (BMMCs), isolated by depletion of CD138^{+} cells using magnetic beads, were cultured for 3 to 6 weeks to generate BMSCs as described.[15] Approval for these studies was obtained from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Cell proliferation and viability assays

The growth inhibitory and antisurvival effects of AZD6244 (ARRY-142886; AstraZeneca International, Macclesfield, United Kingdom) were assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT; Sigma-Aldrich, St Louis, MO) dye absorbance, as well as [^{3}H]thymidine incorporation.[15]

Immunoblotting analysis

To determine whether AZD6244 inhibits baseline and cytokine-induced ERK phosphorylation, MM1S cells were starved and stimulated with indicated cytokines, in the presence or absence of the drug. Cell lysates were immunoblotted using specific antibodies (Abs; Cell Signaling Technology, Beverly, MA). To determine whether AZD6244 altered expression of proteins involved in apoptosis, cell proliferation, survival, and cell-cycle regulation, MM cells were incubated with drug for indicated time intervals, lysed, and subjected to immunoblotting, as in prior studies.[15] To measure AZD6244-induced caspase-dependent apoptosis in the BM milieu, MM1S cells were treated with or without AZD6244 for 24 hours, in the presence or absence of BMSCs; tumor cells (> 95% purity) were harvested, lysed, and immunoblotted using specific Abs, with anti–α-tubulin monoclonal antibody (mAb) as a loading control.

Apoptosis assays

AZD6244-induced MM cell apoptosis was assayed by cell-cycle analysis,[30] annexin V/PI staining, and Caspase-Glo 3 activity assays (Promega, Madison, WI), according to manufacturer's protocols. Cell-cycle analysis and annexin V/PI staining by flow-cytometric analysis were done using Coulter Epics XL with data acquisition software (Cytomics FC500-CXP version 3.0; Beckman Coulter, Hialeah, FL).[30] For cell-cycle analysis, INA-6 cells were cultured at indicated time intervals with AZD6244 (2 μM) or with serial dilutions of AZD6244 for 2 days, washed, stained with annexin V/PI, and analyzed by flow cytometry. To determine whether AZD6244-induced apoptosis is associated with caspase activation, MM lines in 96-well plates were treated with serial concentrations of AZD6244, in the presence or absence of BMSCs, followed by bioluminescent caspase 3 activity assay.

In vitro OC culture

Peripheral blood mononuclear cells (PBMCs) were obtained from MM patients after informed consent was obtained. CD14^{+} OC precursor cells[31] were cultured for 7 to 14 days in ISCOV/10% FCS with receptor activator of NF-κB ligand (RANKL; 50 ng/mL; Peprotech, Rocky Hill, NJ) and macrophage-colony-stimulating factor (M-CSF; 25 ng/mL; Peprotech), in the presence or absence of

AZD6244 (0-2 μM). Total RNA was prepared using the RNeasy kit (Qiagen, Valencia, CA) and subjected to reverse transcriptase–polymerase chain reaction (RT-PCR) using primers 5′ GTTCTTCTGCACATATTGGAAGG 3′ (forward) and 5′ CCAACTCAAGAAGAAAACTGGC 3′ (reverse) to determine expression of OC marker gene cathepsin K (GenBank accession no. NM000396.2). Twenty-two cycles were performed to ensure that the PCR reaction is in the linear range with an MJ Research Thermal Cycler (Waltham, MA) at 94°C for 0.5 minutes, 58°C for 0.5 minutes, 72°C for 0.5 minutes, then 72°C for 7 minutes. OC formation was further characterized for integrin $\alpha_v\beta_3$integrin expression using flow cytometry.

Human plasmacytoma xenograft model

All animal studies were conducted according to protocols approved by the Dana-Farber Cancer Institute Animal Care and Use Committee. The human plasmacytoma xenograft model was performed as previously described.[8] AZD6244 was dissolved in 0.5% hydroxypropyl methyl cellulose/0.1% polysorbate. CB-17 severe combined immunodeficiency (SCID) mice were inoculated subcutaneously in the left flank with 2.5×10^6 OPM1 MM cells in 100 μL PBS. All mice developed palpable tumors approximately 4 days after cell injection; mice (n = 8 per group) were then treated orally twice per day with AZD6244 (25 or 50 mg/kg) or with the control vehicle alone. Tumor size was measured every day in two dimension using a caliper; tumor volume was calculated using the following formula: $V = 0.5 \times a \times b^2$, where "a" and "b" are the long and short diameters of the tumor, respectively. Animals were killed when their tumors reached 2 cm^3 in diameter or when paralysis or major compromise in their quality of life occurred. At the time of the animals' death, tumors were excised. Survival was evaluated from the first day of treatment until death.

Immunocytochemistry

Sections (4 μm) of formalin-fixed tumor tissues with or without AZD6244 were used for pERK staining with an anti-ERK Ab (Cell Signaling Technology). Slides were examined by standard light microscopy and were analyzed using an Olympus BX41 microscope equipped with UPlan FL 40×/0.75 and 20×/0.50 objective lenses (Olympus, Melville, NY). Pictures were taken using Olympus QColor3 and analyzed using QCapture 2.60 software (QImaging, Burnaby, BC). Adobe Photoshop 6.0 (Adobe, San Jose, CA) was used to process images.

Statistical analysis

Statistical significance of differences observed in drug-treated compared with control cultures was determined using Student t test. The minimal level of significance was a P value less than .05. Regression analysis was used to calculate 50% inhibitory concentration (IC$_{50}$) of tested chemicals. The Chou-Talalay method was used to assess synergistic or additive effects of combined therapies.[32] A combination index (CI) less than 1.0 indicates synergism, and CI of 1.0 indicates additive activity.[8,32] The tumor growth inhibition and survival of mice were determined using SigmaPlot analysis software version 9 (Systat Software, San Jose, CA). Overall survival was measured using the Kaplan-Meier and log-rank method.

Results

AZD6244 specifically inhibits ERK activation and induces cytotoxicity in MM cells, regardless of sensitivity to conventional chemotherapy

Baseline and cytokine-induced ERK phosphorylation was first examined in MM1S cells, which have been extensively studied to validate novel (bortezomib and lenalidomide) therapies in MM. MM1S cells were cultured with or without AZD6244 (10 nM) followed by IGF-1 (100 ng/mL) stimulation for indicated time intervals. Total cell lysates were prepared and subjected to immunoblotting using specific antibodies. AZD6244 completely blocked
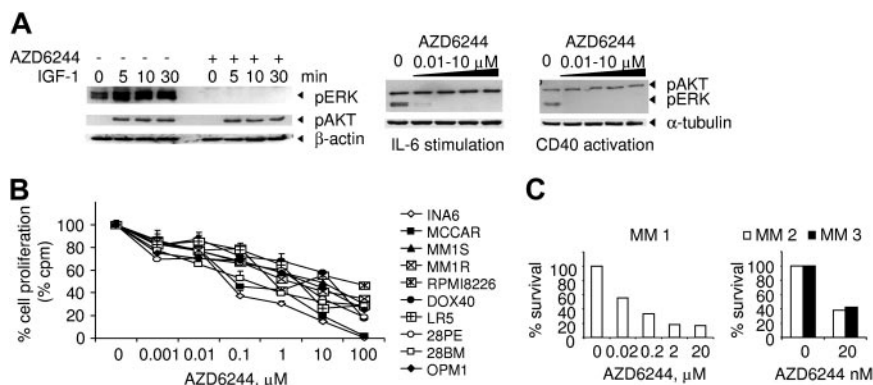
Figure 1. AZD6244 specifically inhibits ERK phosphorylation and induces cytotoxicity in MM lines resistant to conventional chemotherapy, as well as in primary patient MM cells. (A) Serum-starved MM1S MM cells were pretreated with either AZD6244 (10 nM) or control medium for 1 hour and then stimulated with IGF-1 (100 ng/mL) for indicated time intervals (left panel). Similarly, MM1S cells were pretreated with serial dilutions of AZD6244 (0-10 μM) and stimulated with IL-6 (50 ng/mL, middle panel) or sCD40L (5 μg/mL, right panel). Immunoblotting was performed using anti-pAKT and pERK Abs, as well as anti–β-actin or anti–α-tubulin mAbs as loading controls. (B) Ten MM lines, including drug-sensitive RPMI8226, Dox-resistant RPMI8226 (Dox40), melphalan-resistant RPMI8226 (LR5), Dex-sensitive MM1S and -resistant MM1R, IL-6–dependent INA-6, and MCCAR, 28PE, 28BM, and OPM1 cells, were cultured with AZD6244 for 2 days and then pulsed with [^{3}H]thymidine for the last 8 hours for measurement of DNA synthesis. Data represent mean (+ SE) of quadruplicate cultures; cpm indicates counts per minute. (C) Freshly isolated tumor cells from 3 MM patients (MM 1, MM 2, MM3) were cultured with AZD6244 (0.02-20 μM) for 2 days, and cytotoxicity was assessed by MTT assay.

both constitutive and IGF-1–induced ERK phosphorylation (Figure 1A). In contrast, IGF-induced AKT phosphorylation was not inhibited. Similarly, pretreatment with AZD6244 completely blocked IL-6– or CD40L-induced ERK phosphorylation without altering AKT activation. Thus, AZD6244 at concentrations as low as 10 nM selectively inhibits constitutive and cytokine-induced ERK phosphorylation. We next examined the growth inhibitory effect of AZD6244 in a panel of MM lines sensitive or resistant to conventional therapies. MM lines were cultured for 2 days in the presence or absence of AZD6244, and DNA synthesis was then measured. AZD6244 significantly inhibited MM cell proliferation in a dose-dependent fashion (Figure 1B). Importantly, AZD6244 at concentrations as low as 20 nM inhibited growth of CD138$^+$ MM cells from 3 patients (Figure 1C). Responses to AZD6244 and mutation status of RAS genes including NRAS and KRAS (codon 12 and 61) from patients with advanced disease were included in Table 1. These results indicate that AZD6244 is also effective in advanced stages where MM cells are less dependent on factors produced by the microenvironment.

AZD6244 induces MM cell cytotoxicity in the presence of IL-6 and BMSCs

Since IL-6 is an MM growth, survival, and drug-resistance factor,[1] we next determined whether AZD6244 maintains its cytotoxic effects on MM cells in the presence of IL-6. AZD6244 (20 nM) treatment of patient MM cells significantly blocked IL-6–induced growth and survival in MM-patient cells (Figure 2A), evidenced by greater than 2.2-fold reduction in growth and cell viability ($P < .01$). Moreover, AZD6244 also inhibited survival of IL-6–dependent INA-6 MM cells adherent to BMSCs in a dose-dependent fashion (Figure 2B left panel). We next determined whether AZD6244 triggers apoptosis in IL-6–dependent INA-6 cells cultured with exogenous IL-6 or in the context of BMSCs. In a dose-dependent manner, AZD6244 induces apoptosis in cultures of INA-6 MM cells with IL-6 or BMSCs, evidenced by increased caspase 3 activation (Figure 2B right panel). AZD6244 also induces cytotoxicity against patient MM cells cultured with BMSCs (Figure 2C). Consistent with previous studies, MM-cell adhesion to BMSCs augments IL-6 secretion from BMSCs, which is also blocked by AZD6244 (Figure 2D). Finally, AZD6244 has minimal cytotoxic effects on BMSCs derived from MM patients, since it does not inhibit their growth and viability at concentrations (50 μM) that are cytotoxic against MM cells (Figure 2E). These results indicate that neither IL-6 nor BMSCs protect against AZD6244-triggered cytotoxicity in MM-cell lines and patient MM cells.

AZD6244 induces caspase 3–dependent apoptosis in MM cells adherent to BMSCs

To further confirm whether AZD6244 triggers apoptotic signaling in MM cells adherent to BMSCs, we next measured caspase 3 activity in drug-treated Dex-resistant MM1R cells cultured with or without BMSCs. Caspase 3 activity was triggered by AZD6244 in these MM cells in a dose-dependent fashion, even in the presence of BMSCs, whereas caspase 3 activation was not induced in BMSCs (Figure 3A). AZD6244 similarly induced caspase 3 activity in Dex-sensitive MM1S cells adherent to BMSCs (data not shown). Importantly, AZD6244 (200 nM) also inhibits growth of patient MM cells adherent to BMSCs, associated with increased caspase 3 activity (Figure 3B). To confirm targeting of ERK1/2, we performed immunoblotting of lysates from MM1S cells cultured alone or with BMSCs, in the presence or absence of AZD6244. As in our previous studies,[7,8] adhesion of MM1S cells to BMSCs significantly induced phosphorylation of ERK1/2 (Figure 3C). Importantly, AZD6244 inhibits constitutive and adhesion-induced ERK1/2 activation in MM1S cells. Drug (2 μM) also significantly induces cleavage of PARP and caspase 3 in MM1S cells cultured with BMSCs (Figure 3C). These

Table 1. Effects of AZD6244 on patient MM cells

Patient no.	Patient stage	RAS mutations	% viable cells Control	% viable cells AZD6244
1	Refractory	+	78	41
2	Refractory	−	63	42
3	Newly diagnosed	−	72	64
4	Refractory	+	75	45
5	Newly diagnosed	−	77	48
6	Advanced	+	90	46
7	Advanced	+	65	41
8	Advanced	−	71	64
9	Advanced	+	68	34
10	Advanced	−	72	38
11	Advanced	−	87	68
12	Advanced	+	76	32

CD138-purified MM cells from 12 patients were cultured with control or AZD6244 (0.2 μM) medium and were assayed for apoptosis after 5 days. NRAS- and KRAS-activating mutations were examined using an allele-specific amplification method.[33]

Figure 2. AZD6244 induces MM-cell cytotoxicity in the presence of IL-6 or BMSCs. (A) CD138-purified patient MM cells were treated with AZD6244 (20 nM) in the presence or absence of IL-6 (10 ng/mL). Cytotoxicity (growth and viability) was measured by [3H]thymidine uptake and MTT assay. **$P < .01$ compared with control. (B) INA-6 MM cells were treated for 2 days with serial dilutions of AZD6244 (0-10 μM) in the presence or absence of BMSCs, followed by MTT assay (left panel) and caspase 3 activity assay (right panel). Data represent mean (+ SE) of triplicate cultures; au indicates arbitrary unit. (C) Freshly purified CD138+ tumor cells from 3 MM patients were cultured with AZD6244 (200 nM, ■) or control medium (□), alone or with BMSCs. Cytotoxicity was determined by [3H]thymidine incorporation assay (MM1 and MM3) or MTT assay (MM2). (D) MM1S cells were treated with AZD6244 (0.2-20 μM) for 2 days in the presence or absence of BMSCs, and supernatants were assayed for IL-6 by ELISA. (E) BMSCs derived from MM patients were incubated for 2 days with AZD6244 (0.5-500 μM) in triplicate and then subjected to MTT assay. Results represent mean (+ SE) of BMSCs from 3 patients.
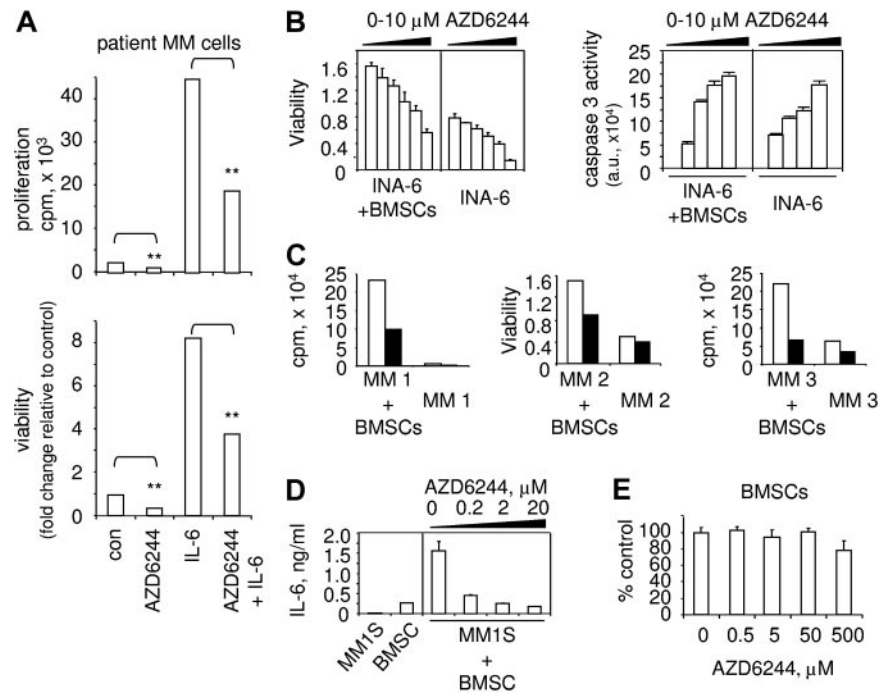
results strongly suggest that ERK inhibition by AZD6244 targets MM cells in the BM microenvironment.

Functional sequelae of ERK inhibition by AZD6244 in MM cells

To delineate the mechanism whereby AZD6244 mediates MM-cell growth inhibition, we next examined the cell-cycle profile of INA-6 cells cultured with control media or AZD6244 (2 μM). As shown in Figure 4A, AZD6244, in a time-dependent manner, induces a progressive increase in sub-G_0/G_1–phase cells. A dose-dependent increment in apoptotic fraction induced by AZD6244 (0.1-10 μM) is further evidenced by increased PI+/annexin V+ INA-6 cells (Figure 4B). Treatment of patient MM cells with AZD6244 (0.2 μM) for 2 days similarly increases PI+/annexin V+ apoptotic cells, confirming that AZD6244 significantly induces cytotoxicity and apoptosis (Figure 4C). We next examined the molecular sequelae triggered by AZD6244 using immunoblotting (Figure 4D). In IL-6–dependent INA-6 MM

cells, AZD6244 significantly up-regulates proapoptotic proteins (BAX, BIM, BNIP3) and negative cell-cycle regulators (p53, p21, p27, p16INK4A, p18INAK4C), whereas factors critical for antiapoptosis/survival and cell-cycle progression/proliferation (Bcl-2, Mcl-1, STAT3, cyclin E1, c-maf) are significantly decreased. Thus, ERK inhibition by AZD6244 in MM cells is associated with both suppression of proliferation/antiapoptosis/survival, as well as induction of proapoptotic proteins.

AZD6244 enhances cytotoxicity of conventional and novel therapies

Since ERK activation mediates MM-cell proliferation and antiapoptosis, we next determined whether AZD6244 could enhance cytotoxicity of conventional (Dex) and novel (bortezomib, lenalidomide, perifosine) agents. INA-6 cells were cultured for 2 days with Dex (25-50 nM) in the presence or absence of AZD6244. AZD6244 enhances growth inhibition triggered by Dex: Dex (25 nM) induces
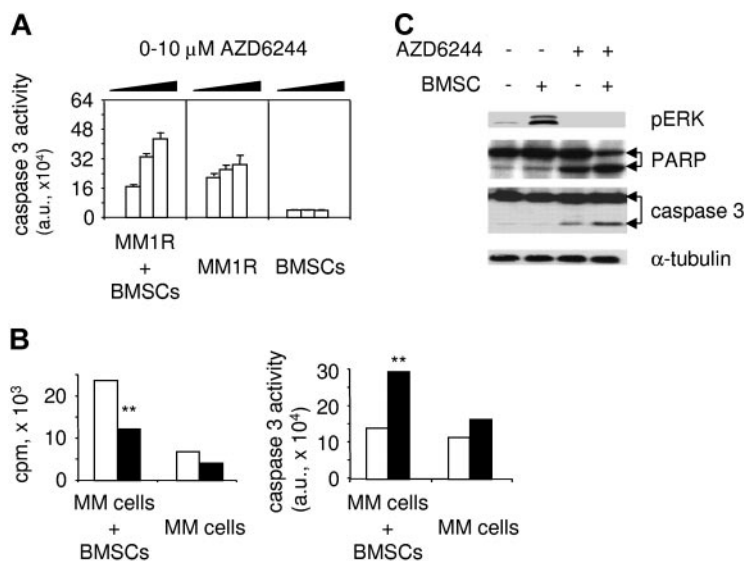

Figure 3. AZD6244 induces apoptotic signaling in MM cells adherent to BMSCs. (A) Dex-resistant MM1R cells were incubated for 2 days with serial dilutions of AZD6244 (0-10 μM), in the presence or absence of BMSCs, followed by caspase 3 activity assays. (B) CD138+ patient MM cells were incubated with AZD6244 (200 nM, ■) or control medium (□), in the presence or absence of BMSCs. Cytotoxicity was assayed by growth inhibition (left panel), and apoptosis was measured by caspase 3 activity assay (right panel). **$P < .01$ compared with control. (C) MM1S cells were cultured in BMSC-coated plates for 24 hours, in the presence or absence of AZD6244. MM1S cells were then harvested, lysed, electrophoresed, and immunoblotted for pERK, PARP, and caspase 3, with α-tubulin as loading control.
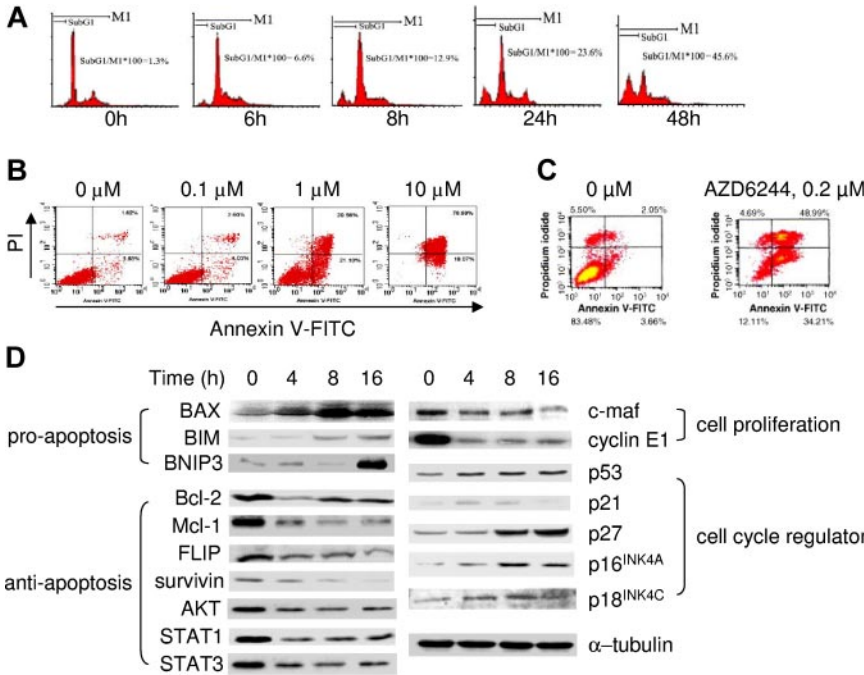
Figure 4. Effect of AZD6244 on cell-cycle profile and functional sequelae of ERK inhibition in MM cells. (A) INA-6 MM cells were treated with AZD6244 (2 μM) for the time intervals indicated. Cell-cycle profile was then evaluated by PI staining using flow cytometry. (B) INA-6 MM cells were treated with AZD6244 (0.1-10 μM) for 2 days, followed by PI/annexin V staining and flow-cytometric analysis. (C) CD138^{+} patient MM cells were incubated for 2 days with control media or AZD6244 (0.2 μM) and then subjected to PI/annexin V staining. (D) INA-6 MM cells in 10% FBS-containing medium were treated with AZD6244 for the intervals indicated and lysed, and whole-cell lysates were then subjected to immunoblotting using specific Abs.

36% cytotoxicity, which is augmented to 80% and 90% cytotoxicity by 0.1 μM and 1 μM of AZD6244, respectively (Figure 5A). Isobologram analysis[32] confirmed synergistic anti-MM activity of AZD6244 and Dex (CI = 0.64; n = 3). Similar results were obtained in 2 additional MM lines: Dex (25 nM) triggers 35% and 65% growth inhibition in MCCAR and MM1S cells, respectively, which is enhanced to 76% and 80%, respectively, by 0.1 μM of AZD6244 (0.1 μM; data not shown; CI = 0.71 and 0.80 for MCCAR and MM1S cells, respectively). Bortezomib and lenalidomide with Dex were recently approved by the Food and Drug Administration for regimens for treatment of patients with relapsed and refractory MM; however, some patients do not respond, and resistance is acquired to these 2 novel agents. We therefore next examined whether AZD6244 enhances cytotoxicity of these novel agents against MM1S cells. AZD6244 increases bortezomib-induced (Figure 5B, CI = 1.0; $P < .04$; n = 3) and lenalidomide-induced (Figure 5C, CI = 1.0; $P < .05$; n = 3) cytotoxicity.

Most recently, we have shown that AKT inhibition by perifosine is associated with significant anti-MM activity in vitro and in vivo,[8] providing the preclinical rationale for a phase 1 clinical trial in MM. Using the pharmacologic ERK inhibitor U0126, we also recently showed that ERK inhibition synergistically augments perifosine-induced cytotoxicity in MM1S and MM1R MM cells, cultured in the presence or absence of BMSCs.[8] In this study, patient MM cells were cultured for 2 days with perifosine (2.5, 5, 7.5 μM), in the presence (0.01, 0.1 μM) or absence of AZD6244, followed by MTT assay. Perifosine induced a dose-dependent increase in AZD6244-induced cytotoxicity (Figure 5D, CI = 0.70). MM patient cells were similarly cultured with BMSCs for 3 days, in the presence or absence of AZD6244 (20 nM), perifosine (7.5 μM), or both, followed by MTT assay. AZD6244 and perifosine trigger synergistic cytotoxicity in adherent patient MM cells (Figure 5E, CI = 0.70). These results indicate that AZD6244 enhances cytotoxicity induced by both conventional and novel agents.
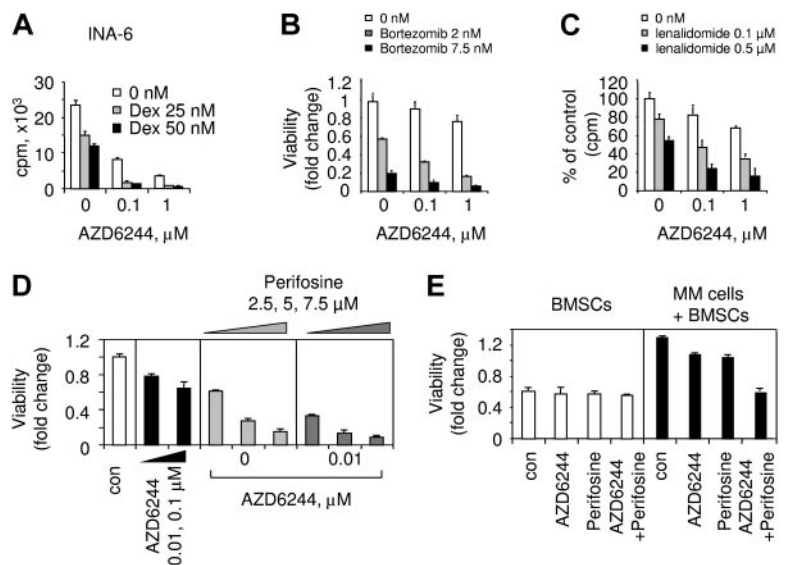

Figure 5. ERK inactivation by AZD6244 enhances cytotoxicity of conventional and novel agents. (A) INA-6 cells were cultured for 2 days with Dex (0-50 nM) in the presence or absence of AZD6244 (0-1 μM), and DNA synthesis was measured. Data represent mean ($+$ SE) of quadruplicate cultures. MM1S cells were cultured for 2 days with (B) bortezomib (0-7.5 nM) and AZD6244 (0-1 μM) or (C) lenalidomide (0-0.5 μM) and AZD6244 (0-1 μM). (D) CD138^{+} patient MM cells were incubated for 3 days with AZD6244 (0-0.1 μM), perifosine (0-7.5 μM), or the combination. (E) CD138^{+} MM-patient cells were cultured with BMSCs in the presence or absence of AZD6244 (20 nM), perifosine (7.5 μM), or the combination. Cytotoxicity (viability) was measured by MTT assay, expressed as fold change to control.

Figure 6. AZD6244 OC formation and transcripts of OC-stimulating factors in MM cells. (A) Adherent monocytic OC precursors from MM-patient PBMCs were incubated with M-CSF/RANKL, in the presence or absence of AZD6244 (0.1 μM), for indicated time intervals. Total cell lysates were prepared and subjected to immunoblotting using anti-pERK Ab, with anti-ERK Ab as a loading control. (B) Adherent OC precursors from MM patient PBMCs were incubated with M-CSF/RANKL in the presence of AZD6244 for 10 days. Total RNA was then prepared and subjected to RT-PCR for cathepsin K (PCR product: 127 bp). RT-PCR for GADPH served as an internal control. (C) PBMCs from MM patients (n = 3) were incubated with M-CSF/RANKL for 14 days, in the presence or absence of AZD6244. OC formation was characterized by integrin $\alpha_v\beta_3$ expression by flow-cytometric analysis. *$P < .05$; **$P < .005$; data represent the mean of 3 experiments (± SE). Multinucleated OCs were induced by M-CSF/RANKL (control), whereas AZD6244 (0.2 μM) blocked OC formation; original magnification × 100. See "Patients, materials, and methods; Immunohistochemistry" for more information on image acquisition. (D) Transcriptional changes of indicated OC-activating factors in MM1S cells following AZD6244 treatment.
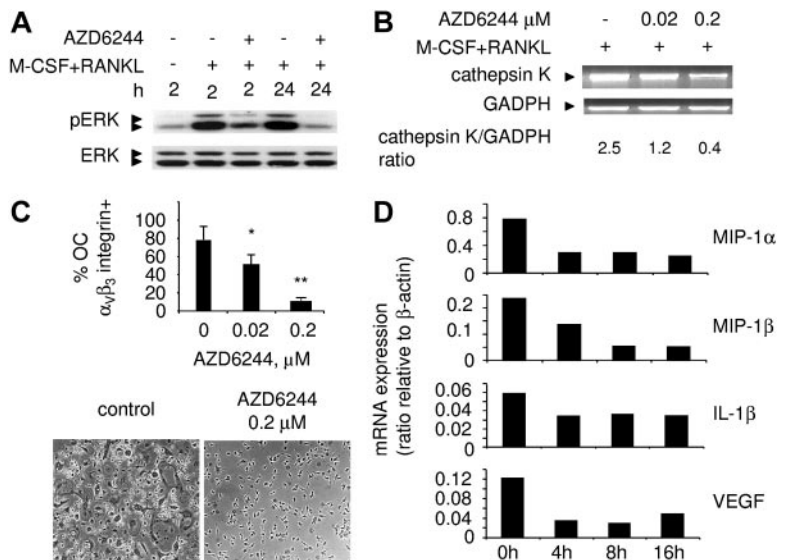
Effect of AZD6244 on OCs in the BM

Since MM is associated with enhanced osteoclast (OC) activity,[34] and M-CSF and RANKL induce differentiation and survival of OC precursor cells via ERK activation,[35] we next examined whether AZD6244 blocks ERK activation induced by these cytokines in CD14+ OC precursor cells. Monocytic cells (CD14+) from MM-patient PBMCs were incubated with M-CSF/RANKL–containing medium, in the presence or absence of AZD6244. Cell lysates were prepared and subjected to immunoblotting using anti-pERK1/2 and ERK1/2 Abs. Phosphorylation of ERK induced by these cytokines as early as 2 hours and sustained to 24 hours is completely blocked by AZD6244 (Figure 6A). We next assessed OC marker gene cathepsin K expression in CD14+ OC precursor cells after culture for 10 days with M-CSF/RANKL, in the presence or absence of AZD6244. AZD6244 decreased cathepsin K expression in a dose-dependent fashion (Figure 6B). Importantly, AZD6244 also inhibits integrin$\alpha_v\beta_3$+ OC formation in a dose-dependent manner: median percentage of mature OCs is decreased from 81% (+4.4) in control medium to 49% (+8.0) and 9.8% (+2.9) with 0.02 and 0.2 μM of AZD6244, respectively (n = 3, $P = .02$; Figure 6C). Interactions of MM cells with BMSCs stimulate transcription and secretion of MM-cell survival and proliferation factors, as well as OC-activating cytokines including macrophage inflammatory protein-1α and -1β (MIP-1α, MIP-1β), IL-1β, and VEGF. Significantly, mRNA expression of these cytokines in MM1S cells was reduced as early as 4 hours following AZD6244 treatment (Figure 6D). AZD6244 similarly decreased mRNA transcripts for OC-

activating cytokines in INA-6 MM cells in a time-dependent fashion (data not shown). Finally, VEGF and MIP-1α secretion from MM1S and INA-6 cells, measured by enzyme-linked immunosorbent assay (ELISA), decreased following AZD6244 treatment, consistent with its effects on mRNA (data not shown). These results indicate that AZD6244 prevents activation of CD14+ OC precursor cells induced by M-CSF and RANKL, thereby blocking OC differentiation.

AZD6244 inhibits human MM-cell growth in vivo in a human plasmacytoma xenograft model

We next examined the in vivo efficacy of AZD6244 using our human plasmacytoma xenograft mouse model with OPM1 MM cells, which have an IC$_{50}$ of 5 μM AZD6244 in vitro (Figure 1B). All mice developed measurable tumors 4 days after injection of tumor cells and were then randomized to receive treatment twice daily with either AZD6244 or control vehicle. Treatment of OPM1 MM-tumor–bearing mice with AZD6244 (25 and 50 mg/kg, n = 8 per dose), but not control vehicle alone (n = 8), significantly inhibits MM-tumor growth ($P = .03$; Figure 7A). OPM1 MM cells grew aggressively in mice, and animals were killed when tumors reached 2 cm³ in diameter. All mice receiving control vehicle were killed at day 10 after treatment. As seen in Figure 7B, survival was significantly prolonged in treated animals versus controls ($P = .02$): the median overall survival was 9 days in the control group and
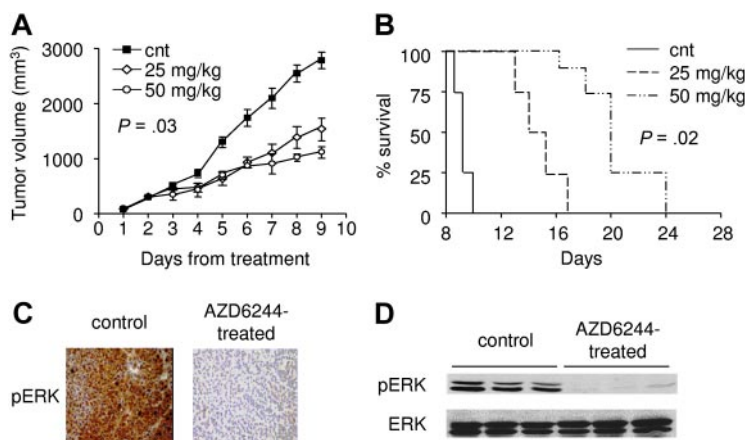

Figure 7. AZD6244 inhibits human plasmacytoma growth and prolongs survival in a murine xenograft model. (A) OPM1 MM cells were injected subcutaneously. Mice (n = 8 per group) received AZD6244 (orally twice per day) or control vehicle (cnt, ■) orally starting on day 1 for 9 days. Significant growth inhibition of OPM1 MM cells was noted in AZD6244-treated (◇ 25 mg/kg, ○ 50 mg/kg) compared with vehicle-treated control mice (n = 8; $P = .03$). Points indicate mean; bars, SE. (B) After OPM1 tumors were measurable, mice were treated with AZD6244 (25 mg/kg, — — —; 50 mg/kg, — - - —) or with control vehicle alone (cnt, —) for 9 consecutive days. Survival was evaluated from the first day of treatment until death or sacrifice (mice were killed when tumors reached 2 cm³ in diameter) using the SigmaPlot (Systat) analysis software ($P = .02$). (C) Representative immunohistochemical staining for phosphorylation of ERK (pERK) in tumor sections from vehicle control- and AZD6244 (25 mg/kg)–treated mice; original magnification × 100. See "Patients, materials, and methods; Immunohistochemistry" for more information on image acquisition. (D) Tumor tissues from mice treated with vehicle control or AZD6244 (25 mg/kg) for 1 day were harvested and processed, and lysates were subjected to immunoblotting using pERK and ERK Ab.

16 days in the AZD6244 (50 mg/kg)–treated cohort. AZD6244 was well tolerated by mice, without significant weight loss (data not shown). Whole tumor-cell tissues and tumor lysates from vehicle control versus AZD6244 (25 mg/kg for 1 day)–treated mice were subjected to immunohistochemical staining and immunoblotting, respectively, to assess in vivo phosphorylation of ERK. Importantly, tumor tissues from AZD6244-treated mice demonstrated significant inhibition of ERK phosphorylation compared with tumor tissues from vehicle control animals (Figure 7C,D).

Discussion

In the BM microenvironment, both the interaction of MM cells with BMSCs and associated secretion of cytokines activate MEK/ERK signaling, which mediates MM-cell proliferation, survival, drug resistance, and angiogenesis.[1] Therefore, blocking this pathway, rather than blocking a single factor, is a promising treatment strategy in MM. Here we show that AZD6244, an orally active benzimidazole inhibitor of MEK/ERK, completely blocks both constitutive and cytokine-induced ERK phosphorylation at concentrations as low as 10 nM without altering AKT activation. Importantly, it inhibits growth and survival of MM lines and patient MM cells, alone or adherent to BMSCs. AZD6244 induces caspase 3 activities in INA-6, MM1R, and MM1S cells bound to BMSCs, without any inducible caspase 3 activity in BMSCs, suggesting minimal cytotoxicity in normal cells and a favorable therapeutic index. Immunoblotting confirms inhibition of ERK phosphorylation and cleavage of caspase 3 and PARP triggered by AZD6244, even in MM1S cells adherent to BMSCs. Thus, AZD6244 overcomes protection conferred by BMSCs in vitro. Importantly, AZD6244 induces significant tumor regression, associated with down-regulation of pERK, and prolongs survival in an aggressive xenograft model of OPM1 MM cells. These data suggest that AZD6244 can overcome cell adhesion–mediated drug resistance (CAM-DR) to conventional therapies.

We demonstrated that the IC_{50} of AZD6244 is 20 nM against primary patient MM cells, which is greater than 10-fold lower than in MM-cell lines. As observed in other solid tumor cells,[28] human MM cells exhibit a wide range of sensitivity to AZD6244, with IC_{50} of 20 to 200 nM in primary MM cells. One potential mechanism to account for differential sensitivity to this class of drugs is RAS gene mutation. For example, INA-6 MM cells with N-RAS mutation[36] are more sensitive to AZD6244 than MM1S and MM1R cell lines without RAS mutation. These results are consistent with reports of gain-of-function RAS or RAF mutations leading to constitutive ERK activation and conferring sensitivity to ERK inhibitors in other solid tumors.[6] Further studies of RAS and RAF gene status in MM lines and patient MM cells are ongoing to define mechanisms of differential responses.

AZD6244-mediated growth inhibition in MM cells was associated with induction of apoptosis and cyclin-dependent kinase inhibitors, as well as down-regulation of cell proliferation. Specifically, it significantly induces proapoptotic proteins (BAX, BIM, BINP3) and inhibits antiapoptotic proteins (Bcl-2, Mcl-1, FLIP) in INA-6 MM cells. It also significantly down-regulates proteins regulating MM-cell proliferation including c-maf. Importantly, AZD6244-inhibited c-maf expression has clinical implications, since this proto-oncogene is overexpressed in greater than 50% of MM cell lines and patient MM cells.[37] Our preliminary results indicate that AZD6244, in a time-dependent fashion, also blocks expression of c-maf target genes including integrin β7 and VEGF

in both INA-6 and MM1S cells (data not shown). Decreased VEGF transcription and secretion is triggered by AZD6244, confirming the role of ERK pathway–regulating angiogenesis in the MM BM microenvironment.[24,38] AZD6244 therefore targets both interactions between MM cells and other cellular components in the BM milieu (ie, altering integrins) as well as the microenvironment (ie, modulating cytokines and angiogenesis).

Interaction of MM cells with the BM microenvironment (BMSCs, OCs) promotes MM-cell growth and disease progression. Here we show that AZD6244 both inhibits cytokine-induced activation of CD14+ osteoclast precursor cells and blocks secretion of OC-activating cytokines from MM cells triggered by their interaction with BMSCs. Specifically, M-CSF and RANKL play critical roles in early development of OCs, and specific mAbs or fusion proteins directed against these cytokines are now being developed to inhibit OC function and related bone complications in MM. Our studies show that AZD6244 blocks sustained ERK activation induced by both cytokines in CD14+ OC precursor cells, thereby preventing OC differentiation. Importantly, transcription and secretion of cytokines that stimulate OC activation (ie, IL-1β, MIP-1α, MIP-1β, and VEGF) from MM cells is markedly reduced by AZD6244 treatment. Furthermore, our preliminary data demonstrate that AZD6244 also inhibits secretion of IL-6, BAFF, and a proliferation-inducing ligand (APRIL) in these OC differentiation cultures (data not shown). Down-regulation of BAFF and APRIL, 2 MM survival factors secreted by OC,[31] induced by AZD6244 treatment provides further evidence that this drug blocks OC formation. Thus, targeting the ERK pathway, rather than targeting a single factor, induces multiple mechanisms to enhance cytotoxicity against MM cells in their BM microenvironment.

Potent in vivo activity of AZD6244 was demonstrated in our aggressive human plasmacytoma model of OPM1 MM cells. Specifically, IC_{50} is 5 μM for AZD6244 against OPM1 cells, indicating that the OPM1 cells are more resistant than either INA-6 or patient MM cells. AZD6244 inhibited growth of OPM1 cells in the xenograft model, confirming its potent anti-MM activity in vivo. These results are in accord with those observed in a pancreatic BxPC3 xenograft model (IC_{50} of 8 μM),[28] further supporting the potential clinical utility of this agent.

In summary, AZD6244 (ARRY-142886) not only targets MM cells directly but also targets the BM microenvironment (cytokines, angiogenesis, OCs). It significantly blocks tumor-cell growth in a human plasmacytoma xenograft model in vivo. Taken together, these results provide the framework for clinical trials of a specific MEK1/2 pathway inhibitor, AZD6244, alone or with combination, to improve patient outcome in MM.

Authorship

Contribution: Y.-T.T. designed and performed experiments, analyzed data, and wrote the manuscript; M.F., X.-F.L., and M.L. performed animal studies; T.H., W.S., X.-F.L., M.R., P.B., A.M., and M.L. performed research and analyzed data; K.P. and P.T. provided valuable reagents; P.R., N.C.M., and I.G. provided patient BM and blood samples; D.C. reviewed the paper; and K.C.A. critically evaluated and edited the manuscript.

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