Sorafenib has potent anti-tumor activity against multiple myeloma in vitro, ex vivo and in vivo, in the 5T33MM mouse model

Running title: Sorafenib is a potent anti-cancer agent against multiple myeloma

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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 (BM) that relies on multiple signaling cascades, including tyrosine kinase activated pathways, to proliferate and evade cell death. Despite emerging new treatment strategies, MM remains at present incurable. Thus, novel approaches targeting several signaling cascades by using the multi-tyrosine kinase inhibitor (TKI), sorafenib, seems a promising treatment approach for MM. Here, we demonstrate that sorafenib induces cell death in MM cell lines and in CD138+ enriched primary MM patient samples in a caspase-dependent and independent manner. Furthermore, sorafenib has a strong anti-tumoral and anti-angiogenic activity in the 5T33MM mouse model leading to increased overall survival. MM 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 MM, is downregulated at the protein level by sorafenib allowing for the execution of cell death, since ectopic overexpression of this protein protects MM cells. Concomitant targeting of Mcl-1 by sorafenib and of Bcl-2/Bcl-xL by the antagonist ABT737 improves the efficacy of sorafenib in MM cell lines and CD138+ enriched primary cells in the presence of BM stromal cells. Altogether, our data support the use of sorafenib as a novel therapeutic modality against human MM and its efficacy maybe potentiated in combination with ABT737.
Introduction

Multiple myeloma (MM) is a neoplastic B cell disorder characterized by proliferation of malignant plasma cells in the bone marrow (BM) where they interact with the BM microenvironment leading to skeletal destruction and angiogenesis (1). MM accounts for 1% of all malignancies and 10% of all hematological malignancies (2). Recently introduced drugs, like thalidomide, lenalidomide, or bortezomib, in the clinic have improved survival and are therefore widely used as a first- and second-line treatment against MM. Albeit the use of these drugs, the 5-year relative survival rate has only marginally increased and MM patients eventually relapse thus further highlighting the unmet medical need for new therapeutic agents (3-5).

MM is characterized by the activation of multiple of tyrosine kinase signaling cascades, that induce three main downstream pathways, the Raf/MEK/ERK1/2, the 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 anti-apoptotic 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 MM. Such a multi-tyrosine kinase inhibitor (TKI) is sorafenib, a novel bi-aryl urea, a type II inhibitor primarily against Raf1, VEGFR and PDGFRβ (11). Sorafenib is already in use in the clinic for the treatment of renal cell carcinoma and hepatocellular cancer (12).

MM pathogenesis, proliferation and response to therapy largely depend on the BM microenvironment (13). The complex interplay between MM cells and stromal cells involves numerous growth factors, most predominantly IGF-1 and IL-6, but also cytokines like tumor necrosis factor α (TNF-α), transforming growth factor β (TGF-β) and vascular endothelial growth factor (VEGF) which are secreted by the MM cells. These cytokines in turn may induce IL-6, insulin-like growth factor 1 (IGF-1) and platelet derived growth factor β (PDGFβ) secretion from
BM stromal cells (BMSCs) (13). Recent advances in the understanding of molecular mechanisms underlying the pathogenesis and progression of MM indicate that the use of tyrosine kinase inhibitors as single agents or used in combinatorial regimes 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 in mice. We have found that sorafenib is a potent inducer of caspase-dependent and independent cell death even in the presence of bone marrow stroma cells. 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 MM cell lines and in primary MM samples.
Materials and Methods

Antibodies and reagents

The pancaspase inhibitor z-VAD-FMK (z-Val-Ala-Asp(OMe)-FMK) (MP biomedicals) was used at 10 μM, 3-methyladenine (3-MA) (Sigma-Aldrich) was used at 5 mM, Necrostatin1 (Sigma-Aldrich) was used at 50 μM, chloroquine (Sigma-Aldrich) at 10 μM, ABT737 (active biochemical Co.) at 10 μM, rapamycin at 1 μM, U0126 (Sigma-Aldrich) at 10 μM, MG132 (Calbiochem) at 4 μM and G418 (Gibco) 600 µg/ml. Sorafenib (Bayer HealthCare) was dissolved in DMSO and unless otherwise indicated, 10μM were 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, phosphor-AKT (Ser473), AKT, Mcl-1, LC3 I/II, Bcl-2, phosphoY705 STAT3, STAT3, phospho-mTOR (Ser2448), mTOR, phosphor-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 Stressgene, Bcl-xL from Transduction Laboratories, 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, AIF and Tom40 from Santa Cruz Biotechnology.

Patient samples

MM BM samples were obtained from the Karolinska University Hospital Solna, Stockholm, Sweden. Patients had a confirmed diagnosis of MM and all were previously untreated except patient 7 who had a relapse and 9-10 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 BM 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 $1 \times 10^6$ cells/ml into 12-well 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 Universitetslaboratoriet), then peripheral blood mononuclear cell (PBMC) were further purify by using Ficoll-Paque Plus density sedimentation (Amersham Biosciences). 2x10$^5$ cells were plated in each well of 12 well plates and treated with 10 µM sorafenib or ABT 737 for 24 and 48 hours. Subsequently the cells harvested and apoptosis was measured by Annexin V staining.

**Cells lines and transfections**

L88, the human BM stromal cell line has been previously described (14). The human MM cell lines OPM-2, U-266, LP1, NCI-H929, Karpas 620 and RPMI 8226 kindly provided by Dr. Brigitte Sola (Université de Caen, France). The bortezomib sensitive RPMI 8226 cells and resistant 8226/7B680 cell lines were provided by Dr. Javier Naval (Universidad de Zaragoza, Spain). All cell lines were authenticated by the LGC standards cell line authentication service. The murine 5T33MMin 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, UK) supplemented with 10% fetal bovine serum (FBS, Hyclone UK), L-glutamate and antibiotics penicillin/streptomycin (Gibco). In all experiments, the cells were cultured in 6 or 12-well plates 24h prior to 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.

Co-culture experiments

L88 cells were plated in 6 or 12 well plate 2 hours before putting cell culture inserts (BD Falcon™) which contain MM 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) (8). Briefly, 2 x 10^5 cells per sample were collected, washed in PBS, pelleted, and re-suspended in incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl₂) containing 1% Annexin V and PI followed by analysis on fluorescence-activated cell sorter (Becton Dickinson) using the Cell Quest 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 performed as previously described and measure by flow cytometry (8).

Immunocytochemistry

The effect of sorafenib on MM cell lines was analysed by staining of mitochondria with MitoTracker (Mol. Probes, Inc.) and co staining with either active Bak antibody or cytochrome c antibody as previously described (9).

Resazurin assay
Purified MM cells (patients 1, 2, 7 and 8) were incubated with sorafenib alone or in combination with Bcl-2 antagonist (ABT 737) for 24 and 48 hours. At harvest, 10% resazurin was added followed by incubation for 2-3 h at 37°C in a humidified 5% CO₂ in-air atmosphere. The resazurin was excited at 530 nm and the emitted light was measured at 590 nm. Analysis of fluorescence was performed using Wallac Victor Multilabel Counter (Wallac, Turku, Finland). 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 x ΔFluorescence (treated wells)/ ΔFluorescence (untreated wells).

Cell cycle analysis

We use NucleoCounter® NC-3000™ from chemometec to analyse cell cycle according to the manufacturer’s specifications. One million cells were harvested and washed by PBS then resuspended in 0.5 mL Solution 10 supplemented with 10 μg/ml DAPI. Cells were incubated at 37°C for 5 minutes then 0.5 ml Solution 11 was added and 30μl of suspended cells was loaded on 2-chamber slide (NC-Slide A2™) and cell cycle was analyzed by using provided software.

Mitochondria Isolation

2 x 10⁷ cells were harvested and mitochondria were isolated using the Mitochondria Isolation Kit (Pierce Biotechnology, USA) according to the manufacturer’s specifications.

Immunoblot analyses

Cells were harvested and homogenized in RIPA lysis buffer (10 mM Tris, pH 7.2, 150 mM NaCl, 1% deoxycholate, 1% Triton, 0.1% SDS, 5 mM EDTA) containing complete protease
inhibitor cocktail (Roche Diagnostics, Meylan, France). After 1h 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 denaturated by boiling and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. After blocking in 5% non-fat dry milk in PBS for 1h 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 anti-actin antibodies.

RNA extraction, cDNA and qRTPCR

The RNA was extracted using Qiagen RNeasy mini kit (Qiagen) and DNase treated (Ambion Turbo DNA-free, Life Technologies). Roughly, 100-500ng of DNase treated RNA was used in the generation of cDNAs using MuMLV (Life Technologies) and a mixture of oligoDt18 with nanomers (IDT technologies). qRTPCR quantification was carried out using the KAPA 2G SyberGreen (Kapa biosystems) on the Applied biosystem 7900HT platform with the following conditions: 95°C 3min, 95°C 3sec, 60°C 30sec. The following primers were used for the amplification of Mcl-1: For: 5’-AACAAAGAGGCTGGGATGGGTTTG, Rev: 5’-AAACCAGCTCCTACTCCAGCAACA. The expression was standardized to the internal control Beta actin: For 5’-AGGTCATCACCATTGGCAATGAG, Rev: 5’-CTTTGCGGATGTCCACGTCA

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 (i.v.) transfer of the diseased marrow in young syngeneic mice(16). C57BL/KaLwRijHsd mice were purchased from Harlan CPB (Horst, The
Netherlands). Female mice, 8 weeks old when used, and 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, two groups of C57BL/KaLwRijHsd mice (n=10) were injected i.v. with 0.5×10^6 5T33MMvv cells; one 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 paraprotein concentration was assessed using standard electrophoretic techniques. BM 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 naïve C57BlKaLwRij mice either with sorafenib 60 mg/kg mouse daily oral gavage or with vehicle. 21 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, hart, lung and liver were fixed and haematoxylin and eosin (HE) 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, San Diego, CA, USA) overnight at 4°C. A biotin-conjugated goat anti–rat antibody was used as secondary antibody (1:75 dilution,
PharMingen, San Diego, CA, USA). A streptavidin-horseradish peroxidase conjugate in combination with tyramide signal amplification (TSA) (NEN Life Science, Boston, MA, USA) 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 mm.

**Statistical analysis**
The statistical analysis was performed by Mann-Whitney U test; P-values < 0.05 were considered significant. All reported p-values are two-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 MM, U-266, LP-1, OPM-2, NCI-H929, RPMI 8226 and Karpas 620 were exposed for 24h to increasing concentration of sorafenib (Sor) and the number of dead cells was measured by Annexin V (AnnV)/Propidium Iodide (PI) stainings (Fig. 1A). Sor 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 MM cell lines with 10 µM Sor for 24, 48 and 72h resulted in progressive eradication of the majority of MM cell lines tested except for the U-266 cells which maintained around 50% viability even after 72h of treatment (Fig. 1B). Cell cycle analysis of the MM cell lines treated with Sor revealed an accumulation of the cells in the G0/G1 phase (Supplementary Fig. 1). In an attempt to characterize the mode of cell death, we examined a number of well-defined apoptotic markers (Fig. 1 C, D, E, F and G). We found that Sor induce 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

Sor-induced cell death was accompanied by activation of the effector caspases 3 and 7 in all the MM cell lines, already observed after 8h of treatment (Fig. 2A and B). Co-administration of the pan-caspase inhibitor zVAD.fmK with Sor led to a marked inhibition of caspase-7 activation and PARP cleavage (cl-PARP) (Fig. 2C). The pancaspase inhibitor blocked cell death in U-266 and RPMI 8226 cells but had little effect in the rest of the MM cell lines (Fig. 2D). In agreement with the flow cytometry data, zVAD.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 LP-1 and OPM-2 is not necrotic since co-treatment with Necrostatin1 did not
attenuate Sor-induced death (Fig. 2F and data not shown, respectively). These data suggests that Sor, in most of the cell lines examined, induces caspase-dependent cell death whereas in LP-1 and OMP2 cells the cell death is caspase-independent.

One of the key mediators of a caspase-independent cell death is the apoptosis-inducing factor (AIF) (17). We therefore examined the possibility that AIF was involved in the cell death signaling cascade induced in LP-1 and OPM-2 cells. In the panel of MM cell lines we assessed the AIF cleavage in response to Sor and found such a post-translational modification in LP-1 and OPM-2 cells (Fig. 2G). Isolation of mitochondria and cytoplasm from LP-1 cells treated with Sor and incubation with an anti-AIF antibody revealed that AIF is released to the cytoplasm in response to Sor treatment (Fig. 2H). The translocation of AIF was further demonstrated by immunofluorescence (Fig. 2I). In LP-1 and OPM-2 cells treated with Sor, AIF was present in the nucleus of 20-30% of the cells that also exhibited cytochrome c release and nuclear condensation. Overall these data demonstrate that both caspase-dependent and independent cell death pathways are activated in MM cells treated with sorafenib.

**Sorafenib induces autophagy in MM 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 4EBP1. Sor treatment of U266, LP-1 and RPMI 8226 cell lines led to the inhibition of 4EBP1 phosphorylation suggesting that the mTOR activity is inhibited in these cell lines (Fig 3A). Importantly, Sor 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 (Fig. 3A, 3B, 5G and 7D).
Pretreatment of LP-1 with chloroquine (CQ), a classical inhibitor of late autophagy, followed by Sor 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). Pre-treatment of MM cell lines with chemical inhibitors of early (3MA) and late autophagy (CQ) on Sor-induced cell death led to a potentiation of Sor-induced cell death in LP-1, OPM-2 and H929 cells (Fig. 3D). Further support on the involvement of mTOR in Sor-induced autophagy came from the experiments with the mTOR inhibitor, rapamycin (Rapa), in combination with Sor. Treatment of LP-1 and OPM-2 cells with Rapa and Sor partially protected these MM cell lines from Sor-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 Sor.

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

It has been previously described that PI3K-AKT and Ras-Raf-MAPK kinase (MEK)-extracellular signal-regulated kinase (ERK1/2) pathway play a critical role in the survival of MM cells (18). We examined the levels of AKT and ERK1/2 serine phosphorylation and the possibility that Sor acts by inhibiting these pathways in the panel of human MM cell lines. OPM-2 cells, which are PTEN null, display a constitutively active AKT that may be partially inhibited by Sor (Fig. 4A). In LP-1, H929 and RPMI 8226 cells, AKT phosphorylation was induced by Sor. ERK1/2 was constitutively active in U-266 and LP-1 cells and this phosphorylation is potently inhibited by Sor 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 MM cells undergoing cell death was also examined (19). LP-1 does not express Bim due biallelic deletion of this gene (20). However, in all remaining MM cell lines of the panel, Bim was downregulated. This is in agreement with the induction of AKT phosphorylation observed in
response to Sor. These data suggest that Bim is not an important mediator of Sor-induced cell death in MM 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 (21, 22). Mcl-1 is one of the major anti-apoptotic proteins expressed in MM cells, and resistance to drug treatment has been associated with high levels of Mcl-1 expression. To determine whether Sor-induced cell death was associated with modulation of Mcl-1 expression, we investigated the effect of Sor 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 8h of treatment (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 Sor (Fig. 4C). Treatment of the MM cell lines with U0126 alone did not induce cell death in the MM cell lines and the combination with Sor did not influence the efficacy of Sor as compared to Sor alone (Fig. 4D). Collectively, these data demonstrate that the observed Mcl-1 downregulation by Sor is independent of the Raf/MEK/ERK1/2 pathway.

To gain further insights into the mechanism by which Sor 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 (23-25). We found that post-translational modifications are not responsible for the downregulation of Mcl-1 levels in cells treated with Sor (Supplementary Fig. 2). Interestingly, despite the decrease in total Mcl-1 protein levels observed in MM cells treated with Sor, Mcl-1 mRNA is induced after 8 and 24h (Fig. 4E). It has been previously shown that the effects of Sor on protein levels maybe mediated by inhibition of phosphorylation of components of the
translational machinery (26). Notably, treatment with Sor 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 Sor 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 Sor-induced cell death in MM cell lines.

Sorafenib induces cell death ex vivo in the presence of BM stromal cells

The efficacy of Sor ex vivo was examined in freshly isolated CD138+MM cells from newly diagnosed MM patients (Table1). The CD138+ enriched primary MM cells from patient 1 and 2 were treated with increasing concentrations of Sor for 24 and 48h, respectively. We found that Sor displayed similar effects on these primary cells as in the cell lines (Fig. 5A). The cytotoxic effects of Sor on peripheral blood mononuclear cells (PBMC) from a healthy donor were measured by Annexin V staining and showed a 20% increase in cell death compared to control after 48h of treatment ((Fig. 5B).

To investigate the role of soluble factors secreted by the tumor microenvironment we used a transwell co-culturing system between L88 BM stromal cells (BMSCs) and U-266, RPMI 8226 and OPM-2 cells. Since, the effect of the stroma on MM cell survival is largely dependent on their response to IL-6, we anticipated the effects of stroma on Sor cytotoxic efficacy to be IL-6 dependent. Indeed, in the IL-6 dependent cells RPMI 8226, Sor-induced cell death was potentiated when co-cultured with BMSCs (Fig. 5C). In contrast the Sor-induced cell death was equally efficient in the IL-6 independent U266 in mono or co-culture. Downstream of the activated receptor tyrosine kinases, a number of common signaling pathways converge, i.e. the
Janus activated kinase (Jak)-signal transducer and activator of transcription (STAT), the phosphatidylinositol 3-kinase (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 co-culture correlated well with the protection mediated by the BMSC from Sor-induced death in RPMI 8226 cells (Fig. 5D). The potentiation of cell death in co-culture 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 Sor (Fig. 5D).

Having established that 10 µM Sor is equally efficient in the treatment of primary MM patient samples as was shown for the cells in the MM cell line panel, samples from 4 additional MM patients were treated in mono-culture or in BMSC co-culture with Sor for 24 and 48h and the levels of cell death were measured by performing CD138+/AnnexinV+ double stainings to immunophenotypically detect MM cells and measure cell death (Fig. 5E and 5F). Sor was very potent in inducing cell death in all the primary samples cultured in mono-culture. The co-culture setting, L88 cells protected the CD138+ primary MM cells from spontaneous cell death but they were still sensitive to Sor treatment (Fig. 5E and 5F). Due to the lack of sufficient amount of cells, samples from patients 5 and 6 were cultured only in the presence of L88 cells for 48h and we found that MM cell from patient 5 were very sensitive to Sor and fifty percent of MM cells from patient 6 died upon sorafenib treatment (Fig. 5F). To determine whether the molecular determinants of Sor efficacy identified in MM cell lines were also found in patient samples, we treated a BM sample, ex vivo, with Sor and examined the proteins levels of Mcl-1, the cleavage of PARP and the levels of autophagy (Fig. 5G). We found that Sor downregulates Mcl-1, induces PARP cleavage and autophagy in these MM primary patient cells, in concordance with the cell line experiments.
ABT737 improves the efficacy of Sorafenib against MM cells in vitro and ex vivo

Together with Mcl-1, Bcl-2 and Bcl-xL constitute the major anti-apoptotic 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 Sor 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 Sor demonstrating the importance of Bcl-2 in the protection of MM cells to Sor-induced cell death (Fig. 6B).

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

The efficacy of this combination therapy was also examined in the co-culture setting with L88 and the U-266, RPMI 8226 and OPM-2 MM cell lines. Even though RPMI 8226 were not sensitive to Sor in the presence of BMSCs (Fig. 5A), ABT737 alone or the combination with Sor with ABT737 overcame this protection and induced high levels of cell death (Fig. 6D). Furthermore, ABT737 potentiated the efficacy of Sor in OMP2 cells, even in the presence of L88 cells (Fig. 6D).

Based on the efficacy observed in MM cell lines treated with the combination of Sor and ABT737, two CD138+ MM 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 Sor with ABT737 showed potent efficacy in killing primary CD138+ enriched MM cells. Collectively, these data show that this rationale-based combination is effective in the treatment of MM cells both in vitro and ex vivo.
Bortezomib (BTZ) is now introduced as a therapeutic modality against MM (27). Despite its promising therapeutic effects, patients develop resistance to this proteasome inhibitor (27). To investigate whether Sor treatment is effective against BTZ resistant cells we utilized a recently published model system of RPMI 8226 cells that have been induced to develop resistance to BTZ (28). BTZ sensitive RPMI 8226 and BTZ resistant RPMI 8226/7B680 were grown in the presence of 80 nM BTZ and treated with either Sor alone or the combination of Sor with ABT737. Importantly, Sor induced cell death in BTZ-resistant RPMI 8226/7B680 cells and the combination with ABT737 improved the cytotoxic potency of Sor (Fig 6F). These results were recapitulated in primary MM cells from patients that have undergone treatment with BTZ and found to be non-responsive (Fig. 6G).

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

To investigate the efficacy of sorafenib in murine MM cells we performed a dose curve and found that it induces dose and time dependent cell death in 5T33MM cells *in vitro* (Supplementary Fig. 3A). We also examined whether the targeted signaling cascades by sorafenib in human cell lines and patient samples, were also observed in the mouse 5T33MM cell line. In agreement with our findings in the human setting we found that sorafenib inhibits ERK phosphorylation, downregulates Mcl-1 levels and induces autophagy in 5T33MM cell line and cells (Supplementary Fig. 3B).

Having shown that Sorafenib significantly induce apoptosis in MM cells *in vitro*, we next examined the *in vivo* efficacy using the 5T33MM mouse model. C57BL/KaLwRijHsd mice inoculated with 5T33MMvv cells were either assigned to receive Sorafenib or vehicle. Mice treated with Sorafenib showed a significant (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 16-34% while
mice who were terminally diseased at earlier time points had a tumor burden in the bone marrow ranging from 52-97%, indicating that the mice at the last 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 BM was observed in the Sorafenib treated mice when compared to the vehicle treated mice (Fig. 7B, 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 Mm cells isolated from the bone marrow of the 5T33MM mice (Fig. 7D). In the haematoxylin and eosin (H&E) stained sections of a bone marrow from a vehicle-treated mouse, the 5T33 myeloma cells have massively invaded the bone marrow accompanied by rich vascular network and no residual normal haematopoiesis (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 microvessel density with the majority of the vessels compressed by the myeloma infiltrate. Treatment with Sorafenib significantly decreased microvessel density (MVD) by 60% in the BM of treated 5T33MMvv mouse, compared to vehicle-treated 5T33MMvv mice (Fig. 7F and G). No adverse side effects or toxicity were observed as evaluated by behavior, body weight, histological and hematological examinations (data not shown).

Discussion

Receptor and non-receptor tyrosine kinase ((R)TKs) activated signaling cascades play a vital role in the survival and proliferation of MM cells (29). The importance of these (R)TKs for MM carcinogenesis and progression is further underlined by the initiation of a number of clinical
trials in which various tyrosine kinase inhibitors (TKIs) are used alone or in combination with conventional and emerging anti-MM agents. Sorafenib has shown promising results against a number of solid tumors (e.g. renal carcinoma and hepatocellular carcinoma); however, its potential efficacy in hematological malignances and especially in MM has received little attention. Also in the light of the recent publication of whole genome sequencing of revealing 12 of the most common \textit{BRAF} mutations in 4\% of MM patients targeting this pathway i.e. the use of sorafenib seems to constitute a promising novel path of therapy (30).

The described molecular targets for sorafenib are Raf1, PDGFR, VEGFR, FLT3 and c-KIT, several of which have been shown to be active in MM (18). The MM cell lines selected represent the genetic heterogeneity seen in MM and sorafenib is likely to target at least one predominant pathway of MM survival. Since 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 LP-1, whereas in OPM-2 the PI3K/AKT pathway is affected by sorafenib. The predominant pathway of survival in U266 is Stat3 while in the OPM-2, a constitutively active AKT, due to PTEN deletion, may be 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 (31). The mechanism and the biological 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 (31). It may also be possible that the observed AKT phosphorylation is mediated by another tyrosine kinase receptor 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 tyrosine kinase receptor and its downstream signaling cascade PI3K/AKT (32).
The anti-tumoral 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 anti-angiogenic effects of sorafenib were found to be partly responsible for the potent anti-tumor activity observed in the clinic (33, 34). In the MM setting, we found that sorafenib significantly decreased microvessel density in the bone marrow of MM mice and indicating that the anti-tumoral 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 MM 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 caspase-independent cell death in the majority of the MM cell lines and MM primary cells tested, results that are in agreement with a previous report (31). Interestingly, LP-1 and OPM-2 cells, even though they exhibited all the classical characteristics of apoptosis, were not rescued by the pre-incubation with the pancaspase inhibitor zVAD.fmk indicating that several pathways of cell death are activated by sorafenib. For example, LP-1 and OPM-2 are two 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 MM 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 tyrosine kinase inhibitors has been described previously for imatinib mesylate (35). In our experimental system, sorafenib induced autophagy in several of the cell lines tested, in primary MM patient samples and in mice. By using chemical inhibitors of early and late autophagy, it became evident that autophagy is induced to protect MM cells from sorafenib-mediated cell death. Importantly, rapamycin, a potent inducer of autophagy, partially protected certain MM 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 MM, may be the combination of sorafenib with autophagy inhibitors such as chloroquine, a combination that at least, in vitro, demonstrated potent cytotoxic activity.

The tumor stroma has been implicated in modulating not only tumor growth but also the acquisition of resistance to cancer therapy. The impact of the tumor stroma in the survival of MM cells in vitro and ex vivo was confirmed in our experiments. In particular, STAT3 was highly phosphorylated /activated in the co-culture setting as compared to the mono-culture setting. A reasonable explanation for this signature is the response in selected MM cell lines to IL-6 produced from the stroma. The molecular re-wiring of MM cell lines and primary cultures grown under the paracrine influence of the BM stroma cells is firstly observed by the lower levels of basal death. This response is well associated with the activation of all the signaling cascades examined, i.e. RAF/MEK/ERK1/2, PI3K/AKT and JAK/STAT3 pathway which promote the induced expression of a number of anti-apoptotic proteins such as Mcl-1 and Bcl-2/Bcl-xL. The responsiveness of the MM cell lines and primary samples to sorafenib co-cultured with BM stromal cells is variable. RPMI 8226 cells and primary cells from patient 3 were protected by sorafenib in the presence of soluble factors secreted by the stromal cells. One explanation for this protection could be the activation of STAT3 by IL-6 and persistence of Mcl-1 and Bcl-xL protein levels even after treatment with sorafenib in the co-culture setting in this cell line. On the contrary, OPM-2 cells and cells from patient 4 were more sensitive to sorafenib when co-cultured with sorafenib and stromal cells, a response that could be mediated by the inhibition of AKT levels and the induction of Bim expression.

The importance of Mcl-1 for MM survival is well described (36). It has been previously reported that sorafenib is highly effective in downregulating Mcl-1 in a number of other experimental models (21, 37). Sorafenib downregulates the protein levels of Mcl-1 in MM, an event critical that we provide evidence for its importance in the execution of the cell death
program (38). Mcl-1 is known to be regulated at the transcriptional, translational and post-translation level. We found that post-translational 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 24h of treatment with sorafenib. The mechanism behind this upregulation is unclear. It is known that the JAK/STAT3 pathway induce the expression of Mcl-1 (39). However since 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 demonstrated that inhibition of translation is the most likely mechanism of Mcl-1 downregulation in leukemic cells (40). 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 MM cells (41). However the combination of sorafenib with ABT737 with the rational to concomitantly target all three major Bcl-2 anti-apoptotic proteins (i.e. Mcl-1, Bcl-2 and Bcl-xL) and thereby tilting the Bcl-2 rheostat towards cell death has not been previously shown. Indeed, we found that in all the MM cell lines and in primary samples tested, the combination of these two drugs had better therapeutic efficacy than treatment of sorafenib or ABT737 alone. Importantly this combination was very effective in primary MM patient samples.

In summary, our study provides compelling evidence for the future evaluation of the multi-tyrosine kinase inhibitor, sorafenib as a new therapeutic strategy for the treatment of MM in the clinic.
Acknowledgments. We would like to thank Carine Seynaeve and Marie Joos Ter Beerst for excellent technical assistance and the following people for kindly providing constructs and cell lines that were used in this study: Dr Patrik Auburger for the Mcl-1 plasmid (Université de Nice Sophia Antipolis, France); Dr Francesco Bertolini (European Institute of Oncology, Italy) for the L88 BM stromal cells; Dr Noboru Mizushima (The Tokyo Metropolitan Institute of Medical Science, Japan) for the LC3-GFP plasmid (42); Dr Katja Pokrovskaja (Karolinska Institutet, Sweden) for the Bcl-2 overexpressing U-266 cells; Dr Javier Naval for the wild type RPMI 8226 and the bortezomib resistant 8226/7B680 cell lines (Department of Bioquimica y Biologia Molecular y Celular Facultad de Ciencias, Universidad de Zaragoza, Spain).

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References


Figure legends

Figure 1. Sorafenib induces cell death in human Multiple Myeloma cell lines. A, Flow cytometric analysis of Annexin V/PI negative U-266, LP-1, OPM-2, NCI-H929, RPMI 8226 and Karpas 620 cells treated with the indicated concentrations of Sor for 24h and measured by flow cytometry (means ± SD, n≥3); B, U-266, LP-1, OPM-2, NCI-H929, RPMI 8226 and Karpas 620 treated with 10 µM Sor for the indicated time points were analysed for Annexin V/PI negative cells by flow cytometry (means ± SD, n≥3). C, Immunofluorescent staining of mitochondrial membrane potential (appearing in red, Mitotracker) and active Bak (appearing in green, FITC) in the indicated cell lines treated with 10 µM Sor for 24h (scale bar = 2 µm). D, Quantitative analysis of active Bak and active caspase-3 stained cells from the indicated cell lines treated with 10 µM Sor for 24h and measured by flow cytometry. E, Immunofluorescent staining of the indicated cell lines for mitochondrial membrane potential (appearing in red, Mitotracker) and cytochrome c (appearing in green, FITC) treated with 10 µM Sor for 24h (scale bar = 2 µm). F, The indicated cell lines were treated with 10 µM Sor for 24h and the percentage of cells with Annexin V/TMRE were quantified. G, Counting of cells with released cytochrome c in the indicated MM cell lines treated with 10 µM Sor for 24h (means ± SD, n: 3 fields of 100 cells).

Figure 2. Sorafenib induces caspase-dependent and independent cell death. A, Immunoblot analysis of active caspase-3 in the indicated MM cell lines treated with 10 µM Sor for 24h. B, Time course of caspase-7 activation detected by immunoblotting in the indicated cell lines treated with 10 µM Sor for 24h. C, Immunoblot analysis of PARP cleavage and caspase-7 activation in U-266 and LP-1 treated with 10 µM Sor in the presence or absence of 10 µM zVAD.fmk. D, Quantitative analysis of Annexin V/PI stained cells measured by flow cytometry in the indicated cell MM lines treated with 10 µM Sor for 24h (means ± SD, n≥3). E, Immunofluorescent analysis of nuclei and Annexin V positive cells and quantification of this
stainings in the indicated MM cell lines upon treatment with 10 µM Sor for 24h in the presence or absence of 10 µM zVAD.fmK. F, Flow cytometric analysis of Annexin V/PI positivity in the indicated cell lines treated with Sor 10µM for 24h in the presence or absence of 10 µM zVAD.fmK or 50 µM Necrostatin1 (Nec1) (means ± SD, n≥3). G, Immunoblot analysis of total and cleaved AIF in the indicated MM cell lines treated with 10 µM Sor for 24h. H, Subcellular fractionation and detection of AIF and TOM40 by immunoblotting in LP-1 cells treated with 10 µM Sor for 24h. I, Immunofluorescence detection of AIF (appearing in green, FITC) and the cytochrome c (appearing in red, Alexa 568) in LP-1 and OMP2 cells treated with 10 µM Sor for 24h. The percentage of dead cells with nuclear AIF (nAIF) or without nuclear AIF (w/o nAIF) was counted.

**Figure 3. Sorafenib induces autophagy in Multiple Myeloma cell lines.** A, Immunoblot analysis of the indicated autophagic markers in U-266, LP-1, OPM-2, NCI-H929, RPMI 8226 treated with 10 µM Sor for 24h; B, Immunofluorescent staining and quantification of LC3 positive cells in the indicated cell lines treated with 10 µM Sor for 24h. C, Immunoblot analysis of p62, LC3 and active caspase-7 in U-266 and LP-1 cells treated with 10 µM Sor for 24h in the presence or absence of 10 µM Chloroquine (CQ) or 10 µM zVAD.fmK. D, Quantitative analysis of Annexin V/PI positive cells in the indicated MM cell lines treated with 10 µM in the presence or absence of 5 mM 3-Methyladenine (3MA) or CQ (means ± SD, n≥3). E, Quantitative analysis of Annexin V/PI positive cells in the indicated MM cell lines treated with 10 µM Sor for 24h in the presence or absence of 1 µM Rapamycin (Rapa) (means ± SD, n≥3).

**Fig. 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 MM cell lines treated with 10 µM Sor for 24h. B, Immunoblot analysis of the Mcl-1 levels in U-266 and LP-1 treated
with 10 µM Sor for the indicated time points. C, Immunoblot analysis of the indicated proteins in U-266 and LP-1 pre-treated with 10 µM U0126 followed by 10 µM Sor, 24h. D, Quantitative analysis of Annexin V/PI positive cells in the indicated MM cell lines pre-treated with 10 µM U0126 followed by 10 µM Sor for 24h (means ± SD, n≥3). E, Quantitative PCR analysis of Mcl-1 in H929 and LP-1 treated with 10 µM Sor for 8 and 24h. F, Immunoblot analysis of phosphorylated and total eIF4E in the panel of MM cell lines treated with 10 µM Sor for 24h; 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 µM Sor for 24h. 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 µM Sor for 24h (means ± SD, n≥3, p<0.05).

Figure 5. BM stromal cells do not rescue MM cell from sorafenib induced cell death in vitro and ex vivo. A, Quantitative analysis of cell viability of CD138+ enriched MM cells from two newly diagnosed patient (Patient 1 and 2) treated with the indicated doses of Sor for 24h and 48h, respectively. B, Quantitative analysis of Annexin V positive cells in PBMC treated with 10 µM Sor for 24 and 48h. C, Quantitative analysis of Annexin V/PI positivity in U-266, RPMI 8226 and OMP2 cultured alone or co-cultured in inserts with L88 BM stromal cells and treated with 10 µM Sor for 24h (means ± SD, n≥3). D, Immunoblot analysis of the indicated proteins in U-266, RPMI 8226 and OPM-2 alone or co-cultured with L88 and treated with 10 µM Sor for 24h. E, Quantitative analysis of Annexin V positive cells of CD138+ enriched MM cells from the indicated newly diagnosed patients treated with the 10 µM Sor for 24h (E) or 48h (F). G, Immunoblot analysis of Mcl-1, cleaved PARP and LC3 lipidation in BM derived CD138 positive cells from a patient in relapse (Patient 7).
Figure 6. Combination of sorafenib with ABT737 in MM treatment. A, Immunoblot analysis of Bcl-2 and Bcl-xL proteins levels in the indicated MM cell lines treated with 10 µM Sor for 24h, B, Quantitative analysis of Annexin V positive cells in mock or Bcl-2 overexpressing U-266 cells treated with the 10 µM Sor for 24h (means ± SD, n≥3, *p<0.05). C, Quantitative analysis of Annexin V/PI positive cells in the indicated MM cell lines treated with 10 µM Sor alone or in combination with 10 µM ABT737 for 24h (means ± SD, n≥3, *p<0.05, **p<0.01). D, Quantitative analysis of Annexin V/PI positive cells in the indicated cell lines cultured alone or co-cultured with L88 and treated with 10 µM Sor in the presence or absence of 10 µM ABT737 for 24h (means ± SD, n≥3). E, Quantitative analysis of cell viability in CD138 positive MM cells from one patient in relapse and one newly diagnosed patient (Patients 7 and 8, respectively) treated with 10 µM Sor alone or in combination with 10 µM ABT737 for 24h and 48h. F, Quantitative analysis of Annexin V positive cells in bortezomib-sensitive RPM 8226 cells and bortezomib resistant RPMI 8226/7B680 cells treated with 10 µM Sor in the presence or absence of 10 µM ABT737 for 48h (means ± SD, n≥3). G, Quantitative analysis of cell viability in CD138 positive MM cells from two bortezomib-refractory patients treated with 10 µM Sor for 24h.

Figure 7. Sorafenib shows potent anti-tumoral activity in the 5T33MM syngeneic mouse model. A. Mice were inoculated with 5T3MM 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 5T33MM cells of total cell number (n=10 for each group, ***P <0.002). D, Immunoblot analysis of the indicated proteins from MM cells isolated from the bone marrow of 5T33MM mice treated with 10 µM Sor for 24h. E, Haematoxylin and eosin stained section of
bone marrow from naïve (left image), vehicle-treated mouse (center image) and from a Sor-treated mouse (right image). 

F, Images from the immunohistochemical stain for CD31 in the bone marrow of myeloma bearing mice treated with either vehicle or Sor. In the area delineated by the rectangle, the bone marrow is not invaded by myeloma cells. 

G, Quantitative analysis of the number of microvessels (microvessel density, MVD), counted by CD31 immunohistochemical staining, in the bone marrow of myeloma bearing mice treated with either vehicle or Sor the mice. (means +/-SD for groups of 9 mice are shown; * P<0.001, **P <0.0005).

**Supplementary Figure 1. Cell cycle analysis of Myeloma cells treated with sorafenib.**

The indicated myeloma cell lines were treated with 10 µM Sor for 24h and cell cycle distribution was analysed by NucleoCounter® NC-3000™ (Chemometec).

**Supplementary Figure 2.** Immunoblot analysis of the indicated proteins in U-266 and LP-1 pre-treated with 10 µM CQ, 10 µM zVAD.fmkk or 4 µM MG132 (for 4h) followed by 10 µM Sor for 24h.

**Supplementary Figure 3.** A, Quantitative analysis of Annexin V/PI positive murine 5T33MMvitro cells treated the indicated concentrations of sorafenib for 24h and 48h; B, Immunoblot analysis of the indicated proteins from the 5T33MMvitro cell line treated with 10 µM Sor for the indicated time points. C, Immunoblot analysis of the indicated proteins from murine 5T33MMvivo treated ex-vivo with 10 µM Sor for 24h.
Table 1. Myeloma patient sample characteristics.

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ISS: International Staging System.
Figure 1

(A) % AnnV- / PI- cells vs Sor (µM)

(B) % AnnV- / PI- cells vs Duration of treatment (h)

(C) U266 and LP-1 cells showing control and Sor (10 µM) treated conditions with DAPI, Mitotracker, and aBak staining.

(D) Bar graph showing % cells with aBak+/aC3+, aBak-/aC3+, and aBak+/aC3- in U266, LP-1, and OPM2 cells.

(E) U266 and LP-1 cells showing control and Sor (10 µM) treated conditions with DAPI, Mitotracker, and Cyt C staining.

(F) Bar graph showing % cells with AnnV+ TMRE- and AnnV+ TMRE+ in U266, LP-1, OPM2, H929, RPMI 8226 cells.

(G) Bar graph showing % cells with released Cyt C in control and Sor treated conditions in U266, LP-1, OPM2, H929, RPMI 8226 cells.
Figure 2

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% cells with dead nuclei

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- DAPI
- AIF
- Cyt c

% dead cells

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Figure 4

A. Western blots showing the expression levels of various proteins under different conditions. The blots include pS-AKT, AKT, pERK1/2, ERK1/2, Mcl-1, BimEL, BimL, BimS, pBAD, BAD, and -actin.

B. Graph showing the percentage of cells with Annexin V+ and PI+ under different conditions. The graph includes data for U266, LP-1, OPM2, H929, and RPMI.

C. Western blots comparing the expression levels of pERK1/2, ERK1/2, Mcl-1, and active caspase-3 between U266 and LP-1 under different conditions.

D. Bar graph showing the fold change in Mcl1 long/actin under different conditions. The graph includes data for U266, LP-1, OPM2, H929, and RPMI.

E. Bar graph showing the fold change in p-eIF4E/actin under different conditions. The graph includes data for U266, LP-1, OPM2, H929, and RPMI.

F. Western blot showing the expression levels of cl-PARP, active PARP, and p-eIF4E/actin under different conditions.

G. Western blot showing the expression levels of Mcl-1, cl-PARP, active caspase-3, and -actin under different conditions.

H. Bar graph showing the fold change in Annexin V+ cells relative to control under different conditions. The graph includes data for PCDNA wt Mcl-1, PCDNA wt Mcl-1, OPM2, and RPMI.
Figure 7

A. Percent survival (%) over time in days for Naive, Vehicle, and Sorafenib (Sor) groups.

B. Box plot showing paraprotein concentration (g/dl) for Naive, Vehicle, and Sorafenib (Sor) groups.

C. Box plot for plasma cytosis (%) showing differences between Naive, Vehicle, and Sorafenib (Sor) groups.

D. Immunoblot analysis of 5T33MM cells with 10% sorafenib (Sor) showing expression levels of pERK1/2, ERK1/2, Mcl-1, LC3-I, GAPDH, and active caspase-3.

E. Histological images of Naive, Vehicle, and Sorafenib (Sor) groups showing the effect on tumor growth.

F. Immunohistochemical analysis showing MVD (microvessel density) in Naive, Vehicle, and Sorafenib (Sor) groups.

G. Bar graph depicting MVD levels (units) for Naive, Vehicle, and Sorafenib (Sor) groups.
Sorafenib has potent anti-tumor activity in multiple myeloma in vitro, ex vivo and in vivo, in the 5T33MM mouse model.

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