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ORIGINAL ARTICLE

The orally available multikinase inhibitor regorafenib (BAY 73-4506) in multiple myeloma

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Abstract

A promising approach to the treatment of multiple myeloma (MM) involves agents that target not only the myeloma cells directly, but also the tumor microenvironment which promotes tumor cell growth, angiogenesis, and MM bone disease. Here we investigate the orally available multikinase inhibitor, regorafenib (BAY 73-4506), for its therapeutic efficacy in MM. Regorafenib is a potent inhibitor of angiogenic (VEGFR 1-3, PDGFR-b) as well as oncogenic (c-KIT, RET, FGFR, Raf) kinases. We show that regorafenib induces apoptosis in all MM cell lines at below clinically achievable concentrations. Regorafenib overcomes the growth advantage conferred by a stroma cell MM and an endothelial cell MM, co-culture systems, and abrogates growth factor-stimulated MEK, ERK, and AKT phosphorylation at nanomolar to micromolar concentrations. Moreover, it inhibits endothelial cell growth and tubule formation, abrogates both VEGF secretion and VEGF-induced MM cell migration, inhibits osteoclastogenesis, and shows synergistic cytotoxicity with dexamethasone, the immunomodulatory drug pomalidomide, and the p110δ inhibitor idelalisib. Most importantly, regorafenib significantly delays tumor growth in a xenograft mouse model of human MM. These results provide the rationale for further clinical evaluation of regorafenib, alone and in combination, in the treatment of MM.

Keywords Multiple myeloma · Regorafenib · Cell proliferation · Antiangiogenesis effects · Osteoclasts

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Introduction

Multiple myeloma (MM) is the second most common hematological malignancy and is characterized by the malignant transformation of plasma cells within the bone marrow. Although novel drugs targeting MM cells and their bone marrow (BM) microenvironment have shown promising clinical results and significantly improved progression-free (PFS) and overall survival (OS), new treatment modalities are urgently needed [1, 2].

The interactions between MM cells and the BM microenvironment are mediated through both direct and indirect mechanisms: direct contact induces increased angiogenesis, tumor growth, survival, and drug resistance, and is regulated by autocrine and paracrine loops; the indirect pathway involves cytokines such as vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and insulin-like growth factor 1 (IGF1) produced and secreted in the BM microenvironment. The Ras/Raf/MEK/ERK, JAK/STAT3, phosphoinositol-3-kinase (PI3K)/AKT, and NFκB pathways integrate the downstream signaling of these cytokines [2] mediating cell cycle

progression, enhanced cell motility, and inactivation of pro-apoptotic pathways, highlighting the importance of targeting MM cells in their BM milieu [3–6].

Regorafenib is an orally available diphenylurea inhibitor of multiple kinases including angiogenic (VEGFR 1-3, PDGFR-b, TIE2) as well as oncogenic (c-KIT, RET, FGFR, Raf-1, BRAF) kinases [7]. The efficacy of regorafenib in solid tumors has been demonstrated in various mouse models [7], and numerous clinical trials have shown a broad spectrum of antitumor activity in solid tumors. The favorable results of the CORRECT phase III clinical trial led to regulatory approval of regorafenib in the USA and Europe for treatment of metastatic colorectal cancer (CRC) resistant to standard therapy [8]. Moreover, the approval of regorafenib for second-line treatment of refractory hepatocellular carcinoma (HCC) is anticipated based upon the recent results of the RESORCE phase III clinical trial showing that regorafenib improved OS in previously treated patients with unresectable cancer [9]. In soft tissue sarcoma, regorafenib showed promising antitumor activity, leading to an improvement in progression-free survival [10]. To date, regorafenib has not been investigated in hematologic malignancies; in this study, we characterize in vitro and in vivo activity of regorafenib in MM.

Materials and methods

Materials

Regorafenib was provided by Bayer HealthCare Pharmaceuticals (Montville, NJ). Antibodies against p-MEK1/2 (Ser217/221), p-p38 (Thr180/Tyr182), p-STAT3 (Tyr705), STAT3 and p-AKT (Thr308), caspase 3 and 9, Mcl-1, Bcl-2, c-Myc, CHOP, PARP, p-eIF2alpha (Ser51), and IRE1alpha were obtained from Cell Signaling Technology (Beverly, MA); antibodies against p-ERK (Thr981) and ERK-2 were from Santa Cruz Biotechnologies (Santa Cruz, CA). CAL-101 (GS-1101), melphalan, carfilzomib, and vorinostat were purchased from Selleck Chemicals (Houston, TX).

Cell cultures

All human MM cell lines were purchased from ATCC (Manassas, VA, USA) (KMS12 PE, KMS12 BM, U266, NCI-H929, RPMI-8226, OPM-1, OPM-2, S6B45, KMS11, LR5, and Dox40). MM1.S and MM1.R were established by S. Rosen, and INA-6 was originally provided by M. Gramatzki and R. Burger. Cells lines were cultured as previously described [11]. Human umbilical vein endothelial cells (HUVECs) (ATCC, Manassas, VA, USA) were maintained in EGM-2MV media (Clonetics BioWhittaker, Walkersville, MD) containing 2% fetal bovine serum (FBS). Bone marrow stromal cell cultures (BMSCs) and bone marrow plasma cells (BMPCs) were derived from relapsed/newly diagnosed MM patients (Jerome Lipper Multiple Myeloma Center, Harvard University, Boston, MA, USA). Written informed consent of MM patients was obtained with approval of the institutional ethics committee according to the Declaration of Helsinki. BMSCs were cultured in RPMI and 20% FBS after separation of mononuclear cells via Ficoll-Paque gradient. BMPCs had been purified by CD138 magnetic bead-activated cell sorting. BMSCs/BMPCs were cultured in 96-well plates (0.5×10^4 cells/cm^2). Medium was changed twice weekly. Supernatant of co-cultures was collected and stored at − 80 °C. All cell lines are regularly authenticated by fingerprinting before backup freezing and are kept less than 4 months in culture, as previously described [12].

Cytotoxicity and cell proliferation assays

The cytotoxic effects of regorafenib, CAL-101, melphalan, and carfilzomib on MM cells after incubation for 48 h was assessed using the MTT assay, as previously described [11]. Cell survival was estimated as the percentage of the value of untreated controls. Cell proliferation was assessed by measuring [^{3}H]-thymidine uptake, as previously described [13]. MM cells were cultured with or without BMSCs or HUVECs and treated with control media or with regorafenib. Proliferation was measured after 24 h. [^{3}H]-thymidine was added during the last 8 h of incubation.

DNA fragmentation assay

Induction of apoptosis was assessed by a DNA fragmentation assay (Cell Death ELISA, Roche, Indianapolis, IN) according to the manufacturer's instructions.

Transwell migration assay

Growth factor-deprived MM.1S cells in increasing concentrations of regorafenib were stimulated for migration by exposure (4 h) to VEGF (10 ng/ml) (+ fibronectin, 10 μg/ml), added to the lower chamber of a modified Boyden chamber, as previously described [11]. VEGF was purchased from R&D Systems (Minneapolis, MN, USA). Human plasma fibronectin was obtained from Invitrogen (Massachusetts, MA, USA).

In vitro angiogenesis assay

The antiangiogenic properties of regorafenib were evaluated using an in vitro angiogenesis assay kit (Chemicon, Temecula, CA), according to the manufacturer's instructions. For tubule formation assay, HUVECs were pre-mixed with different concentrations of regorafenib in EGM-2 and added on top of the ECMatrix™. Tubule formation was evaluated using an

inverted light fluorescence microscope at ×4 to ×10 magnification (Olympus, Lake Success, NY). Photographs are representative of each group of three independent experiments.

Cell lysis and western blotting

Cell lysis and western blot analysis were done as described in prior studies [13].

Osteoclast formation assay

Osteoclasts (OCLs) were generated in vitro using peripheral blood mononuclear cells (PBMCs) from MM patients. For OCL formation assays, PBMCs were separated by Ficoll-Paque gradient, and non-adherent cells were cultured in 6- or 96-well plates (0.5×10^6 cells/cm^2), as previously described [14, 15]. OCLs were generated by culturing cells for 14–21 days in α-MEM containing 10% FBS, 1% penicillin-streptomycin (Mediatech Inc., Herndon, VA), and 25 ng/ml of macrophage colony-stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN) and RANKL (50 ng/ml) (PeproTech, Rocky Hill, NJ). After 2 weeks of incubation, OCLs in both the control and treated groups were fixed with citrate-acetone solution and stained for tartrate-resistant acid phosphatase (TRAP) using an acid phosphatase leukocyte staining kit (Sigma Chemical, Saint Louis, MO, USA). TRAP-positive OCLs containing three or more nuclei per cell were enumerated using an inverted microscope. Images were obtained using a Leica DM IL microscope (Leica Microsystems, Wetzlar, Germany), and were acquired through IM50 software (Leica Microsystems Imaging Solutions, Cambridge, UK).

Xenograft mouse model

To determine the in vivo anti-MM activity of regorafenib, beige-nude Xid mice (Jackson Laboratory, Bar Harbor, ME, USA) were inoculated subcutaneously with 3×10^6 MM1.S cells in 100 μl RPMI 1640 medium together with 100 μl Matrigel (Becton Dickinson Biosciences, Bedford, MA). Treatment by oral gavage with vehicle alone or 10 or 30 mg/kg BAY was started when tumors were measurable, after assigning mice into treatment or control groups ($n = 6$ per group). For administration to mice, regorafenib was formulated as a solution in PEG400/125 mM aqueous methanesulfonic acid (80/20) and given daily by oral gavage. The control group received the carrier alone at the same schedule and using the same route of administration. Tumor burden was assessed every alternate day using a caliper (calculated volume $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$), and body weight was evaluated three times a week. Mice were sacrificed at a tumor size of 2 cm in diameter, or when the mice became moribund. Survival was evaluated from the first day of treatment until death. All animal studies were approved by the Dana-Farber Animal Care and Use Committee.

Statistical analysis

Statistical differences in the measurements between the regorafenib-treated and control mice were determined using an unpaired Student *t* test. The threshold for significance was *P* less than 0.05. The combinatorial effects achieved using regorafenib with other drugs were analyzed using the CalcuSyn 2.1 software.

Results

Regorafenib inhibits proliferation and survival of MM cell lines and patient cells

We first examined the ability of regorafenib to suppress MM cell proliferation (KMS12 BM, RPMI 8226, OPM1, OPM2, MM1.R, DOX40, LR5, INA6, S6B45, KMS11) and survival (MM1.S, RPMI 8226, U266, KMS11, OPM2, NCI-H929, S6B45, MM1.R, LR5, DOX40) in MM cell lines and BMPCs ($n = 4$). Cells were cultured with control media or with regorafenib at indicated concentrations ranging from 0.5 to 20 μM. An early, dose-related effect of regorafenib on cell proliferation was detectable at 24 h (Fig. 1a). Furthermore, cell survival was markedly reduced after 48 h of exposure, with a median inhibitory concentration of around 2.5 μM (range 1–3 μM) (Fig. 1b) in all MM cell lines tested, including those resistant to conventional chemotherapies, as well as in BMPCs (Fig. 1c).

Regorafenib overcomes MM cell proliferation stimulated by the BM microenvironment

Given the protective effects of the tumor microenvironment against MM cytotoxicity of various agents, we next investigated whether regorafenib can overcome this effect. Tumor cells in the microenvironment were stimulated in vitro by co-culturing either BMSCs or HUVECs with either MM1.S or KMS11 cells. Although BMSCs and HUVECs stimulated the growth of MM cells, regorafenib effectively blocked this proliferative response in a dose-dependent manner (Fig. 2a–d), as assessed by [^{3}H]-thymidine uptake. The mean effective inhibitory concentrations of regorafenib in the BMSC/MM cell co-culture system (BMSC/MM1.S $EC_{50} = 3.6$ μM; BMSC/KMS11 $EC_{50} = 2.9$ μM; $n = 4$, respectively) were comparable to those observed in the absence of BMSCs, indicating that

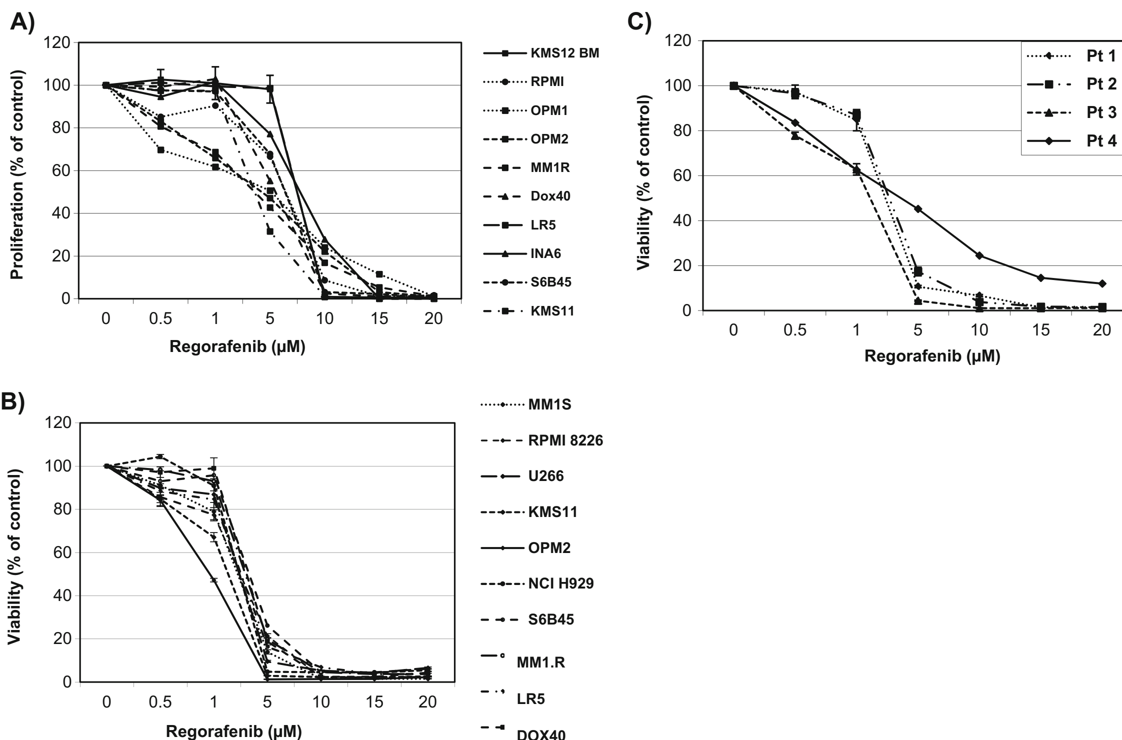

Fig. 1 Dose- related effect of regorafenib on viability and proliferation of MM cell lines and patient cells. To evaluate the effect of regorafenib on cell proliferation (**a**) and viability (**b, c**), MM cell lines, MM cell lines resistant to conventional chemotherapeutics, and MM patient cells were cultured with control media or with the compound at indicated concentrations. Cell viability was assessed using MTT colorimetric assay after 48 h, cell proliferation was measured using [^{3}H] thymidine- uptake after 24 h. Results were estimated as percentage of the value of untreated controls. Data shown are mean +/− SD of experiments performed in triplicate

regorafenib can abrogate the protective effect of the MM microenvironment. Furthermore, the mean inhibitory concentration required in the HUVEC/MM cell combination appeared even lower (HUVEC/MM1.S EC_{50} =

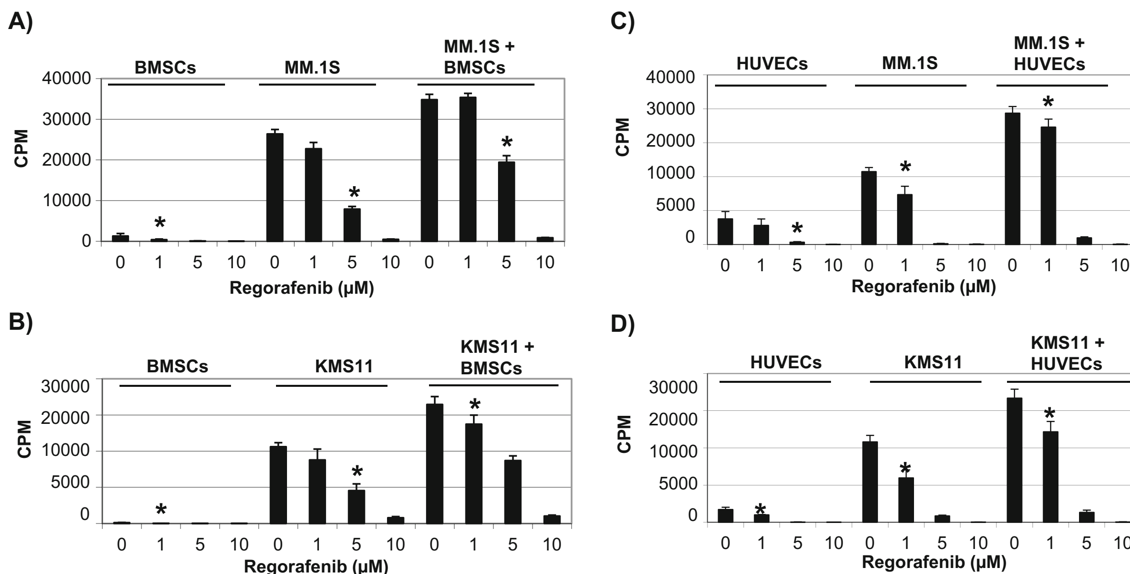

Fig. 2 Regorafenib inhibits proliferation of MM cells adherent to BMSCs. (a, b) MM cell lines were cultured with or without BMSCs or **(c, d)** HUVECs. Cells were treated with control media or with the indicated concentrations of regorafenib, and proliferation was measured using [^{3}H] thymidine- uptake after 48 h. Data shown are mean +/− SD of experiments performed in triplicate. *$P < 0.005$

1.1 μM; HUVEC/KMS11 EC_{50} = 1.1 μM; n = 4, respectively).

Regorafenib triggers antiangiogenic activity by suppressing VEGF secretion, VEGF-induced tubule formation, and migration

Given the importance of VEGF in MM cell survival and progression as well as in angiogenesis, we next investigated the effects of regorafenib on VEGF secretion and VEGF-induced migration and tubule formation in the BMSC/MM cell co-culture system. To assess VEGF-induced migration, we seeded growth factor-deprived MM1.S cells into a modified Boyden Chamber with media supplemented by VEGF at different concentrations (0, 3, 5, 10 ng/ml). Treatment with regorafenib for 4 h significantly inhibited MM cell migration in a dose-dependent manner (*$P < 0.005$) (Fig. 3a). To evaluate BMSC-induced VEGF secretion, MM cells (MM1.S, KMS11) were seeded alone or in combination with BMSCs, with or without regorafenib at concentrations from 1 to 10 μM. Analyzing cell culture supernatants showed that regorafenib significantly inhibited VEGF secretion in a dose-dependent manner (*$P < 0.005$) (Fig. 3b, c). To evaluate the direct inhibition of angiogenesis by regorafenib, we assessed tubule formation by endothelial cells on Matrigel. Our results show that regorafenib blocks endothelial cell tubule formation in a dose-dependent manner (Fig. 3d), starting at concentrations less than 1 μM (Fig. 3e) (*$P < 0.005$, **$P < 0.0001$). Taken together, these results demonstrate that regorafenib inhibits VEGF secretion, VEGF-induced MM cell migration triggered by MM cell interaction with the microenvironment, and endothelial cell tubule formation in vitro.

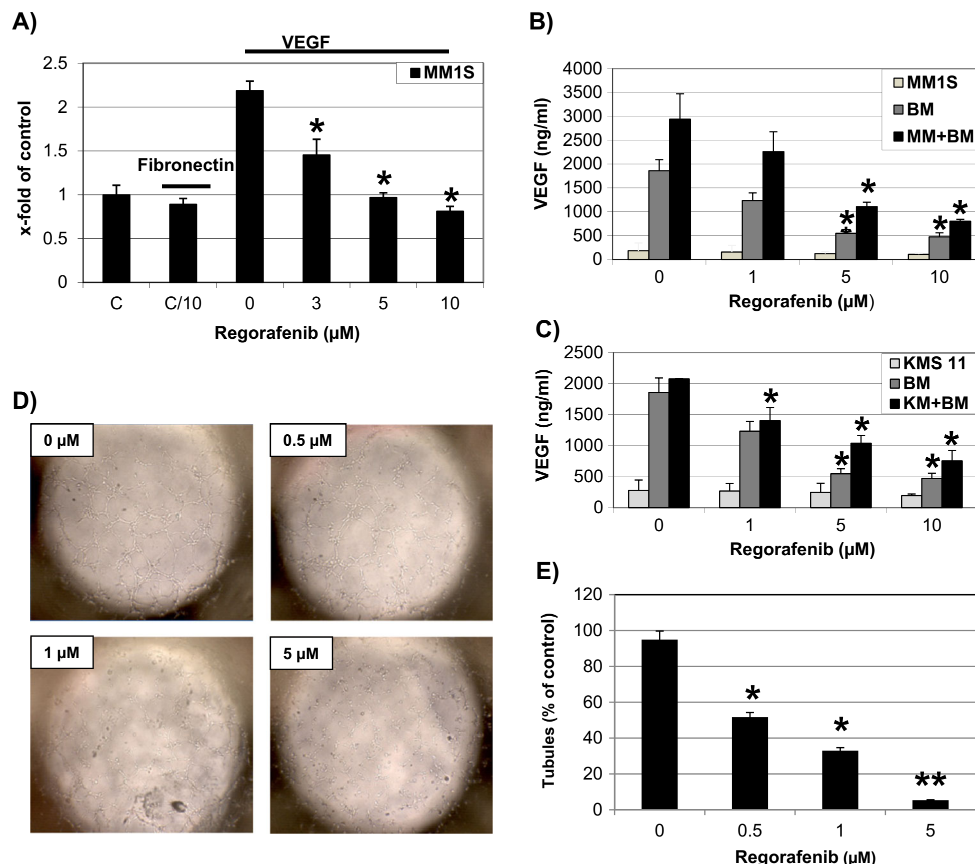

Fig. 3 Regorafenib inhibits VEGF-induced migration. (a) Growth-factor deprived MM1.S cells were stimulated for migration by exposure (4 h) to VEGF (10 ng/ml) (control +Fibronectin 0, 10 μg/ml), added to the lower chamber of a modified Boyden chamber. Treatment with the indicated concentrations of regorafenib (0, 3, 5, 10 μM) efficiently inhibits the migration induced by VEGF, measured by in vitroangiogenesis assay. Data shown are mean +/− SD of experiments performed in triplicate. *$P < 0.005$. **(b, c) Regorafenib inhibits BMSC-induced VEGF-secretion.** In MM1.S or KMS11, BM and MM/BM co-cultures systems, regorafenib efficiently inhibits VEGF-secretion, measured from supernatant of cell cultures. VEGF-concentration was analyzed using an in vitroangiogenesis assay kit (Chemicon, Temecula, CA), according to manufacturer's instructions. Data shown are mean +/− SD of experiments performed in triplicate. *$P < 0.005$. **(d, e) Regorafenib inhibits endothelial tubule-formation.** HUVECs were premixed with indicated concentrations of regorafenib in EGM-2 and added on top of the ECMatrix™. Tubule formation was evaluated and enumerated using an inverted light fluorescence microscope at ×4 to ×10 magnification. Photographs are representative of each group of 3 independent experiments. Complete tubular ring formations are shown as mean +/− SD of experiments performed in triplicate. *$P < 0.005$, **$P < 0.0001$

Signaling inhibition by regorafenib

To characterize the molecular mechanisms underlying the efficacy of regorafenib in MM, we next treated MM cells with regorafenib for 4 h, followed by cell lysis and western blotting. Regorafenib treatment resulted in profound abrogation of mitogen-activated protein kinase (MAPK) signaling, evidenced by dephosphorylation of MEK and ERK in a dose-dependent manner (Fig. 4a). Furthermore, the stimulatory effect of VEGF/ fibronectin on MEK/ERK and the MAPK-p38-signaling pathway was blocked by regorafenib at micromolar concentrations (Fig. 4b). Importantly, regorafenib inhibited IL-6-induced signaling cascades including STAT3, AKT, and MEK/ERK (Fig. 4c). We further observed apoptosis induced after treatment with regorafenib at different concentrations evidenced by an increase of caspase 3 and PARP cleavage (suppl. Fig. 1). These results suggest that regorafenib inhibits pathways mediating growth, survival, and drug resistance in MM.

Regorafenib inhibits osteoclastogenesis

Osteolytic bone disease in MM is caused by enhanced OCL activation and inhibition of osteoblast function. Here, we assessed the effect of regorafenib on osteoclastogenesis. Incubation with regorafenib resulted in a significant dose-dependent decrease of multinucleated TRAP-positive cells (**$P < 0.0001$) (Fig. 5a, b). To exclude non-specific drug toxicity on PBMCs or monocytic precursors as well as both early and differentiated OCLs, we cultured PBMCs in the presence of RANKL and M-CSF for 1, 8, and 14 days. Regorafenib was added for 72 h at indicated concentrations on days 1, 8, and 14. Assessment of cell survival showed that regorafenib did not induce non-specific short-term toxicity on PBMC or OCL cultures at various stages of differentiation (Fig. 5c). The lack of unspecific toxicity against non-malignant cell types indicates a therapeutic window. In addition, these results indicate that regorafenib inhibits osteoclastogenesis, as evidenced by blockade of the M-CSF/RANKL-triggered differentiation of mononuclear cells into TRAP-positive OCLs.

Regorafenib delays tumor growth in a xenograft mouse model

We next assessed whether regorafenib also abrogates tumor growth in vivo using beige-nude Xid mice inoculated with MM cell line (MM1.S cells). Regorafenib treatment by daily oral gavage was started when tumors were measurable, tumor burden was assessed every alternate day, and body weight was evaluated three times each week.

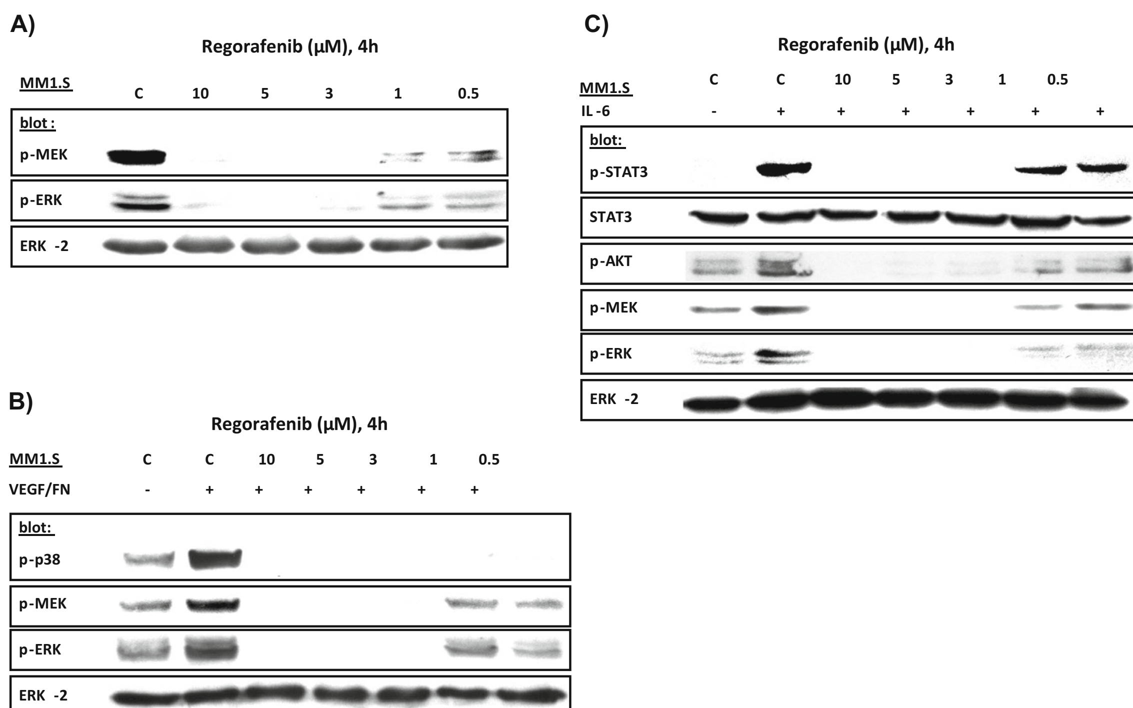

Fig. 4 Regorafenib inhibits cell signaling in MM cells. (**a-c**) MM cells were exposed to increasing concentrations of regorafenib for 4 h, followed by immunoblot analysis of whole cell lysates with indicated antibodies. **(b, c)** MM cells were stimulated with indicated cytokines for 1 h prior to exposure to regorafenib

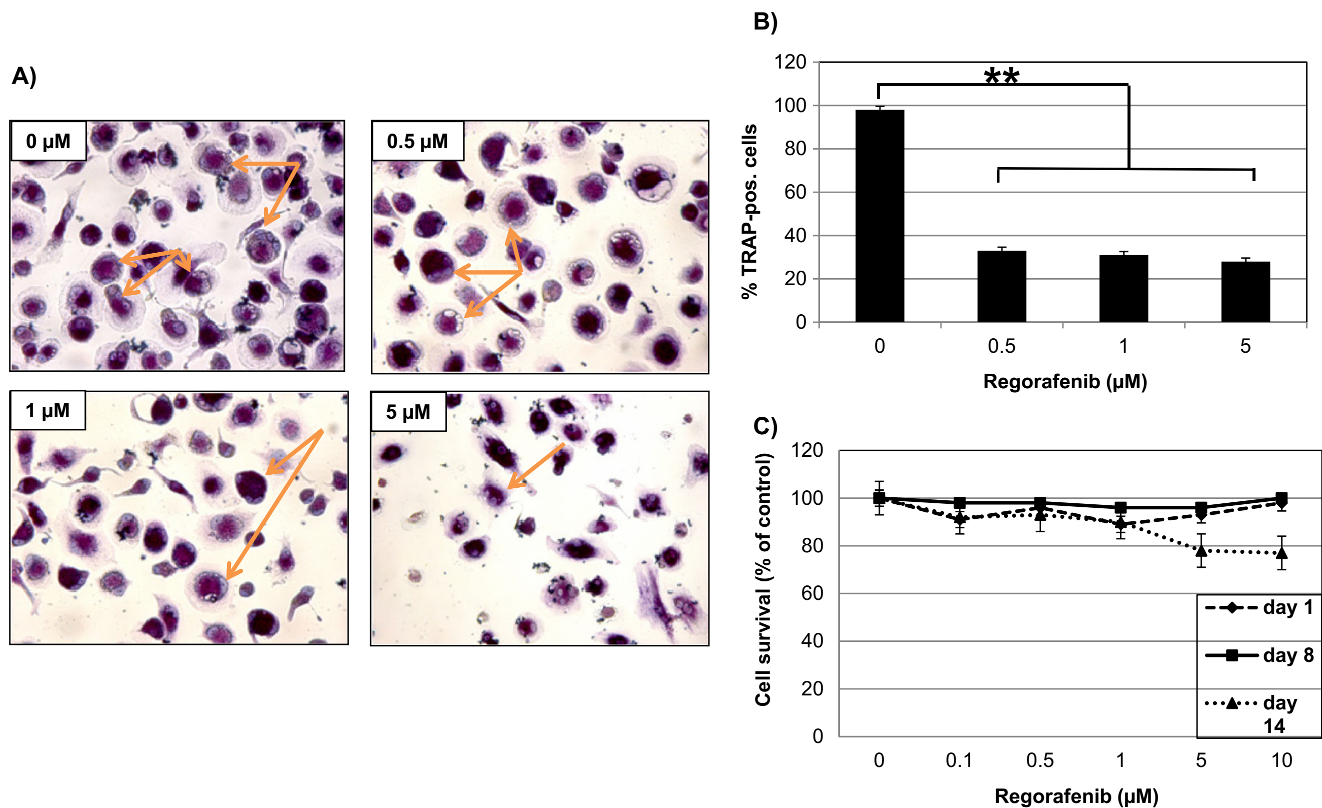

Fig. 5 **Regorafenib inhibits osteoclast differentiation. (a, b)** PBMCs were cultured with regorafenib in the presence of RANKL (50 ng/ml) and M-CSF (25 ng/ml) for 14 days. TRAP staining was performed, and TRAP-positive OCLs containing three or more nuclei per cell were enumerated using an inverted microscope. Incubation with regorafenib resulted in a dose-dependent decrease in multinucleated TRAP-positive cells. Photographs are representative of each group of three independent experiments. Arrows show examples of multinucleated cells. Data shown are mean +/− SD of experiments performed in triplicate. $^{**}P < 0.0001$. **(c) Regorafenib exhibits only marginal short-term toxicity on osteoclast cultures.** PBMCs were cultured in the presence of RANKL and M-CSF for 1, 8 and 14 days. Regorafenib was added at day 1, 8 and 14 for 72 h at indicated concentrations to evaluate toxicity of regorafenib on monocytic precursors, pre- and differentiated osteoclasts. After 4 h of incubation, cell survival was assessed by MTT-assay. Data shown are mean +/− SD of experiments performed in triplicate

Our analysis showed a significant delay in tumor cell growth at doses of 3 and 10 mg compared to the control group ($^{**}P < 0.0001$, respectively) (Fig. 6a). Body weight remained stable in all groups over the course of treatment (Fig. 6b) while there was a trend to longer survival of treated mice compared to the control group ($P = 0.06$) (Fig. 6c). Therefore, our results indicate that regorafenib abrogates tumor cell growth in vivo in a MM xenograft model.

Regorafenib triggers synergistic and additive cytotoxicity

Finally, we examined the combinatorial effects of low doses of regorafenib (1 and 2 μM) with standard-of-care compounds, such as the proteasome inhibitor carfilzomib (0.002 and 0.004 μM), the immunomodulatory drug pomalidomide (0.2 and 0.4 μM), and the corticosteroid dexamethasone (0.01 and 0.02 μM), in MM1.S cells. According to the classification proposed by Chou and Talalay et al. [16–18], carfilzomib showed dose-dependent synergistic inhibitory activity at 0.004 μM, but only additive or even moderate antagonistic effects at 0.002 μM of carfilzomib (Table 1 (a)), while combinations with pomalidomide (Table 1 (b)) or dexamethasone (Table 1 (c)) both showed synergistic efficacy.

To test for simultaneous inhibition of both the PI3K/AKT and MEK/ERK signaling cascades, we assessed the combination of regorafenib with the phosphoinositol-3-kinase inhibitor CAL-101 in MM1.S. Interestingly, synergistic effects were seen with regorafenib (1 and 2 μM) in combination with CAL-101 (idelalisib, 5 and 20 μM) (Table 1 (d)), associated with a complete abrogation of both signaling pathways (Suppl. Fig. 2). This combination was further validated in U266 cells, a cell line known to harbor an activating BRAF mutation (p.K601N) with sensitivity to BRAF inhibition by dabrafenib. The pan-RAF inhibitor regorafenib showed synergistic activity with the PI3K inhibitor CAL-101 in U266 cells. These results suggest that CAL-101, pomalidomide,

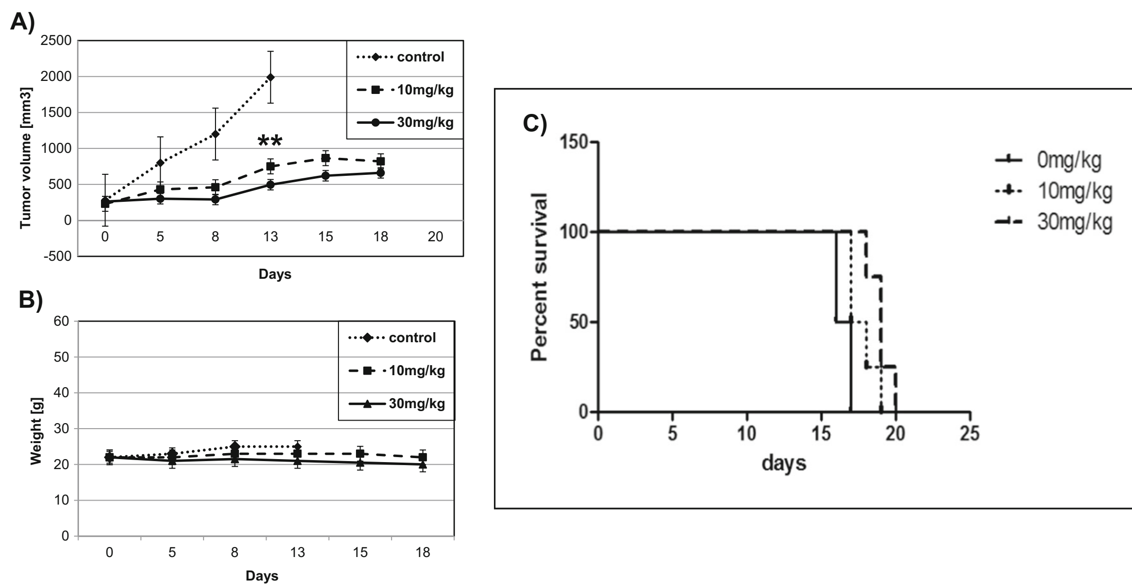

Fig. 6 Regorafenib inhibits MM tumor cell growth in vivo. Beige-nude Xid mice were inoculated s.c. with 3×10^6 MM1.S cells. Treatment was started when tumors were measurable, by oral gavage, solvent only, or 10 or 30 mg/kg regorafenib, respectively. **a)** Tumor burden was assessed every alternate day (**$P < 0.0001$). **b**) Body weight was evaluated 3×/week. Data shown are mean +/− SD of experiments performed in triplicate. **c)** Kaplan-Meier-blot shows a trend to longer survival of treated mice compared to control group ($P = 0.06$). Mice were sacrificed at a tumor size of 2 cm in diameter, or when mice became moribund

and dexamethasone might be useful combination partners with regorafenib.

Discussion

Mechanism of antimyeloma activity of regorafenib

The development of new therapeutic strategies in MM requires a clear understanding of disease biology and the mechanisms of action of available therapies. Interactions between MM cells and BMSCs or extracellular matrix proteins are mediated through cell surface receptors. These interactions modulate BMSC function by increasing cytokine secretion (IL-6, VEGF, IGF1, TNF-α, and others), which in turn activate distinct pathways in MM-mediating MM cell proliferation, survival, and drug resistance [19, 20]. These cytokines activate the Ras/Raf/MEK/ERK signaling pathways [20], JAK/STAT3 [21]· and p38 mitogen-activated protein kinase (MAPK), another member of the MAPK family [3, 22]. Preclinical data established that regorafenib triggers inhibitory activity on pathways (e.g., on VEGFR1-3, c-KIT, TIE-2, PDGFR-β, FGFR-1, RET, RAF-1, BRAF, and p38-MAPK) mediating cell proliferation, survival, drug resistance, and disease progression [3, 5, 6, 23]. Here we show potent inhibitory activity of regorafenib on cell growth and survival, on induction of apoptosis, and on key signaling events in a wide range of MM cell lines and BMPCs, at concentrations well below those that were achieved in patient plasma in phase I clinical trials in solid tumors. Plasma exposure of regorafenib after treatment with 160 mg/day for 21 days has shown a maximum concentration (C_{max}) of 3.450 mg h^{-1} l^{-1} (7.1 μM) [24]. We also found that regorafenib overcomes the protective effect of the bone marrow microenvironment, and that the stimulatory effect of HUVECs on MM cells may even sensitize MM cells to regorafenib.

Antiangiogenesis

VEGF is a regulator of physiologic endothelial cell growth, permeability, and migration in vitro and in vivo, and plays an essential role in MM pathogenesis. VEGF secretion is mediated through autocrine and paracrine mechanisms, and binding of MM cells to BMSCs enhances both IL-6 and VEGF secretion. VEGF triggers IL-6 secretion from BMSCs, which in turn enhances VEGF secretion by the MM cells, promoting MM cell survival, proliferation, and neovascularization of the BM [25, 26]. VEGF thereby triggers MM cell growth and migration by activating the Raf-MEK-ERK pathway [6]. Our data show that regorafenib has direct inhibitory effects on VEGF-induced MM cell migration and on VEGF secretion within a MM-BMSC co-culture. We also observed that regorafenib abrogates VEGF-triggered neovascularization by blocking endothelial tubule formation. Although regorafenib is a known inhibitor of angiogenic (VEGFR 1-3, PDGFR-b, TIE2) kinases [7], the mechanism leading to reduced VEGF secretion in the co-culture model remain to be further defined.

Table 1 Regorafenib shows synergistic and additive cytotoxicity

Regorafenib (μM)	Carfilzomib (μM)	Fa	CI
(a) MM1.S			
1	0.002	0.67	1.03
1	0.004	0.99	0.57
2	0.002	0.71	1.24
2	0.004	0.99	0.52
(b) MM1.S			
1	0.20	0.45	0.65
1	0.40	0.50	0.59
2	0.20	0.49	0.37
2	0.40	0.51	0.53
(c) MM1.S			
1	0.010	0.29	0.58
1	0.020	0.38	0.30
2	0.010	0.49	0.11
2	0.020	0.41	0.09
(d) MM1.S			
1	5	0.36	0.69
1	20	0.58	0.69
2	5	0.45	0.81
2	20	0.63	0.76
(e) U266			
1	5	0.10	0.75
1	20	0.18	0.78
2	5	0.21	0.83
2	20	0.24	0.92

MM1.S cells were treated with regorafenib (1 and 2 μM) and either (a) carfilzomib (0.002 and 0.004 μM), (b) pomalidomide (0.2 and 0.4 μM), (c) dexamethasone (0.01 and 0.02 μM), or (d) CAL-101 (5 and 20 μM) for 48 h. (e) U266 cells were treated with regorafenib (1and 2 μM) and CAL-101 (5 and 20 μM) for 48 h. Cytotoxicity was assessed by MTT assay. CI and Fa were calculated by CalcuSyn software package. CI < 0.1 = very strong synergism; CI 0.1–0.3 = strong synergism; CI 0.3–0.7 = synergism; CI 0.7–0.85 = moderate synergism; CI 0.85–0.9 = slight synergism; CI 0.9–1.1 = nearly additive; CI 1.1–1.2 = slight antagonism; CI 1.20–1.45 = moderate antagonism.

CI combination index, *Fa* affected fraction

Inhibition of osteoclastogenesis

Osteolytic bone disease remains a major source of morbidity, occurring in 70–80% of MM patients, and is associated with severe bone pain, pathologic fractures, paralysis through nerve compression, hypercalcemia, and death [27]. As previously shown, osteoclastogenesis can be blocked by novel antimyeloma agents [14, 15]. The signal transduction pathways modulating osteoclastogenesis have been extensively studied: PU.1 plays a critical role in the early determination phase of osteoclastogenesis, whereas activation of PI3K, MAPK-p38, and MAPK-MEK/ERK mediates OCL survival and differentiation [28] to multinucleated, mature OCLs. We here observe a dose-dependent inhibition of TRAP-positive, multinucleated cells in the presence of regorafenib, which may impact MM patients' quality of life by preventing the development of new osteolytic lesions.

Regorafenib in combination

Combination regimens of two or more compounds have been proven to lead to better response rates and longer survival of MM patients. We show dose-dependent combinatorial effects when regorafenib was tested together with carfilzomib, a second-generation proteasome inhibitor that strongly induces ER stress responses and that is currently approved for the treatment of relapsed MM when used in combination with dexamethasone with or without lenalidomide [29]. The moderate antagonistic effect was only seen at the lower concentration of carfilzomib. Since there are different concentrations available of carfilzomib for clinical use, choosing a higher concentration might be of advantage. Furthermore, when pomalidomide or dexamethasone was combined with regorafenib, we observed synergistic cytotoxic effects. Pomalidomide, a novel immunomodulatory drug, has recently been approved for relapsed/refractory myeloma based on results from the Nimbus trial, an international phase III clinical trial. [30] .

The PI3K/AKT pathway is involved in MM growth, survival, and drug resistance and is considered a target for the development of new drugs [31–34]. The p110δ isoform is mainly expressed in leucocytes and in most lymphoid tumors. The inhibitor of p110δ, CAL-101 (GS-1101, idelalisib), has achieved remarkable clinical response in some B-cell malignancies with manageable toxicity, and has been approved for the treatment of chronic lymphocytic leukemia [35–37]. CAL-101 is under evaluation in the treatment of MM and showed inhibitory activity on MM cell lines and patient cells via downregulation of AKT and ERK phosphorylation [38]. Remarkably, when regorafenib and CAL-101 were combined, we observed synergistic effects in MM1.S and U266 cells, simultaneously targeting both the PI3K/AKT and MEK/ERK signaling cascades, suggesting the concept of dual inhibition of compensatory survival pathways to be of potential clinical efficacy.

Clinical studies of regorafenib and analogues

Following promising pre-clinical data in solid malignancies, regorafenib had become the focus of numerous clinical trials. Its efficacy and tolerability in two large phase III trials led to the rapid approval of regorafenib for use in the treatment of metastatic CRC and metastatic GIST. First, the CORRECT trial was an international, multicenter, randomized, placebo-controlled phase III trial focused on regorafenib monotherapy in 760 patients with metastatic colorectal cancer that had

progressed after all approved standard treatments. Patients receiving regorafenib showed a significantly better median OS of 6.4 versus 5.0 months in the placebo group. The most commonly reported grade 3 and higher adverse events related to regorafenib were hand-foot reaction (83%), fatigue (17%), diarrhea (36%), hypertension (36%), and rash or desquamation (29%) [8]; second, the international, multicenter, randomized, placebo-controlled GRID phase III trial investigated the efficacy of regorafenib in advanced GIST [39]. Median PFS was significantly longer in patients receiving regorafenib (4.8 months) when compared to the placebo group (0.9 months), and side-effect profiles were comparable to those seen in the CORRECT trial. More recently, the results of a randomized phase II clinical trial of regorafenib versus placebo in advanced soft tissue sarcoma also showed promising antitumor effects [10].

Currently, regorafenib is awaiting approval following the promising results of the RESORCE trial, a multicenter clinical phase III trial for patients with HCC who progressed on treatment with sorafenib. The trial enrolled 573 patients (regorafenib = 379; placebo = 194) and reported a median OS of 10.6 versus 7.8 months with a response rate for regorafenib versus placebo of 65.2 versus 36.1%, respectively ($P < 0.001$) [9].

Conclusions

We here provide pre-clinical data on the efficacy of regorafenib in MM. Our data show that regorafenib has potent anti-MM activity and provide the basis for its clinical evaluation, as a single agent or in combination-based regimens, to improve patient outcome in MM.

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Compliance with ethical standards Written informed consent of MM patients was obtained with approval of the institutional ethics committee according to the Declaration of Helsinki.

Conflict of interest The authors declare that they have no conflicts of interest.

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