Sorafenib Has Potent Antitumor Activity against Multiple Myeloma In Vitro, Ex Vivo, and In Vivo in the 5T33MM Mouse Model

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Abstract
Multiple myeloma (MM) is a B-cell malignancy characterized by the expansion of clonal plasma blasts/plasma cells within the bone marrow that relies on multiple signaling cascades, including tyrosine kinase activated pathways, to proliferate and evade cell death. Despite emerging new treatment strategies, multiple myeloma remains at present incurable. Thus, novel approaches targeting several signaling cascades by using the multi-tyrosine kinase inhibitor (TKI), sorafenib, seem a promising treatment approach for multiple myeloma. Here, we show that sorafenib induces cell death in multiple myeloma cell lines and in CD138+ enriched primary multiple myeloma patient samples in a caspase-dependent and -independent manner. Furthermore, sorafenib has a strong antitumoral and -angiogenic activity in the 5T33MM mouse model leading to increased overall survival. Multiple myeloma cells undergo autophagy in response to sorafenib, and inhibition of this cytoprotective pathway potentiated the efficacy of this TKI. Mcl-1, a survival factor in multiple myeloma, is downregulated at the protein level by sorafenib allowing for the execution of cell death, as ectopic overexpression of this protein protects multiple myeloma cells. Concomitant targeting of Mcl-1 by sorafenib and of Bcl-2/Bcl-xL by the antagonist ABT737 improves the efficacy of sorafenib in multiple myeloma cell lines and CD138+ enriched primary cells in the presence of bone marrow stromal cells. Altogether, our data support the use of sorafenib as a novel therapeutic modality against human multiple myeloma, and its efficacy may be potentiated in combination with ABT737.

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Introduction
Multiple myeloma (MM) is a neoplastic B-cell disorder characterized by proliferation of malignant plasma cells in the bone marrow where they interact with the bone marrow microenvironment leading to skeletal destruction and angiogenesis (1).

Multiple myeloma accounts for 1% of all malignancies and 10% of all hematologic malignancies (2). Recently introduced drugs, such as thalidomide, lenalidomide, or bortezomib, in the clinic have improved survival and are therefore widely used as a first- and second-line treatment against multiple myeloma. Albeit the use of these drugs, the 5-year relative survival rate has only marginally increased and patients with multiple myeloma eventually relapse, thus further highlighting the unmet medical need for new therapeutic agents (3–5).

Multiple myeloma is characterized by the activation of multiple tyrosine kinase signaling cascades, that induce 3 main downstream pathways, the Raf/MEK/ERK1/2, the phosphoinositide 3-kinase (PI3K)/AKT, and the Jak/STAT3. One of the main downstream effects of these cascades is the inhibition of cell death, mainly by the overexpression of antiapoptotic Bcl-2 family members such as Mcl-1, Bcl-2, and Bcl-xL, as well as the downregulation of pro-apoptotic BH3-only proteins such as Bim (6–10).

Targeting of multiple activated tyrosine kinase signaling cascades with chemical inhibitors maybe a promising therapeutic strategy against multiple myeloma. Such a multi-tyrosine kinase inhibitor (TKI) is sorafenib, a novel bi-aryl urea, a type II inhibitor primarily against Raf1, VEGF receptor (VEGFR), and platelet-derived growth factor receptor...
Sorafenib Is a Potent Anticancer Agent against Multiple Myeloma

Multiple myeloma pathogenesis, proliferation, and response to therapy largely depend on the bone marrow microenvironment (13). The complex interplay between multiple myeloma cells and stromal cells involves numerous growth factors, most predominantly insulin-like growth factor (IGF)-I and interleukin (IL)-6, but also cytokines such as TNF-α, TGF-β, and VEGF, which are secreted by the multiple myeloma cells. These cytokines in turn may induce IL-6, IL-1, and PDGFβ secretion from bone marrow stromal cells (BMSC; ref. 13). Recent advances in the understanding of molecular mechanisms underlying the pathogenesis and progression of multiple myeloma indicate that the use of TKIs as single agents or used in combinatorial regimens may constitute a good therapeutic approach.

In the present study, we investigated the efficacy of sorafenib as a multiple myeloma anticancer agent in cell lines, patient samples, and mice. We have found that sorafenib is a potent inducer of caspase-dependent and -independent cell death even in the presence of BMSCs. Furthermore, we found that autophagy is cytoprotective and that the usage of autophagy inhibitors such as chloroquine enhances the cytotoxic effects of sorafenib. The efficacy of sorafenib can also be improved by the combination with the Bcl-2/Bcl-xL antagonist ABT737 in inducing cell death in the multiple myeloma cell lines and in primary multiple myeloma samples.

Materials and Methods

Antibodies and reagents

The pan-caspase inhibitor z-VAD-FMK [z-Val-Ala-Asp (OMe)-FMK] from MP Biomedicals was used at 10 μmol/L, 3-methyladenine (3-MA; Sigma-Aldrich) was used at 5 mmol/L, necrostatin1 (Sigma-Aldrich) was used at 50 μmol/L, chloroquine (Sigma-Aldrich) at 50 μmol/L, ABT737 (Active Biochem Co.) at 10 μmol/L, MG132 (Calbiochem) at 4 μmol/L, and G418 (Gibco) 600 μg/mL. Sorafenib (Bayer HealthCare) was dissolved in dimethyl sulfoxide and unless otherwise indicated, 10 μmol/L was used for the experiments.

The primary antibodies used in this study, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, cleaved caspase-3, cleaved caspase-7, cleaved-PARP, phospho-AKT (Ser473), AKT, Mcl-1, LC3 I/II, Bcl-2, phospho-Y705 STAT3, STAT3, phospho-mTOR (Ser2448), mTOR, phospho-4E-BP1(Thr 37/46), 4E-BP1, phosphor-eIF4E(Ser209), eIF4E, phospho-BAD(Ser112), and BAD, were obtained from Cell Signaling Technology; mouse Mcl-1 from Rockland; p62 from Abnova; Bim from Stressgen; Bcl-xL from Transduction Laboratories; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Abcam; β-actin from Sigma-Aldrich; active Bak (Ab-1) from Oncogene Research Products; LC3 from Abgent; active caspase-3 from BD Europe; cytochrome c from BD Pharmingen; and AIF and Tom40 from Santa Cruz Biotechnology.

Patient samples

Multiple myeloma bone marrow samples were obtained from the Karolinska University Hospital Solna, Stockholm, Sweden. Patients had a confirmed diagnosis of multiple myeloma, and all were previously untreated except patient 7 who had a relapse and 9 and 10 who were previously treated with bortezomib with no particular therapeutic benefit (Table 1). The study was approved by the regional ethics committee, and all patients gave their informed consent in accordance with the Declaration of Helsinki. Heparinized bone marrow samples were obtained and mononuclear cells separated by Ficoll-Paque Plus density sedimentation (Amersham Biosciences) were purified with EasySep Human CD138 Selection Kit according to manufacturer’s protocol (StemCell Technologies). The purified tumor cells were seeded at 1 x 10⁶ cells/mL into 12- or 48-well plates followed by immediate addition of the anti-cancer drugs.

Peripheral blood mononuclear cell isolation

One unit of buffy coat of healthy donor was purchased from Karolinska University laboratory (Karolinska Universitetslaboratori, then peripheral blood mononuclear cells (PBMC)

<table>
<thead>
<tr>
<th>No.</th>
<th>Age/Sex</th>
<th>M protein isotype</th>
<th>Disease stage, ISS</th>
<th>M component, g/L</th>
<th>Clinical status</th>
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<td>II</td>
<td>46</td>
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<tr>
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<td>67/female</td>
<td>IgG-κ</td>
<td>III</td>
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<td>Bortezomib-resistant</td>
</tr>
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</table>

Abbreviation: ISS, International Staging System.
were further purified by using Ficol-Paque Plus density sedimentation (Amersham Biosciences). A total of 2 $\times$ 10$^5$ cells were plated in each well of 12-well plates and treated with 10 $\mu$mol/L sorafenib or ABT737 for 24 and 48 hours. Subsequently, the cells were harvested and apoptosis was measured by Annexin V staining.

**Cells lines and transfections**

L88, the human BMSC line, has been previously described (14). The human multiple myeloma cell lines OPM-2, U-266, LPI, NCI-H929, Karpas 620, and RPMI-8226 were kindly provided by Dr. Brigitte Sola (Université de Caen, Caen, France). The bortezomib-sensitive RPMI-8226 cells and bortezomib-resistant 8226/7B680 cell lines were provided by Dr. Javier Naval (Universidad de Zaragoza, Saragossa, Spain). All cell lines were authenticated by the LGC standards cell line authentication service. The murine 5T33MM vitro cells were cultured as described previously (15). Cells were maintained in a 37°C, 5% CO$_2$ fully humidified incubator and were grown in RPMI-1640 (HyClone) supplemented with 10% FBS (HyClone), l-glutamate, and antibiotics penicillin/streptomycin (Gibco). In all experiments, the cells were cultured in 6- or 12-well plates 24 hours before treatment.

The pcDNA3.3 and MCL1-WT were introduced into OPM-2 and RPMI-8226 cells by DMRIE-C (Invitrogen) according to manufacturer’s instructions. Mcl-1 wild-type plasmid was produced by cloning of Mcl-1 segment of Mcl-1PGEX plasmid.

**Coculture experiments**

L88 cells were plated in 6- or 12-well plate 2 hours before putting cell culture inserts (BD Falcon) in the wells, which contained multiple myeloma cell lines. The cells were incubated together overnight before treatment.

**Assessment of apoptosis and immunostainings for flow cytometry**

Exposure of phosphatidylserine as a marker of apoptosis was assessed by Annexin V-FLUOS (Roche; ref. 8). Briefly, 2 $\times$ 10$^5$ cells per sample were collected, washed in PBS, pelleted, and re-suspended in incubation buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl, 5 mmol/L CaCl$_2$) containing 1% Annexin V and propidium iodide (PI) followed by analysis on fluorescence-activated cell sorter Calibur flow cytometer (Becton Dickinson) using the CellQuest software. To detect sorafenib changes in mitochondrial membrane potential, cell were stained with tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes Inc.) as previously described (8). To measure the pro-apoptotic activation of Bak and caspase-3, intracellular stainings were conducted as previously described and measured by flow cytometry (8).

**Immunocytochemistry**

The effect of sorafenib on multiple myeloma cell lines was analyzed by staining of mitochondria with MitoTracker (Molecular Probes Inc.) and costaining with either active Bak antibody or cytochrome c antibody as previously described (9).

**Resazurin assay**

Purified multiple myeloma cells (patients 1, 2, 7, and 8) were incubated with sorafenib alone or in combination with Bcl-2 antagonist (ABT737) for 24 and 48 hours. At harvest, 10% resazurin was added followed by incubation for 2 to 3 hours at 37°C in a humidified 5% CO$_2$ in-air atmosphere. The resazurin was excited at 530 nm, and the emitted light was measured at 590 nm. Analysis of fluorescence was conducted using Wallac Victor Multilabel Counter (Wallac). Mean was calculated from duplicate or triplicate wells and subtracted from mean of blank wells resulting in ΔFluorescence. The relative number of viable cells was expressed as percentage of untreated control and calculated as 100 × ΔFluorescence (treated wells)/ΔFluorescence (untreated wells).

**Cell-cycle analysis**

We used NucleoCounter NC-3000 from ChemoMetec to analyze cell cycle according to the manufacturer’s specifications. One million cells were harvested and washed by PBS then re-suspended in 0.5 mL solution 10 supplemented with 10 μg/mL 4’,6-diamidino-2-phenylindole (DAPI). Cells were incubated at 37°C for 5 minutes and then 0.5 mL solution 11 was added and 30 μL of suspended cells was loaded on 2-chamber slide (NC-Slide A2) and cell cycle was analyzed by using provided software.

**Mitochondria isolation**

A total of 2 $\times$ 10$^6$ cells were harvested, and mitochondria were isolated using the Mitochondria Isolation Kit (Pierce Biotechnology) according to the manufacturer’s specifications.

**Immunoblot analyses**

Cells were harvested and homogenized in radioimmunoprecipitation (RIPA) lysis buffer (10 mmol/L Tris, pH 7.2, 150 mmol/L NaCl, 1% deoxycholate, 1% Triton, 0.1% SDS, 5 mmol/L EDTA) containing complete protease inhibitor cocktail (Roche Diagnostics). After 1 hour on ice, samples were sonicated and protein quantification was carried out using a Bio-Rad protein assay. Equal amounts of soluble proteins (15–30 μg) were denatured by boiling and resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking in 5% non-fat dry milk in PBS for 1 hour and probing with a specific primary antibody and a horseradish peroxidase–conjugated secondary antibody, the protein bands were detected by chemiluminescence (Supersignal, Pierce) and X-ray film exposure (Kodak). Protein loading was normalized by using anti-GAPDH or antiactin antibodies.

**RNA extraction, cDNA, and quantitative reverse transcriptase PCR**

The RNA was extracted using Qiagen RNeasy mini kit (Qiagen) and DNase treated (Ambion Turbo DNA-free, Life Technologies). Roughly, 100 to 500 ng of DNase-treated RNA was used in the generation of cDNAs using MuMIV (Life Technologies) and a mixture of oligoD18 with nanomers (IDT technologies). Quantitative reverse transcriptase PCR quantification was carried out using the KAPA 2G SyberGreen (Kapa biosystems) on the Applied Biosystems 7900HT platform with the following conditions: 95°C for 3 minutes, 95°C
for 3 seconds, and 60 °C for 30 seconds. The following primers were used for the amplification of Mcl-1: For: 5'-AACAAA-GAGGCTGAGGGATTGTG, Rev: 5'-AAACAGCTTCTACTC-CAGCACA. The expression was standardized to the internal control β-actin: For: 5'-AGGTCTACATTGCAATGAG, Rev: 5'CTTGGGATGTCCAGTCA

Animals and 5T33MMvv multiple myeloma model

The 5T33MM model originated spontaneously in aging C57BL/KaLwRij mice and has since been propagated in vivo by intravenous transfer of the diseased marrow in young syngeneic mice (16). C57BL/KaLwRijHsd mice were purchased from Harlan CPB. Female mice, 8 weeks old when used, were housed and treated following the conditions approved by the Ethical Committee for Animal Experiments, VUB (license no. LA1230281).

In vivo analysis of tumor burden

To study the effects of sorafenib, on myeloma progression, 2 groups of C57BL/KaLwRijHsd mice (n = 10) were injected i.v. with 0.5 x 10⁷ 5T33MMvv cells; 1 group of 10 naive mice was included as negative control. Three days after tumor cell inoculation, mice were treated with either sorafenib (daily, oral gavage, 60 mg/kg) or vehicle (1:4 dilution of 50:50 Sigma Cremophor EL, 95% ethanol) until the vehicle-treated 5T33MM-bearing mice showed signs of morbidity. Serum M component was assessed using standard electrophoretic techniques. Bone marrow tumor burden was assessed by determining plasmacytosis on cytosmears (15). A similar experimental setup was used to assess the effects of sorafenib treatment on overall survival. Mice were treated daily and were sacrificed upon signs of morbidity. As a control for possible side effects, we have treated naive C57BL/KaLwRij mice either with sorafenib 60 mg/kg mouse daily oral gavage or with vehicle. Twenty-one days after treatment, mice were weighted, sacrificed, and different samples taken. Peripheral blood was analyzed for white and red blood cell count; samples of skin, kidney, spleen, heart, lung, and liver were fixed, and hematoxylin and eosin (H&E)-stained sections were evaluated.

Assessment of microvessel density

Microvessel density (MVD) was determined by CD31 staining as previously described (7). Briefly, mouse femurs were fixed in zinc fixative for 48 hours, decalcified for 48 hours, and embedded in paraffin. After blocking with normal goat serum, sections were incubated with a rat anti-CD31 antibody (PECAM-1; 1:10; Pharmingen) overnight at 4 °C. A biotin-conjugated goat anti-rat antibody was used as secondary antibody (1:75 dilution, Pharmingen). A streptavidin–horseradish peroxidase conjugate in combination with tyramide signal amplification (TSA; NEN Life Science) was used for detection. In the area with the highest blood vessel density (hot spot), the number of blood vessels was counted per 0.22 mm².

Statistical analysis

The statistical analysis was conducted by Mann–Whitney U test; P < 0.05 was considered significant. All reported P values are 2-sided. For the survival assays, Kaplan–Meier analysis was used.

Results

Sorafenib induces cell death in a panel of human multiple myeloma cells

The panel of human cell lines, representative of multiple myeloma, U-266, LP1, OPM-2, NCI-H929, RPMI-8226, and Karpas 620, were exposed for 24 hours to increasing concentration of sorafenib, and the number of dead cells was measured by Annexin V (AnnV)/PI staining (Fig. 1A). Sorafenib induced cell death in all cell lines of the panel with RPMI-8226 being the most sensitive and U-266 being the least sensitive. Treatment of the multiple myeloma cell lines with 10 μmol/L sorafenib for 24, 48, and 72 hours resulted in progressive eradication of the majority of multiple myeloma cell lines tested except for the U-266 cells, which maintained around 50% viability even after 72 hours of treatment (Fig. 1B). Cell-cycle analysis of the multiple myeloma cell lines treated with sorafenib revealed an accumulation of the cells in the G0–G1 phase (Supplementary Fig. S1). In an attempt to characterize the mode of cell death, we examined a number of well-defined apoptotic markers (Fig. 1C–G). We found that sorafenib induced apoptotic cell death characterized by mitochondrial depolarization, cytochrome c release, Bak and caspase-3 activation, and nuclear condensation/fragmentation.

Sorafenib induces caspase-dependent and -independent cell death

Sorafenib-induced cell death was accompanied by activation of the effector caspase-3 and -7 in all the multiple myeloma cell lines, already observed after 8 hours of treatment (Fig. 2A and B). Coadministration of the pan-caspase inhibitor z-VAD-FMK with sorafenib led to a marked inhibition of caspase-7 activation and PARP cleavage (z-PARP; Fig. 2C). The pan-caspase inhibitor blocked cell death in U-266 and RPMI-8226 cells but had little effect in the rest of the multiple myeloma cell lines (Fig. 2D). In agreement with the flow cytometric data, z-VAD-FMK could partly block the caspase-dependent nuclear fragmentation and rescue RPMI-8226 cells but was largely ineffective in blocking nuclear condensation and phosphatidyl exposure in OPM-2 cells (Fig. 2E). The caspase-independent cell death observed in LP1 and OPM-2 is not necrotic, as cotreatment with Necrostatin1 did not attenuate sorafenib-induced death (Fig. 2F and data not shown, respectively). These data suggest that sorafenib, in most of the cell lines examined, induces caspase-dependent cell death, whereas in LP1 and OPM-2 cells, the cell death is caspase-independent.

One of the key mediators of a caspase-independent cell death is the apoptosis-inducing factor (AIF; ref. 17). We therefore examined the possibility that AIF was involved in the cell death signaling cascade induced in LP1 and OPM-2 cells. In the panel of multiple myeloma cell lines, we assessed the AIF cleavage in response to sorafenib and found such a posttranslational modification in LP1 and OPM-2 cells (Fig. 2G). Isolation of mitochondria and cytoplasm from LP1 cells treated with sorafenib and incubation with an anti-AIF antibody revealed that AIF is released to the cytoplasm in response to sorafenib treatment (Fig. 2H). The translocation of AIF was further shown by immunofluorescence (Fig. 2I). In LP1 and

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OPM-2 cells treated with sorafenib, AIF was present in the nucleus of 20% to 30% of the cells that also exhibited cytochrome c release and nuclear condensation. Overall, these data show that both caspase-dependent and -independent cell death pathways are activated in multiple myeloma cells treated with sorafenib.

**Sorafenib induces autophagy in multiple myeloma cells**

Autophagy is generally considered to be a cytoprotective pathway and protects cancer cells by removing damaged organelles and providing nutrients. In this study, we wanted to investigate the role of autophagy in sorafenib-induced cell death. One of the main negative modulators of autophagy is...
mTOR, activation of which is characterized by the phosphorylation of one of its direct downstream target 4E-BP1. Sora- fenib treatment of U-266, LP1, and RPMI-8226 cell lines led to the inhibition of 4E-BP1 phosphorylation, suggesting that the mTOR activity is inhibited in these cell lines (Fig. 3A). Importantly, sorafenib induced autophagy in LP1 and RPMI-8226 cells in patient samples and in mice as determined by p62 degradation, LC3 lipidation, and by immunofluorescence for the detection of LC3-GFP–positive foci (Figs. 3A and B, 5G, and 7D). Pretreatment of LP1 with chloroquine, a classical inhibitor of late autophagy, followed by sorafenib led to the accumulation of autophagosomes as judged by the levels of...
LC3-II and an increase in the levels of active caspase-7 (Fig. 3C). Pretreatment of multiple myeloma cell lines with chemical inhibitors of early (3-MA) and late autophagy (chloroquine) on sorafenib-induced cell death led to a potentiation of sorafenib-induced cell death in LP1, OPM-2, and H929 cells (Fig. 3D). Further support on the involvement of mTOR in sorafenib-induced autophagy came from the experiments with the mTOR inhibitor, rapamycin, in combination with sorafenib. Treatment of LP1 and OPM-2 cells with rapamycin and sorafenib partially protected these multiple myeloma cell lines from sorafenib-induced cell death (Fig. 3E). Together, these data indicate that sorafenib-induced autophagy is cytoprotective, and inhibition of this pathway can potentiate the efficacy of sorafenib.

**Downregulation of Mcl-1 protein levels is critical for sorafenib-induced cell death**

It has been previously described that PI3K/AKT and Ras/Raf/MEK kinase (MEK)/extracellular signal–regulated kinase (ERK) 1/2 pathways play a critical role in the survival of multiple myeloma cells (18). We examined the levels of AKT and ERK1/2 serine phosphorylation and the possibility that sorafenib acts by inhibiting these pathways in the panel of human multiple myeloma cell lines. OPM-2 cells, which are PTEN-null, display a constitutively active AKT that may be partially inhibited by sorafenib (Fig. 4A). In LP1, H929, and RPMI-8226 cells, AKT phosphorylation was induced by sorafenib. ERK1/2 was constitutively active in U-266 and LP1 cells and this phosphorylation is potently inhibited by sorafenib, whereas in the rest of the cell lines, ERK1/2 phosphorylation was not dramatically altered (Fig. 4A). Bim, a BH3-only protein that is known to play a critical role in multiple myeloma cells undergoing cell death, was also examined (19). LP1 does not express Bim due to biallelic deletion of this gene (7). However, in all remaining multiple myeloma cell lines of the panel, Bim was downregulated. This is in agreement with the induction of AKT phosphorylation observed in response to sorafenib. These data suggest that Bim is not an important mediator of sorafenib-induced cell death in multiple myeloma cells.

The cytoprotective effect of Ras/Raf/MEK/ERK signaling cascade is partly mediated by the inhibition of degradation and subsequent stabilization of Mcl-1 proteins levels (20, 21). Mcl-1 is one of the major antiapoptotic proteins expressed in multiple myeloma cells, and resistance to drug treatment has been associated with high levels of Mcl-1 expression. To determine whether sorafenib-induced cell death was associated with modulation of Mcl-1 expression, we investigated the effect of sorafenib on Mcl-1 protein levels by Western blotting. Mcl-1 protein levels were downregulated in all the cell lines of the panel, an effect observed already at 8 hours of treatment.
Figure 4. Sorafenib-mediated Mcl-1 downregulation is required for the induction of cell death. A, immunoblot analysis of the indicated proteins in the panel of multiple myeloma cell lines treated with 10 μmol/L sorafenib (Sor) for 24 hours. B, immunoblot analysis of the Mcl-1 levels in U-266 and LP1 treated with 10 μmol/L sorafenib for the indicated time points. C, immunoblot analysis of the indicated proteins in U-266 and LP1 pretreated with 10 μmol/L U0126 followed by 10 μmol/L sorafenib for 24 hours. D, quantitative analysis of Annexin V/PI-positive cells in the indicated multiple myeloma cell lines pretreated with 10 μmol/L U0126 followed by 10 μmol/L sorafenib for 24 hours (means ± SD, n ≥ 3). E, quantitative PCR analysis of Mcl-1 in H929 and LP1 treated with 10 μmol/L sorafenib for 8 and 24 hours. F, immunoblot analysis of phosphorylated and total eIF4E in the panel of multiple myeloma cell lines treated with 10 μmol/L sorafenib for 24 hours. G, immunoblot analysis of Mcl-1, caspase-3, and cleaved PARP in RPMI-8226 cells transiently transfected with either pCDNA3.3 or Mcl-1 and treated with 10 μmol/L sorafenib for 24 hours. H, quantitative analysis of Annexin V–positive in OPM-2 and RPMI-8226 cells transiently transfected with either pCDNA3.3 or Mcl-1 and treated with 10 μmol/L sorafenib for 24 hours (means ± SD, n ≥ 3, P < 0.05).

(Fig. 4A and B). To determine whether the decrease of Mcl-1 protein levels was due to the inhibition of ERK1/2, a chemical inhibitor of MEK/ERK1/2 signaling cascade, U0126, was used. As expected, U0126 inhibited ERK1/2 phosphorylation but had no or little effect on Mcl-1 levels neither alone nor in combination with sorafenib (Fig. 4C). Treatment of the multiple myeloma cell lines with U0126 alone did not induce cell death in the multiple myeloma cell lines, and the combination with sorafenib did not influence the efficacy of sorafenib as compared with sorafenib alone (Fig. 4D). Collectively, these data show that the observed Mcl-1 downregulation by sorafenib is independent of the Raf/MEK/ERK1/2 pathway.

To gain further insights into the mechanism by which sorafenib induces the downregulation of Mcl-1, a number of signaling cascades known to regulate Mcl-1—including transcription, translation, caspase cleavage, proteosomal, and in some cases, autophagosomal degradation—were investigated (22–24). We found that posttranslational modifications are not...
responsible for the downregulation of Mcl-1 levels in cells treated with sorafenib (Supplementary Fig. S2). Interestingly, despite the decrease in total Mcl-1 protein levels observed in multiple myeloma cells treated with sorafenib, Mcl-1 mRNA is induced after 8 and 24 hours (Fig. 4E). It has been previously shown that the effects of sorafenib on protein levels may be mediated by inhibition of phosphorylation of components of the translational machinery (25). Notably, treatment with sorafenib led to an inhibition of the phosphorylation and activation of eIF4E, a key player in the initiation of translation (Fig. 4F).

To determine the functional importance of Mcl-1 downregulation in sorafenib-induced cell death, we transfected OPM-2 and RPMI cells with a construct overexpressing Mcl-1. We found that it can substantially protect OPM-2 cells and partially protect RPMI-8226 cells against apoptosis here determined as exposure of PS, caspase-3 activation, and PARP cleavage (Fig. 4G and H). These data suggest that downregulation of Mcl-1 is required for the induction of sorafenib-induced cell death in multiple myeloma cell lines.

**Sorafenib induces cell death ex vivo in the presence of BMSCs**

The efficacy of sorafenib ex vivo was examined in freshly isolated CD138⁺ multiple myeloma cells from newly diagnosed patients with multiple myeloma (Table 1). The CD138⁺-enriched primary multiple myeloma cells from patients 1 and 2 were treated with increasing concentrations of sorafenib for 24 and 48 hours, respectively. We found that sorafenib displayed similar effects on these primary cells as in the cell lines (Fig. 5A). The cytotoxic effects of sorafenib on PBMCs from a healthy donor were measured by Annexin V staining and showed a 20% increase in cell death compared with control after 48 hours of treatment (Fig. 5B).

To investigate the role of soluble factors secreted by the tumor microenvironment, we used a Transwell coculturing

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**Figure 5.** BMSCs do not rescue multiple myeloma cells from sorafenib (Sor)-induced cell death in vitro and ex vivo. A, quantitative analysis of cell viability of CD138⁺-enriched multiple myeloma cells from 2 newly diagnosed patients (patients 1 and 2) treated with the indicated doses of sorafenib for 24 h and 48 hours, respectively. B, quantitative analysis of Annexin V-positive cells in PBMCs treated with 10 μmol/L sorafenib for 24 and 48 hours. C, quantitative analysis of Annexin V/PI positivity in U-266, RPMI-8226, and OPM-2 cultured alone or cocultured in inserts with L88 BMSCs and treated with 10 μmol/L sorafenib for 24 hours (means ± SD, n ≥ 3). D, immunoblot analysis of the indicated proteins in U-266, RPMI-8226, and OPM-2 alone or cocultured with L88 and treated with 10 μmol/L sorafenib for 24 hours. E, quantitative analysis of Annexin V-positive cells of CD138⁺-enriched multiple myeloma cells from the indicated newly diagnosed patients treated with the 10 μmol/L sorafenib for 24 hours (E) or 48 hours (F). G, immunoblot analysis of Mcl-1, cleaved PARP, and LC3 lipidation in bone marrow–derived CD138⁺-positive cells from a patient in relapse (patient 7).
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system between L88 BMSCs and U-266, RPMI-8226, and OPM-2 cells. Because the effect of the stroma on multiple myeloma cell survival is largely dependent on their response to IL-6, we anticipated the effects of stroma on sorafenib cytotoxic efficacy to be IL-6–dependent. Indeed, in the IL-6–dependent RPMI-8226 cells, sorafenib-induced cell death was potentiated when cocultured with BMSCs (Fig. 5C). In contrast, the sorafenib-induced cell death was equally efficient in the IL-6–independent U-266 in mono- or coculture. Downstream of the activated receptor tyrosine kinases (RTK), a number of common signaling pathways converge, that is, the Janus-activated kinase (Jak)/STAT, the PI3K/AKT, and the aforementioned RAF/MEK/ERK1/2, all of which were upregulated in the presence of BMSCs (Fig. 5D). Importantly, the levels of STAT3 phosphorylation, Bcl-xL and Mcl-1 expression in the mono- and coculture correlated well with the protection mediated by the BMSCs from sorafenib-induced death in RPMI-8226 cells (Fig. 5D). The potentiation of cell death in coculture of OPM-2 with L88 cells was associated with the inhibition of AKT phosphorylation, downregulation of Bcl-xL, and the sustained expression of Bim protein levels in response to sorafenib (Fig. 5D).

Having established that 10 μmol/L sorafenib is equally efficient in the treatment of primary multiple myeloma patient samples as was shown for the cells in the multiple myeloma cell line panel, samples from 4 additional patients with multiple myeloma were treated in monoculture or in BMSCs cocultured with sorafenib for 24 and 48 hours, and the levels of cell death were measured by conducting CD138+/Annexin V+ double stainings to immunophenotypically detect multiple myeloma cells and measure cell death (Fig. 5E and F). In the monoculture setting, sorafenib was very potent in inducing cell death in all the primary samples. In the coculture setting, L88 cells protected the CD138+ primary multiple myeloma cells from spontaneous cell death but they were still sensitive to sorafenib treatment (Fig. 5E and F). Because of the lack of sufficient amount of cells, samples from patients 5 and 6 were cultured only in the presence of L88 cells for 48 hours, and we found that multiple myeloma cell from patient 5 were very sensitive to sorafenib, and 50% of multiple myeloma cells from patient 6 died upon sorafenib treatment (Fig. 5F). To determine whether the molecular determinants of sorafenib efficacy identified in multiple myeloma cell lines were also found in patient samples, we treated a bone marrow sample, ex vivo, with sorafenib and examined the proteins levels of Mcl-1, the cleavage of PARP, and the levels of autophagy (Fig. 5G). We found that sorafenib downregulates Mcl-1 and induces PARP cleavage and autophagy in these multiple myeloma primary patient cells, in concordance with the cell line experiments.

**ABT737 improves the efficacy of sorafenib against multiple myeloma cells in vitro and ex vivo**

Together with Mcl-1, Bcl-2 and Bcl-xL constitute the major antiapoptotic Bcl-2 proteins known to be involved in the acquisition of resistance to therapy. The protein levels of Bcl-2 and Bcl-xL did not change in response to sorafenib with the exception of Bcl-xL that decreased in the OPM-2 cells (Fig. 6A). Ectopic expression of Bcl-2 in the U-266 cells resulted in partial resistance to sorafenib showing the importance of Bcl-2 in the protection of multiple myeloma cells to sorafenib-induced cell death (Fig. 6B).

Because sorafenib potently downregulates Mcl-1 protein levels, we hypothesized that a combination with a Bcl-2 antagonist would lead to the concomitant targeting of all 3 major antiapoptotic proteins in multiple myeloma cells and thereby potentiate the efficacy of sorafenib. The multiple myeloma cell lines were treated with either sorafenib alone or in combination with ABT737 (Fig. 6C). LP1 and OPM-2 cells became more sensitive to sorafenib in the presence of ABT737, whereas NCI-H929 and Karpas 620 cell lines were as sensitive to sorafenib as to ABT737 alone.

The efficacy of this combination therapy was also examined in the coculture setting with L88 and the U-266, RPMI-8226, and OPM-2 multiple myeloma cell lines. Even though RPMI-8226 was not sensitive to sorafenib in the presence of BMSCs (Fig. 5A), ABT737 alone or the combination with sorafenib with ABT737 overcame this protection and induced high levels of cell death (Fig. 6D). Furthermore, ABT737 potentiated the efficacy of sorafenib in OPM-2 cells, even in the presence of L88 cells (Fig. 6D).

On the basis of the efficacy observed in multiple myeloma cell lines treated with the combination of sorafenib and ABT737, 2 CD138+ multiple myeloma patient samples, one in relapse (patient 7) and one newly diagnosed (patient 8), were treated similarly and cell death was measured (Fig. 6E). The combination of sorafenib with ABT737 showed potent efficacy in killing primary CD138+-enriched multiple myeloma cells. Collectively, these data show that this rationale-based combination is effective in the treatment of multiple myeloma cells both in vitro and ex vivo.

Bortezomib is now introduced as a therapeutic modality against multiple myeloma (26). Despite its promising therapeutic effects, patients develop resistance to this proteasome inhibitor (26). To investigate whether sorafenib treatment is effective against bortezomib-resistant cells, we used a recently published model system of RPMI-8226 cells that have been induced to develop resistance to bortezomib (27). Bortezomib–sensitive RPMI-8226 and bortezomib-resistant RPMI-8226/7B680 were grown in the presence of 80 nmol/L bortezomib and treated with either sorafenib alone or the combination of sorafenib with ABT737. Importantly, sorafenib induced cell death in bortezomib-resistant RPMI-8226/7B680 cells and the combination with ABT737 improved the cytotoxic potency of sorafenib (Fig. 6F). These results were recapitulated in primary multiple myeloma cells from patients who have undergone treatment with bortezomib and found to be nonresponsive (Fig. 6G).

**In vivo therapeutic effects of sorafenib on 5T33MM model**

To investigate the efficacy of sorafenib in murine multiple myeloma cells, we evaluated a dose curve and found that it induces dose- and time-dependent cell death in 5T33MM cells in vitro (Supplementary Fig. S3A). We also examined whether the targeted signaling cascades by sorafenib in human cell lines and patient samples were also observed in the mouse 5T33MM model.

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In agreement with our findings in the human setting, we found that sorafenib inhibits ERK phosphorylation, down-regulates Mcl-1 levels, and induces autophagy in 5T33MM cell line and cells (Supplementary Fig. S3B).

Having shown that sorafenib significantly induces apoptosis in multiple myeloma cells in vitro, we next examined the in vivo efficacy using the 5T33MM mouse model. C57BL/KaLwRijHsd mice inoculated with 5T33MMv cells were either assigned to receive sorafenib or vehicle. Mice treated with sorafenib showed a significantly \( P < 0.001 \) increased survival (Fig. 7A). The experiment was terminated at day 63. At that moment, 4 mice of the sorafenib-treated group were still alive. These mice were sacrificed at day 66. Tumor burden was ranging from 16\% to 34\%, whereas mice who were terminally diseased at earlier time points had a tumor burden in the bone marrow ranging from 52\% to 97\%, indicating that the mice at the last

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**Figure 6.** Combination of sorafenib (Sor) with ABT737 in multiple myeloma treatment. A, immunoblot analysis of Bcl-2 and Bcl-xL proteins levels in the indicated multiple myeloma cell lines treated with 10 \( \mu \)mol/L sorafenib for 24 hours. B, quantitative analysis of Annexin V–positive cells in mock or Bcl-2-overexpressing U-266 cells treated with the 10 \( \mu \)mol/L sorafenib for 24 hours (means ± SD, \( n \geq 3 \); *, \( P < 0.05 \)). C, quantitative analysis of Annexin V/PDI–positive cells in the indicated multiple myeloma cell lines treated with 10 \( \mu \)mol/L sorafenib alone or in combination with 10 \( \mu \)mol/L ABT737 for 24 hours (means ± SD, \( n \geq 3 \); *, \( P < 0.05 \); **, \( P < 0.01 \)). D, quantitative analysis of Annexin V/PDI–positive cells in the indicated cell lines cultured alone or cocultured with L88 and treated with 10 \( \mu \)mol/L sorafenib in the presence or absence of 10 \( \mu \)mol/L ABT737 for 24 hours (means ± SD, \( n \geq 3 \); *, \( P < 0.05 \)), **, \( P < 0.01 \)). E, quantitative analysis of cell viability in CD138–positive multiple myeloma cells from 1 patient in relapse and 1 newly diagnosed patient (patients 7 and 8, respectively) treated with 10 \( \mu \)mol/L sorafenib alone or in combination with 10 \( \mu \)mol/L ABT737 for 24 h and 48 hours (means ± SD, \( n \geq 3 \)). G, quantitative analysis of cell viability in CD138–positive multiple myeloma cells from 2 bortezomib-refractory patients treated with 10 \( \mu \)mol/L sorafenib for 24 hours.

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points had a significant reduced tumor development. These data were paralleled by a similar decrease in serum M component. In another in vivo experiment, where all mice were sacrificed the day that the first vehicle mice showed signs of morbidity, a significant reduction in serum paraprotein concentrations and plasmacytosis in the bone marrow was observed in the sorafenib-treated mice when compared with the vehicle-treated mice (Fig. 7B and C). We examined whether the sorafenib molecular targets identified in human cell lines and patient samples were also observed in the mouse 5T33MM. In agreement with our findings in the human setting, we found that sorafenib inhibits ERK phosphorylation, induces caspase activation and autophagy in multiple myeloma cells isolated from the bone marrow of the 5T33MM mice (Fig. 7D).

Figure 7. Sorafenib (Sor) shows potent antitumoral activity in the 5T33MM syngeneic mouse model. A, mice were inoculated with 5T33MM cells and treated with sorafenib or vehicle. Naive mice were included as controls. Upon signs of morbidity, mice were sacrificed. B, treatment effects on serum M protein concentration, determined by serum electrophoresis (n = 10 for each group; ***, P < 0.002). C, therapeutic effects on tumor load in the bone marrow. Data are expressed as percentage of 5T33MM cells of total cell number (n = 10 for each group; ***, P < 0.002). D, immunoblot analysis of the indicated proteins from multiple myeloma cells isolated from the bone marrow of 5T33MM mice treated with 10 μmol/L sorafenib for 24 hours. E, H&E-stained section of bone marrow from naive (left image), vehicle-treated mouse (middle image), and from a sorafenib-treated mouse (right image). F, images from the immuno-histochimical stain for CD31 in the bone marrow of myeloma-bearing mice treated with either vehicle or sorafenib. In the area delineated by the rectangle, the bone marrow is not invaded by myeloma cells. G, quantitative analysis of the number of microvessels (MVD), counted by CD31 immunohistochimical staining, in the bone marrow of myeloma-bearing mice treated with either vehicle or sorafenib (means ± SD for groups of 9 mice are shown; *, P < 0.01; **, P < 0.0005).
bone marrow accompanied by rich vascular network and no residual normal hematopoiesis (Fig, 7E). In the H&E-stained section of a bone marrow of a mouse treated with sorafenib, there is almost no residual tumor present (Fig, 7E). In the bone marrow of a myeloma-bearing mouse, there is an increased MVD with the majority of the vessels compressed by the myeloma infiltrate. Treatment with sorafenib significantly decreased MVD by 60% in the bone marrow of treated 5T33MMv mouse, compared with vehicle-treated 5T33MMv mice (Fig, 7F and G). No adverse side effects or toxicity were observed as evaluated by behavior, body weight, histologic, and hematologic examinations (data not shown).

Discussion

RTK- and non-RTK–activated signaling cascades play a vital role in the survival and proliferation of multiple myeloma cells (28). The importance of these RTKs for multiple myeloma carcinogenesis and progression is further underlined by the initiation of a number of clinical trials in which various TKIs are used alone or in combination with conventional and emerging anti–multiple myeloma agents. Sorafenib has shown promising results against a number of solid tumors (e.g., renal carcinoma and hepatocellular carcinoma); however, its potential efficacy in hematologic malignances and especially in multiple myeloma has received little attention. Also, in the light of the recent publication of whole-genome sequencing of multiple myeloma (18). The multiple myeloma cell lines examined represent the genetic heterogeneity shown to be active in multiple myeloma (18). The multiple myeloma cell lines selected represent the genetic heterogeneity seen in multiple myeloma and sorafenib is likely to target at least one predominant pathway of multiple myeloma survival. Because the majority of the (R)TK signaling cascades converge at core downstream pathways, such as Raf/MEK/ERK1/2, PI3K/AKT, Jak/STAT, we focused, in this study, on the effects of sorafenib on these pathways. Our results show that the Raf/MEK/ERK1/2 pathway is targeted in U-266 and LP1, whereas in OPM-2, the PI3K/AKT pathway is affected by sorafenib. The predominant pathway of survival in U-266 is STAT3, whereas in OPM-2, a constitutively active AKT, due to PTEN deletion, may be a part of the underlying observation. One intriguing result is that sorafenib induces AKT phosphorylation in the majority of the cell lines examined as it has been previously described (30). The mechanism and the biologic significance of such an induction are not known. It may signify the extensive cross-talk between the PI3K/AKT and the Raf/MEK/ERK pathway and the Ras-mediated activation of the PI3K/AKT pathway (30). It may also be possible that the observed AKT phosphorylation is mediated by another RTK that is activated upon treatment with sorafenib. It has been recently shown that sorafenib-mediated inhibition of Raf phosphorylation may lead to the activation of the c-MET RTK and its downstream signaling cascade PI3K/AKT (31).

The antitumoral efficacy of sorafenib may also depend to the potent inhibition of VEGFR and angiogenesis. This has been previously shown for renal cell carcinoma and hepatocellular carcinoma where the antiangiogenic effects of sorafenib were found to be partly responsible for the potent antitumor activity observed in the clinic (32, 33). In the multiple myeloma setting, we found that sorafenib significantly decreased MVD in the bone marrow of multiple myeloma mice and indicating that the antitumoral effects of sorafenib may, at least in part, be mediated by inhibition of angiogenesis. It is thus likely that it is the combination of direct cytotoxic effects inflicted on multiple myeloma cells and decreased vascularization in the bone marrow that are responsible for the therapeutic efficacy of sorafenib.

In this study, we found that sorafenib induces both caspase-dependent and -independent cell death in the majority of the multiple myeloma cell lines and multiple myeloma primary cells tested, results that are in agreement with a previous report (30). Interestingly, LP1 and OPM-2 cells, even though they exhibited all the classical characteristics of apoptosis, were not rescued by the pre-incubation with the pan-caspase inhibitor z-VAD-fmk, indicating that several pathways of cell death are activated by sorafenib. For example, LP1 and OPM-2 are 2 cell lines in which AIF, a mediator of caspase-independent cell death, is cleaved, released from the mitochondria, and translocated to the nucleus. The differences in the responses to sorafenib observed in this panel of multiple myeloma cell lines as a consequence of genetic heterogeneity including the presence of Ras mutations and other genetic alterations is the current focus of our further investigations.

The activation of autophagy by TKIs has been described previously for imatinib mesylate (34). In our experimental system, sorafenib induced autophagy in several of the cell lines tested, primary multiple myeloma patient samples, and mice. By using chemical inhibitors of early and late autophagy, it became evident that autophagy is induced to protect multiple myeloma cells from sorafenib-mediated cell death. Importantly, rapamycin, a potent inducer of autophagy, partially protected certain multiple myeloma cell lines from sorafenib-induced cell death, presumably by alleviating the damage inflicted by sorafenib. From these data, we conclude that a novel therapeutic strategy against multiple myeloma may be the combination of sorafenib with autophagy inhibitors such as chloroquine, a combination that at least, in vitro, showed potent cytotoxic activity.

The tumor stroma has been implicated in modulating not only the tumor growth but also the acquisition of resistance to cancer therapy. The impact of the tumor stroma in the survival of multiple myeloma cells in vitro and ex vivo was confirmed in our experiments. In particular, STAT3 was highly phosphorylated/activated in the coculture setting than in the monoculture setting. A reasonable explanation for this signature is the response in selected multiple myeloma cell lines to IL-6 produced from the stroma. The molecular re-wiring of multiple myeloma cell lines and primary cultures grown under the paracrine influence of the BMSCs is firstly observed by the lower levels of basal death. This response is well associated with the activation of all the signaling cascades examined, that
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The importance of Mcl-1 for multiple myeloma survival is well described (35). It has been previously reported that sorafenib is highly effective in downregulating Mcl-1 in a number of other experimental models (20, 36). Sorafenib downregulates the protein levels of Mcl-1 in multiple myeloma, an event critical that we provide evidence for its importance in the execution of the cell death program (37). Mcl-1 is known to be regulated at the transcriptional, translational, and post-translation level. We found that posttranslational modifications such as caspase cleavage or proteosomal degradation were not responsible for the downregulation of Mcl-1 protein levels induced by sorafenib. Intriguingly, quantitative measurement of Mcl-1 mRNA levels showed a transcriptional upregulation after 8 and 24 hours of treatment with sorafenib. It is known that the JAK/STAT3 pathway induces the expression of Mcl-1 (38). However, in the majority of our cell lines, STAT3 was not phosphorylated with the exception of U-266, it is unlikely that this pathway is responsible for this effect. Translational mechanisms are most likely to be mediating the downregulation of Mcl-1 in response to sorafenib. It was recently shown that inhibition of translation is the most likely mechanism of Mcl-1 downregulation in leukemic cells (39). In agreement with these results, we found that sorafenib inhibits the phosphorylation of eIF4E, a key initiation of protein translation. The mechanism and the specificity of translational inhibition of Mcl-1 in our system requires further investigation.

It has been previously described that ABT737 is a potent inducer of cell death in multiple myeloma cells (40). However, the combination of sorafenib with ABT737 with the rationale to concomitantly target all 3 major Bcl-2 antiapoptotic proteins (i.e., Mcl-1, Bcl-2, and Bcl-xL) and thereby tilting the Bcl-2 rheostat toward cell death has not been previously shown. Indeed, we found that in all the multiple myeloma cell lines and in primary samples tested, the combination of these 2 drugs had better therapeutic efficacy than treatment of sorafenib or ABT737 alone. Importantly, this combination was very effective in primary multiple myeloma patient samples.

In summary, our study provides compelling evidence for the future evaluation of the multi-TKI sorafenib as a new therapeutic strategy for the treatment of multiple myeloma in the clinic.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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