The Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitor PTK787/ZK222584 Inhibits Growth and Migration of Multiple Myeloma Cells in the Bone Marrow Microenvironment


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ABSTRACT

Our prior studies show that multiple myeloma (MM) cell lines and patient cells express high-affinity vascular endothelial growth factor (VEGF) receptor (VEGFR) Flt-1 but not Flk-1/KDR. Moreover, these studies have shown that VEGF induces proliferation and migration of MM cells, and we have begun to delineate the signaling cascades mediating these sequelae. In this study, we examined the activity of PTK787/ZK222584 (PTK787), a molecule designed to bind specifically to the tyrosine kinase domain of VEGFR and inhibit angiogenesis. We show that PTK787 acts both directly on MM cells and in the bone marrow microenvironment. Specifically, PTK787 (1–5 μM) inhibits proliferation of MM cells by 50%, as assayed by [3H]thymidine uptake. This effect of PTK787 is dose dependent in both MM cell lines and patient cells that are both sensitive and resistant to conventional therapy. PTK787 enhances the inhibitory effect of dexamethasone on growth of MM cells and can overcome the protective effect of interleukin 6 (IL-6) against dexamethasone-induced apoptosis. PTK787 (1 μM) also blocks VEGF-induced migration of MM cells across an extracellular matrix. Importantly, PTK787 also inhibits the increased MM cell proliferation and increased IL-6 and VEGF secretion in cultures of MM cells adherent to bone marrow stem cells. These findings therefore demonstrate that PTK787 both acts directly on MM cells and prohibits paracrine IL-6-mediated MM cell growth in the bone marrow milieu. The demonstrated anti-MM activity of PTK787, coupled with its antiangiogenic effects, provides the framework for clinical trials of this agent to overcome drug resistance and improve outcome in MM.

INTRODUCTION

MM remains incurable, despite the use of conventional and high-dose therapies. However, drugs targeting not only the MM cell but also the BM microenvironment can overcome drug resistance both in vitro and in clinical studies. For example, thalidomide and its immunomodulatory derivatives (1), the proteasome inhibitor PS341 (2), and arsenic trioxide (3–5) directly induce apoptosis or G1 growth arrest in drug-resistant MM cell lines and patient MM cells. In addition, these drugs inhibit the transcription, secretion, and actions of cytokines (such as IL-6, VEGF, and TNF-α) promoting MM cell growth, adhesion, survival, drug resistance, and migration in the BM microenvironment.

Elevated serum levels of VEGF have been reported in patients with MM (6) and correlate with both increased angiogenesis in MM BM (7–10) and a higher plasma cell labeling index (8, 9). We have shown recently that binding of MM cells to BMSCs markedly up-regulates VEGF secretion (11). Moreover, VEGF secreted by MM cells triggers IL-6 production from BMSCs (12), thereby augmenting paracrine MM cell growth. Importantly, we have also shown recently that VEGF acts directly on MM cells. Specifically, VEGF receptor-1 protein (Flt-1), but not the VEGF receptor-2 (Flk-1, or KDR), is expressed on MM cell lines and patient cells, and endogenous VEGF stimulates in vitro proliferation and migration of MM cells through an extracellular matrix (13, 14). These data show that VEGF, in addition to stimulating BM angiogenesis, also plays an important role in both autocrine and paracrine growth of MM cells. They provide the framework for targeting VEGF and Flt-1 in novel MM therapeutics.

PTK787/ZK222584 (PTK787) is a tyrosine kinase inhibitor initially designed to inhibit VEGF signal transduction by binding directly to the ATP-binding sites of VEGFRs. The drug is most specific for KDR (IC50 37 nm) but can also inhibit Flt-1 (77 nm) and Flt-4 (660 nm; Ref. 15). It also has activity against other type III tyrosine kinase receptors, including c-Kit (730 nm), platelet-derived growth factor receptor-β (580 nm), and c-Fms (1.4 nm). In contrast, it has no inhibitory activity on EGF receptor, v-abl, c-src, or PKC-α (IC50 > 10 μM). In mice, a single oral dose of PTK787 (50 mg/kg) can achieve plasma levels of 30 μg/ml at 1 h, with levels remaining ≥1 μM at 8 h after ingestion. Moreover, daily oral doses as high as 100 mg/kg and serum levels ≥10 μM have been maintained chronically without significant side effects (15). Importantly, PTK787 inhibits growth of several human carcinomas transplanted orthotopically into mice, including the A431 epidermoid carcinoma, LS174T colon carcinoma, HT-29 colon carcinoma, and PC-3 prostate carcinoma (15). In addition, the drug inhibits the development of pleural effusions generated by injection of the non-small cell lung carcinoma cell line PC14PE6 (16), prevents the development of malignant ascites and tumor cell growth of ovarian carcinomas (17), and blocks lung and lymph node metastases of renal carcinoma cells (18). PTK787 does not have a direct effect on any of these tumor cells but does reduce vessel density in tumor tissues, suggesting that its primary mode of action in these cells is through inhibition of angiogenesis.

In this study, we investigated the effects of PTK787 both directly on MM cells and in the BM microenvironment. We found that PTK787 directly inhibits proliferation of MM cell lines and patient MM cells that express Flt-1. In addition, PTK787 inhibits MM cell migration, assayed via transwell cell migration. This agent enhances anti-MM activity of Dex and can overcome the protective effect of IL-6 against Dex-induced MM cell apoptosis. Importantly, PTK787...
can inhibit the secretion of IL-6 induced by binding of MM cells to BMSCs as well as the resultant proliferation of adherent MM cells. Taken together, these observations show that PTK787 acts directly on MM cells and in the BM milieu, providing the framework for derived clinical trials of PTK787 to improve patient outcome in MM.

MATERIALS AND METHODS

MM-derived Cell Lines and Patient Cells. RPMI 8226 and U266 human MM cell lines were obtained from the American Type Culture Collection (Rockville, MD). Dextr-sensitive MM.1S and Dextr-resistant MM.1R human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). RPMI 8226 human MM cell line resistant to Dox was kindly provided by Dr. William Dalton (Moffitt Cancer Center, Tampa, FL). ARP-1 MM cell line was a gift from Dr. Joshua Epstein (University of Arkansas for Medical Sciences, Little Rock, AK). All human MM cell lines were cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO), containing 10% FBS, 2 mM l-glutamine (Life Technologies, Inc., Grand Island, NY), 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.). Patient MM cells were purified from BM samples, as described previously (19), and were ≥95% CD38+ and 95% CD138−. PBMCs were obtained from healthy donors by subjecting peripheral blood to standard Ficoll-Hypaque (Pharmacia-Amersham, Uppsala, Sweden) gradient separation. PBMCs were stimulated by exposing cells to 5 µg/ml PHA (Sigma) and 500 units/ml IL-2 (Sigma) for 48 h in the presence or absence of PTK787.

BMSCs. BMSCs were prepared from aspirates of MM patients as well as healthy donors as described previously (11, 20). Cells were cultured in Iscove’s modified Dulbecco’s medium containing 20% FBS, 2 mM l-glutamine, and 100 µg/ml penicillin/streptomycin. HUVECs. HUVEC P168 cells were purchased from Clonetics and BioWhittaker and were maintained in EGM-2MV medium (Clonetics and BioWhittaker). PTK787/ZK222584 VEGFR Inhibitor. PTK787/ZK222584 (PTK787) was developed as a joint venture between Novartis AG (Basel, Switzerland) and Schering AG (Berlin, Germany) and is provided by Novartis AG. The compound was dissolved in DMSO (Sigma) and stored as a 100 mM stock solution at −20°C until use. For all assays, the compound was diluted in culture medium to concentrations ranging from 0.01 to 100 µM. The concentration of DMSO was diluted to 0.1% for all assays.

Detection of VEGFRs (Flt-1, Flt-4, and KDR) and c-Kit by RT-PCR. Total RNA was extracted from cell lines using the RNeasy Mini kit (Qiagen, Valencia, CA). RT-PCR was performed in a thermal cycler (MJ Research, Watertown, MA) using 5 µg of total RNA, and 50 pmol each of forward and reverse primers. RNA was amplified over 30 cycles using Superscript One-Step RT-PCR with Platinum Taq (Life Technologies, Inc., Gaithersburg, MD). Primers to detect Flt-1, KDR, and Flt-4 have been used and described in previous papers (21). Primers to detect Flt-1, KDR, and Flt-4 have been used and described in previous papers (21). Primers to detect Flt-1, KDR, and Flt-4 have been used and described in previous papers (21). Primers to detect Flt-1, KDR, and Flt-4 have been used and described in previous papers (21).

VEGF Inhibitor in Myeloma

Affymetrix huGene U95A2V arrays were hybridized with biotinylated in vitro transcription products (10 µg/chip) for 16 h at 45°C using the manufacturer’s hybridization buffer. Fluidic station 400 (Affymetrix) was used for washing and staining the arrays. Because of the size-reduced hybridization features, a three-step protocol was used to enhance detection of the hybridized biotinylated RNA: incubation with streptavidin-phycocerythrin conjugate, lactoperoxidase detection anti-streptavidin goat biotinylated Ab (Vector Laboratories, Burlingame, CA), and staining again with streptavidin-phycocerythrin conjugate. The DNA chips were then analyzed using the Gene Array Scanner (Affymetrix). The excitation source was an argon ion laser, and the emission was detected by a photomultiplier tube through a 570-nm long pass filter. Digitized image data were processed using version 4.0 of the GeneChip software (Affymetrix). Image data files were processed using the DNA Chip Analyzer (24). Genes differentially modulated with a 95% confidence level were considered significant. For MM patient data, GeneChip Microarray Suite 4.0 was used, and ≥1.5-fold changes, when compared with plasma cell expression levels, were considered significant. Internal controls of housekeeping genes and test chip trial were run prior to test samples.

Immunoprecipitation and Western Blot Analysis. MM cells were starved for 12 h in RPMI with 2% FBS and then incubated for 1 h in RPMI 1640 without FBS in the presence PTK787 or DMSO control. These cells were subsequently stimulated with 100 ng/ml VEGF165, as described previously (13). Cells were then lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and a protease inhibitor mixture (Boehringer Mannheim). Lysates were either analyzed directly on SDS-PAGE gel or incubated overnight with an Ab against Flt-1, as well as protein G plus-Agarose (both from Santa Cruz Biotechnology, Santa Cruz, CA). Whole cell lysates (30 µg/lane) or immunoprecipitates were analyzed on an 8–10% SDS-PAGE gel; transferred onto Hybond C Super paper (Ammersham, Arlington Heights, IL); probed with a murine MoAb against phospho-ERK, a murine MoAb against phospho-tyrosine residues (4G10, provided by Dr. Tom Roberts, Dana-Farber Cancer Institute), or Abs against Flt-1 or ERK2 (Santa Cruz); and detected using an horseradish peroxidase-conjugated antimurine or anti rabbit Ab (both from Santa Cruz) and enhanced chemiluminescence (ECL) substrate solution (Ammersham).

Proliferation and Cell Viability Assays. MM cells were first starved for 12 h in RPMI 1640 containing 2% FBS and then plated into 96-well microtiter plates (Costar, Cambridge, MA) in the presence of drug or DMSO control. Experiments were also performed in the presence or absence of VEGF165 (50 ng/ml; R&D Systems, Minneapolis, MN). Proliferation was measured by the incorporation of [3H]thymidine (NEN Products, Boston, MA). Specifically, cells were pulsed with [3H]thymidine (0.5 µCi/well) for the last 6 h of 48-h cultures, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted using a LKB Betaplate counter (Wallac, Gaithersburg, MD). Measurement of cell viability was performed colorimetrically by MTS assay, using the CellTiter96 AQueous One Solution Reagent (Promega Corp., Madison, WI). Cells were exposed to the MTS for the last 2 h of 48-h cultures, and absorbance was measured using an ELISA plate reader (Molecular Devices Corp., Sunnyvale, CA) at an absorbance of 490 nm.

Cell Cycle Analysis. MM cells (1 × 10^6) cells) were cultured in the presence of PTK787 or DMSO control for 24, 48, and 72 h. Cells were then washed with PBS, fixed with 70% ethanol, and treated with RNase (Sigma). Cells were next stained with propidium iodide (5 µg/ml), and the cell cycle profile was determined using the M software on an Epics flow cytometer (Coulter Immunology, Hialeah, FL).

Proliferation of MM Cells in an Adhesion System. BMSCs (1 × 10^4) cells/well) were plated into 96-well microtiter plates and incubated at 37°C for 24 h in Iscove’s medium (20% FBS). MM cells were then added to the BMSC-containing wells (5 × 10^4 cells/well) in the presence of drug or DMSO control. When MM.1S cells were used, both BMSCs and MM cells were starved for 12 h in RPMI 1640 containing 2% FBS. When patient PCL cells were cultured in RPMI 1640 containing 2% FBS and then incubated for 1 h in RPMI 1640 without FBS in the presence PTK787 or DMSO control. These cells were subsequently stimulated with 100 ng/ml VEGF165, as described previously (13). Cells were then lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and a protease inhibitor mixture (Boehringer Mannheim). Lysates were either analyzed directly on SDS-PAGE gel or incubated overnight with an Ab against Flt-1, as well as protein G plus-Agarose (both from Santa Cruz Biotechnology, Santa Cruz, CA). Whole cell lysates (30 µg/lane) or immunoprecipitates were analyzed on an 8–10% SDS-PAGE gel; transferred onto Hybond C Super paper (Ammersham, Arlington Heights, IL); probed with a murine MoAb against phospho-ERK, a murine MoAb against phospho-tyrosine residues (4G10, provided by Dr. Tom Roberts, Dana-Farber Cancer Institute), or Abs against Flt-1 or ERK2 (Santa Cruz); and detected using an horseradish peroxidase-conjugated antimurine or anti rabbit Ab (both from Santa Cruz) and enhanced chemiluminescence (ECL) substrate solution (Ammersham).
were used, the cocultures were performed in RPMI 1640 containing 10% FBS. BMSCs and MM cells were also cultured separately to serve as controls.  

After 48 h, proliferation and cell viability were analyzed as described above. To ensure that all cells were collected for the proliferation assay, 10× trypsin (Sigma) was added to each well 10 min before harvesting.

**Transwell Cell Migration Assay**. The migration assay was performed in a modified Boyden chamber system, as described in our previous study (13), using a 24-well plate with 8-μm pore size inserts. Before the assay, the upper and lower chambers were precoated with fibronectin (10 μg/ml). MM.1S cells were starved in 2% RPMI 1640 for 6 h and then treated with drug or DMSO control for 4 h. MM cells (2 × 10⁶ cells/ml) were then placed into the upper chamber of the transwell system. To the lower chamber was added RPMI (1% FBS), 10 ng/ml of VEGF165, or an activating MoAb to CD40 (10 ng/ml). The plates were then incubated at 37°C for 6 h, and cells in the lower chamber were then harvested. The number of viable migrated cells was gated and measured using a Beckman Coulter counter, model ZBII (Beckman Coulter).

A migration index was calculated to compare migration of cells relative to control. The migration index was defined as the percentage of live migrated cells in the sample (±drug and ±VEGF), divided by the percentage of live migrated cells in the control (no drug or VEGF).

**Measurement of Cytokine Concentrations**. Cytokine levels were measured in supernatants from the coculture system described above. VEGF and IL-6 concentrations were measured using commercially available ELISA kits (R&D Systems).

**Statistical Analysis**. Nonparametric tests and a log-linear model were used to analyze the data. This includes the Wilcoxon rank-sum test for two-group comparisons and the Joncheere-Terpstra trend test for assessing effects on proliferation and survival as well as VEGF- and CD40-induced migration of MM.1S cells over doses of PTK787. A migration index was calculated to compare migration of cells relative to control at concentrations as high as 20 μM (P = 0.99). Even when PBMCs were stimulated using PHA and IL-2, PTK787 only inhibited proliferation at high doses (>10 μM; P < 0.0001). Although [3H]thymidine uptake by BMSCs was inhibited by PTK787, the constitutive BMSC proliferation was low, and the drug effect was not significant (data not shown).

Because we have shown previously that the mitogen-activated protein kinase-ERK pathway partially mediates VEGF-induced proliferation (13), we investigated whether PTK787-associated inhibition of MM cell proliferation correlated with reduction in ERK phosphorylation. As seen in Fig. 2B, VEGF triggers phosphorylation of ERK proteins in MM.1S cells, which is inhibited by PTK787.

We next examined the cell cycle profile changes in MM.1S cells induced by PTK787. Cells were cultured for 48 h in the presence or absence of PTK787, fixed, and stained with propidium iodide. As seen in Fig. 2C, PTK787 triggers a significant decrease in cells in G₁ (66.5% versus 13.8%; P < 0.001 by Wilcoxon test), with a corresponding increase of cells in G₂-M (18.5% versus 59.1%; P < 0.001 by Wilcoxon test).

**PTK787 Inhibits VEGF-induced Migration of MM Cells**. We have shown previously that VEGF induces migration of MM cells through an extracellular matrix (13) and therefore next evaluated whether PTK787 inhibits VEGF-induced cell migration. As seen in Fig. 3, VEGF increases migration of MM.1S cells (P = 0.01), and addition of 1 μM PTK787 returned migration to control levels (P = 0.50). Higher concentrations of PTK787 (5 and 10 μM) further reduced both baseline, as well as VEGF-induced, migration of MM.1S cells (P < 0.001). We have also shown recently that CD40 activation induces migration of MM cells, as well as increased secretion of VEGF from tumor cells (25). However, in contrast to VEGF-induced migration, CD40-triggered migration did not return to control levels even at PTK787 concentrations of 10 μM (P = 0.04).

**Effect of PTK787 on Proliferation MM.1S Cells in the Presence of Dex and IL-6**. Because Dex is a principal agent for treatment of MM, we next determined whether PTK787 added to the effect of Dex on MM cells in vitro. As shown in Fig. 4A, both PTK787 and Dex significantly blocked proliferation of MM.1S cells (P < 0.0001), and their growth-inhibitory effects were additive.

IL-6 is a major growth and survival factor for MM cells and can protect MM cells against Dex-induced apoptosis. We therefore next examined whether PTK787 could overcome these effects. MM.1S cells were incubated for 24 h with VEGF (50 ng/ml), in the presence or absence of Dex (0.1 or 1 μM), PTK (5 μM), or IL-6 (20 and 100 ng/ml; Fig. 4B). Dex and PTK787 significantly inhibited proliferation of MM.1S cells (assessed by [3H]thymidine uptake at 24 h;
P < 0.001, respectively) and increased cell death (evidenced by MTS assay; P = 0.001 and P = 0.025, respectively). IL-6 increases MM.1S proliferation significantly, compared with a DMSO control (P < 0.001), and protected MM.1S cells against Dex (survival without IL-6 versus with IL-6; P < 0.001). Interestingly, the addition of IL-6 to cells already exposed to PTK787 further inhibited MM.1S proliferation (P < 0.001) and increased cell death (P < 0.003). Addition-ally, the combination of PTK787 and Dex totally abrogated the ability of IL-6 to promote growth and to protect against Dex-induced apoptosis in MM.1S cells (P = 0.33 for proliferation and P = 0.5 for survival). These effects were noted at Dex concentrations ranging from 0.1 to 1 μM. Moreover, even high (100 ng/ml) concentrations of IL-6 did not rescue MM.1S cells from growth inhibition by the combination of Dex and PTK. Importantly, PTK787 also similarly inhibited proliferation and survival of Dex-resistant MM.1R cells (Fig. 4C); in contrast to MM.1S cells, Dex did not enhance this effect.

Effect of PTK787 on Proliferation of MM.1S Cells Cultured with BMSCs. We have demonstrated previously that adherence of MM cells to BMSCs induces nuclear factor-κB-dependent transcription and secretion of IL-6 (in BMSCs), with an associated proliferation of adherent MM cells (26). Moreover, we have also shown that TNF-α, transforming growth factor-β, and VEGF secreted by MM cells up-regulated IL-6 secretion from BMSCs, with related increased MM cell proliferation (2, 26–28). To examine the effect of PTK787 on paracrine MM cell growth, we first determined whether PTK787 inhibited proliferation of MM cells adherent to BMSCs. Coculture of MM.1S cells and patient PCL cells with BMSCs increased the growth of tumor cells 1.8–2.8-fold (Fig. 5A). Importantly, PTK (5 μM) decreased proliferation, even of MM.1S cells and patient PCL cells adherent to BMSCs (P < 0.001 for both MM.1S and PCL cells). These effects were observed whether the BMSCs were derived from a normal donor or patient BM (data not shown).

We next evaluated whether PTK787 abrogated the increased secretion of IL-6 in BMSCs triggered by MM cell adhesion. IL-6 levels were measured using an ELISA assay in supernatants from 48-h cultures of MM.1S cells with BMSCs. As seen in Fig. 5B, IL-6 secretion increased in cultures of MM cells and BMSCs compared with cultures of BMSCs alone (P < 0.001 at PTK = 0 and 1 μM, respectively).
P = 0.02 at PTK = 5 μM, and P = 0.04 at PTK = 10 μM). IL-6 secretion from MM.1S cells was below detectable limits (data not shown). Importantly, the addition of PTK787 blocked the incremental increase in IL-6 secretion in cultures of MM cells adherent to BMSCs (P < 0.001), correlating with its ability to inhibit proliferation even of adherent MM cells. Viability of BMSCs remained high (95%), assessed by both trypan blue exclusion and MTS assay, suggesting that PTK787 reduces secretion of IL-6 without killing BMSCs (data not shown). As with IL-6, PTK787 also inhibited the increased VEGF secretion (P = 0.001) triggered by adherence of MM.1S cells to BMSCs (Fig. 5B, right panel).

DISCUSSION

The role of VEGF in MM cell growth has only been defined recently. We and others have previously delineated mechanisms of interaction between MM cells and the BM microenvironment, as well as the biological sequelae promoting MM cell growth, survival, and drug resistance. Specifically, binding of MM cells to BMSCs triggers production of both VEGF and IL-6 from BMSCs (11, 26). In addition, IL-6 production by BMSCs is also induced by VEGF, transforming growth factor-β, and TNF-α secreted by MM cells; IL-6, in turn, triggers VEGF production (12, 13, 28, 29). The principal source of IL-6 appears to be BMSCs, whereas VEGF is secreted from both BMSCs and MM cells. These studies suggest that paracrine mechanisms involving both IL-6 and VEGF play an important role in MM cell growth and survival.

Although BMSCs have been reported previously to express VEGFRs (12), the expression of these receptors on MM cells appears to be variable. Previous reports have not detected Flt-1 and KDR expression in MM cell lines (12, 30). However, we have shown recently that VEGFR-1 (Flt-1), but not KDR, is expressed on the MM.1S cell line as well as on some patient MM cells (13). In MM cells expressing Flt-1, VEGF induces proliferation via the MEK-1/ERK pathway and migration across an extracellular matrix through a PKC-dependent, ERK-independent pathway. The ability of VEGF to
bars, Migrated cells were counted on a Beckman Coulter counter, and a migration index was calculated. A lower chamber without any VEGF or CD40 Abs to CD40 (10 ng/ml), and cells were then allowed to migrate to the lower chamber. To the lower wells were added either VEGF (10 ng/ml) or activating factors, the ligand for c-Kit, does not increase proliferation of MM cells. We cannot rule out the possibility that PTK787 partially acts both on BMSCs and on Flt-1-expressing MM cells to investigate the role of the VEGFR inhibitor PTK787 as a potential therapy for patients with MM.

We first investigated the expression of Flt-1 RNA in various MM cells, using RT-PCR. We found that Flt-1 was significantly expressed in MM cell lines, including MM.1S, MM.1R, RPMI 8226, and ARP1, as well as tumor cells derived from MM and PCL patients. Interestingly, RPMI-Dox40, a Dox-resistant derivative of RPMI 8226 MM cells, did not express Flt-1. Whether loss of Flt-1 confers resistance of MM cells to conventional therapy or is a phenotypic marker of resistance is currently under investigation. We also probed for expression of other tyrosine kinases inhibited by PTK787. We found that all MM cells express c-Kit but lack KDR. Most MM cells do not express Flt-4.

The ubiquitous expression of c-Kit by MM cells suggested another potential target for PTK787, as well as a potential role for other c-Kit tyrosine kinase inhibitors such as STI571 (Gleevec), in inhibiting MM cell growth. We cannot rule out the possibility that PTK787 partially acts through inhibition of c-Kit. However, we have performed in vitro studies using Gleevec on our MM cells and have found Gleevec to be less effective than PTK787 in inhibiting cell growth. We have not been able to detect c-Kit by Western blot; and stem cell factor, the ligand for c-Kit, does not increase proliferation of MM cells (data not shown). Gene array analysis of MM patient samples also indicates that Flt-1, but not c-Kit, is overexpressed in MM cells. Together, these results favor Flt-1, rather than c-Kit, as a target for PTK787.

We next looked at the direct effects of PTK787 on VEGF-induced sequelae in MM cells. To confirm that Flt-1 was functional on MM cells, we showed that PTK787 inhibits VEGF-induced tyrosine phosphorylation of Flt-1. We found that PTK787 (1–5 μM) inhibited proliferation in MM cell lines and that this decrease in proliferation was correlated (in MM.1S cells) with reduction in downstream ERK phosphorylation. Interestingly, the one cell line that lacked Flt-1 (RPMI-Dox40) had a higher IC₅₀ (>10 μM) than Flt-1-expressing MM cell lines. Importantly, our studies show that PTK787 abrogates proliferation both in the presence or absence of VEGF, which may reflect a low level of constitutive activity by Flt-1. Because we have shown previously that VEGF induces migration of MM cells through an extracellular matrix via a PKC pathway (13, 14), we also investigated the effect of PTK787 on VEGF-induced MM cell migration. Importantly, VEGF-induced migration of MM.1S cells is decreased to baseline levels by 1 μM PTK787. Our demonstration that CD40-induced migration of MM.1S cells is not similarly affected by PTK787 suggests that PTK787 is specific for VEGFR, and that CD40-induced migration occurs via a mechanism that does not involve VEGF.

Having shown direct effects of PTK787 on MM cells, we next investigated whether its effects on MM cells could be enhanced by conventional anti-MM therapies such as Dex. We found that Dex and PTK787 inhibited proliferation of MM.1S cells, and that their inhibitory effects were additive. Because we and others have shown that IL-6 stimulates growth and survival of MM cells (31–33), we investigated whether PTK787 inhibited the MM cell proliferation triggered by exogenous IL-6. Importantly, PTK787 overcame the stimulatory effects of IL-6 on MM cell growth. Moreover, PTK787 overcame the protective effect of IL-6 against Dex-induced apoptosis, even at high (100 ng/ml) IL-6 concentrations. We have shown previously that IL-6 confered resistance to Dex via activation of SHP2, a protein phosphatase, and phosphatidylinositol 3-kinase/AKT signaling (34, 35).
and are now assessing whether VEGF acts in a similar manner. These data further suggest that a combination of Dex and PTK787 may have enhanced anti-MM clinical activity.

The effect of the BM microenvironment on MM cell growth is well established, and we therefore next investigated the effect of PTK787 on paracrine growth of MM cells in the BM milieu. We first examined the effect of PTK787 on IL-6 and VEGF production in a coculture system of MM cells and BMSCs and demonstrated a significant reduction of both IL-6 and VEGF production in the presence of PTK787. Importantly, PTK787 abrogated the increase in proliferation of MM cells when cocultured with BMSCs. The significance of these findings is multifold: (a) PTK787 can interrupt the paracrine interaction between MM cells and BMSCs through down-regulation of both IL-6 and VEGF secretion. Reduced levels of IL-6 result in reduced proliferation of MM cells and an increased susceptibility to Dex-induced apoptosis (36). The fact that PTK787 inhibits MM cell proliferation in a coculture system suggests that it can overcome protection conferred by adherence of MM cells to BMSCs; (c) PTK787 may improve dendritic cell antigen-presenting function in MM.

In summary, we have shown that PTK787 at clinically achievable concentrations acts both directly on MM cells and in the BM milieu to inhibit MM cell growth and survival and overcome drug resistance. These observations, coupled with its lack of major toxicity in preclinical mouse models at serum levels ≥10 μM, provide the framework for derived clinical trials of PTK787 in MM patients.

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