Molecular Medicine

Sorafenib-Induced Apoptosis of Chronic Lymphocytic Leukemia Cells Is Associated with Downregulation of RAF and Myeloid Cell Leukemia Sequence 1 (Mcl-1)

Jessie-F Fecteau,[1] Ila S Bharati,[1] Morgan O'Hayre,[2] Tracy M Handel,[2] Thomas J Kipps,[1,3] and Davorka Messmer[1]

[1]Moores Cancer Center, University of California San Diego, La Jolla, California, United States of America; [2]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, California, United States of America; and [3]School of Medicine, University of California San Diego, La Jolla, California, United States of America

We have previously shown that sorafenib, a multikinase inhibitor, exhibits cytotoxic effects on chronic lymphocytic leukemia (CLL) cells. Because the cellular microenvironment can protect CLL cells from drug-induced apoptosis, it is important to evaluate the effect of novel drugs in this context. Here we characterized the in vitro cytotoxic effects of sorafenib on CLL cells and the underlying mechanism in the presence of marrow stromal cells (MSCs) and nurselike cells (NLCs). One single dose of 10 μmol/L or the repeated addition of 1 μmol/L sorafenib caused caspase-dependent apoptosis and reduced levels of phosphorylated B-RAF, C-RAF, extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription 3 (STAT3) and myeloid cell leukemia sequence 1 (Mcl-1) in CLL cells in the presence of the microenvironment. We show that the RAF/mitogen-activated protein kinase kinase (MEK)/ERK pathway can modulate Mcl-1 expression and contribute to CLL cell viability, thereby associating sorafenib cytotoxicity to its impact on RAF and Mcl-1. To evaluate if the other targets of sorafenib can affect CLL cell viability and contribute to sorafenib-mediated cytotoxicity, we tested the sensitivity of CLL cells to several kinase inhibitors specific for these targets. Our data show that RAF and vascular endothelial growth factor receptor (VEGFR) but not KIT, platelet-derived growth factor receptor (PDGFR) and FMS-like tyrosine kinase 3 (FLT3) are critical for CLL cell viability. Taken together, our data suggest that sorafenib exerts its cytotoxic effect likely via inhibition of the VEGFR and RAF/MEK/ERK pathways, both of which can modulate Mcl-1 expression in CLL cells. Furthermore, sorafenib induced apoptosis of CLL cells from fludarabine refractory patients in the presence of NLCs or MSCs. Our results warrant further clinical exploration of sorafenib in CLL.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world, leading to approximately 5,000 deaths annually (1). CLL is characterized by an accumulation of monoclonal mature B cells in the blood, secondary lymphoid tissues and the marrow. Despite major advances in the field, there is no curative therapy for CLL to date, and new strategies are

needed (2). Current treatment approaches aim at achieving minimal residual disease, which is associated with superior long-term outcome (3). The frontline therapy for CLL is the purine analog fludarabine. However, 30% of patients treated with fludarabine do not achieve complete remission, even when used in combination with other agents (2). To improve this outcome, other treatment avenues, such as those

targeting pathways downstream of the B-cell receptor, are currently being evaluated in preclinical and early clinical trials (3).

More recently, new therapeutic strategies have been designed to abrogate the prosurvival interaction of CLL cells with their microenvironment and the related signaling pathways. Accessory cells such as nurselike cells (NLCs) and marrow stromal cells (MSCs) protect CLL cells from drug-induced apoptosis in vitro (4). Thus, it has been postulated that CLL cells receive survival signals from these accessory cells, which constitute part of the CLL B-cell microenvironment in secondary lymphoid tissue and marrow (5,6). Such niches could protect leukemia cells from spontaneous or drug-induced apoptosis in vivo. Therefore, it is essential when in-

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vestigating novel CLL drugs *in vitro* to test them in the context of the microenvironment.

Exposure of CLL cells to proteins released from cells of the microenvironment causes activation of the extracellular signal-regulated kinase (ERK) signaling pathway, which is an important mediator of CLL cell survival (7,8) and thus an attractive drug target. These proteins include chemokine (C-X-C motif) ligand 12 (CXCL12) and chemokine (C-C motif) ligand 19/21 (CCL19/CCL21), which signal through their respective receptors, CXCR4 and CCR7. We have previously shown that sorafenib (BAY 43-9006, Nexavar), an orally active multikinase inhibitor that targets RAF kinases, as well as several receptor tyrosine kinases (9), prevents CXCL12-mediated upregulation of the active form of mitogen-activated protein kinase kinase (MEK) and ERK in CLL cells and causes cell death (7). Sorafenib causes apoptosis in leukemia cell lines and in blast cells from patients with acute myeloid leukemia (10) and displays a broad-spectrum antitumor activity in colon, breast and non–small-cell lung cancer xenograft models (11). Sorafenib was approved by the U.S. Food and Drug Administration for the treatment of patients with advanced renal cell carcinoma and unresectable hepatocellular carcinoma (12). As the first drug to improve the survival of patients with hepatocellular carcinoma, sorafenib is currently being tested in clinical trials for its efficacy in the treatment of other solid tumors such as thyroid carcinoma (12). Here we investigated the mechanism of sorafenib-mediated CLL cytotoxicity in the context of the cellular microenvironment.

MATERIALS AND METHODS

Isolation and Purification of CLL B Cells

Blood samples were collected from patients at the Moores University of California San Diego (UCSD) Cancer Center who satisfied diagnostic and immuno-phenotypic criteria for common B-cell CLL after providing written informed consent in compliance with the Declaration of Helsinki (13) and the institutional review board of UCSD. Peripheral blood mononuclear cells (PBMCs) were isolated from CLL patients by density centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), resuspended in 90% fetal calf serum (Omega Scientific, Tarzana, CA, USA) and 10% dimethyl-sulfoxide (DMSO) (Sigma) for viable storage in liquid nitrogen. If not otherwise indicated, the CLL cells were isolated from thawed PBMCs via negative selection using anti-CD2 and anti-CD14 magnetic beads (Miltenyi Biotechnology, Auburn, CA, USA).

Patients deemed refractory to fludarabine were defined as showing less than a partial response after completing a fludarabine regimen (14). Among the four patients selected, three presented with progressive disease and one presented with a stable disease after treatment. Cytogenetic analyses were available for three of the four patients tested and revealed that two patients presented no chromosomal abnormalities and one presented with 12q trisomy.

Reagents

Sorafenib was purchased from LC Laboratories (Woburn, MA, USA) and solubilized in DMSO. DMSO was used in all experiments as a vehicle control. Fludarabine monophosphate (F-ara-A), the MEK inhibitor PD98059 and the RAF inhibitor GW5074 were obtained from Sigma, whereas the caspase inhibitor Z-VAD-FMK was purchased from BD. The B-RAF and C-RAF inhibitor KG5, the control kinase inhibitor KG1 and vatalanib were provided by D Cheresh (University of California San Diego).

Generation of NLCs

PBMCs were isolated from the blood of normal volunteers (anonymously purchased from the San Diego Blood Bank) over a Ficoll density gradient (GE Healthcare, Piscataway, NJ, USA). CD14+ monocytes were isolated from PBMCs by positive selection using anti-CD14 beads (Miltenyi Biotech) following the manufacturer's instructions. To generate NLCs, 1.25×10^5/well CD14+ cells were cocultured with 3×10^6/well purified CLL B cells in 1 mL media in a 24-well plate (BD, Franklin Lakes, NJ, USA) in culture media (RPMI 1640 supplemented with 10 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid [HEPES] [GIBCO-BRL]), penicillin (100 U/mL)-streptomycin (100 μg/mL) (GIBCO-BRL) and 10% pooled human serum (Omega Scientific) for 12 d. At this point, CLL B cells were gently washed off, and the adherent NLCs were used for coculture experiments using freshly purified CLL cells.

Generation of MSCs

Mononuclear cells from marrow aspirates of CLL patients were isolated after centrifugation over a Ficoll density gradient. The cells were seeded at a density of 2×10^6 cells/mL in DMEM (Mediatech, Manassas, VA, USA) supplemented with 10% fetal calf serum (Omega Scientific), 10 mmol/L HEPES, 100 U/mL penicillin and 100 μg/mL streptomycin and cultured at 5% O_2 (using a Sanyo incubator, MCO-18M) for approximately 3 wks, with media renewal every week. The adherent MSCs were expanded and used for the coculture assays after two to six passages. MSCs were seeded between 5,000 and 10,000 cells/well in 24-well plates at least 1 d before the addition of CLL cells. Purified CLL cells were seeded at 1×10^6 cells/mL (1 mL/well) in the same media used to generate MSCs and were cultured at 5% O_2 with or without 10 μmol/L sorafenib and the appropriate DMSO control. Measurement of cell viability was performed at the indicated time points as described below. For MSC conditioned media preparation, MSCs were plated between 2,000 and 3,000 cells/cm^2 and incubated in the media described above for 7–9 d at 5% O_2, at which point the supernatant was collected, spun down and frozen at –20°C until further use. One hundred percent of conditioned media was used for CLL cell stimulation.
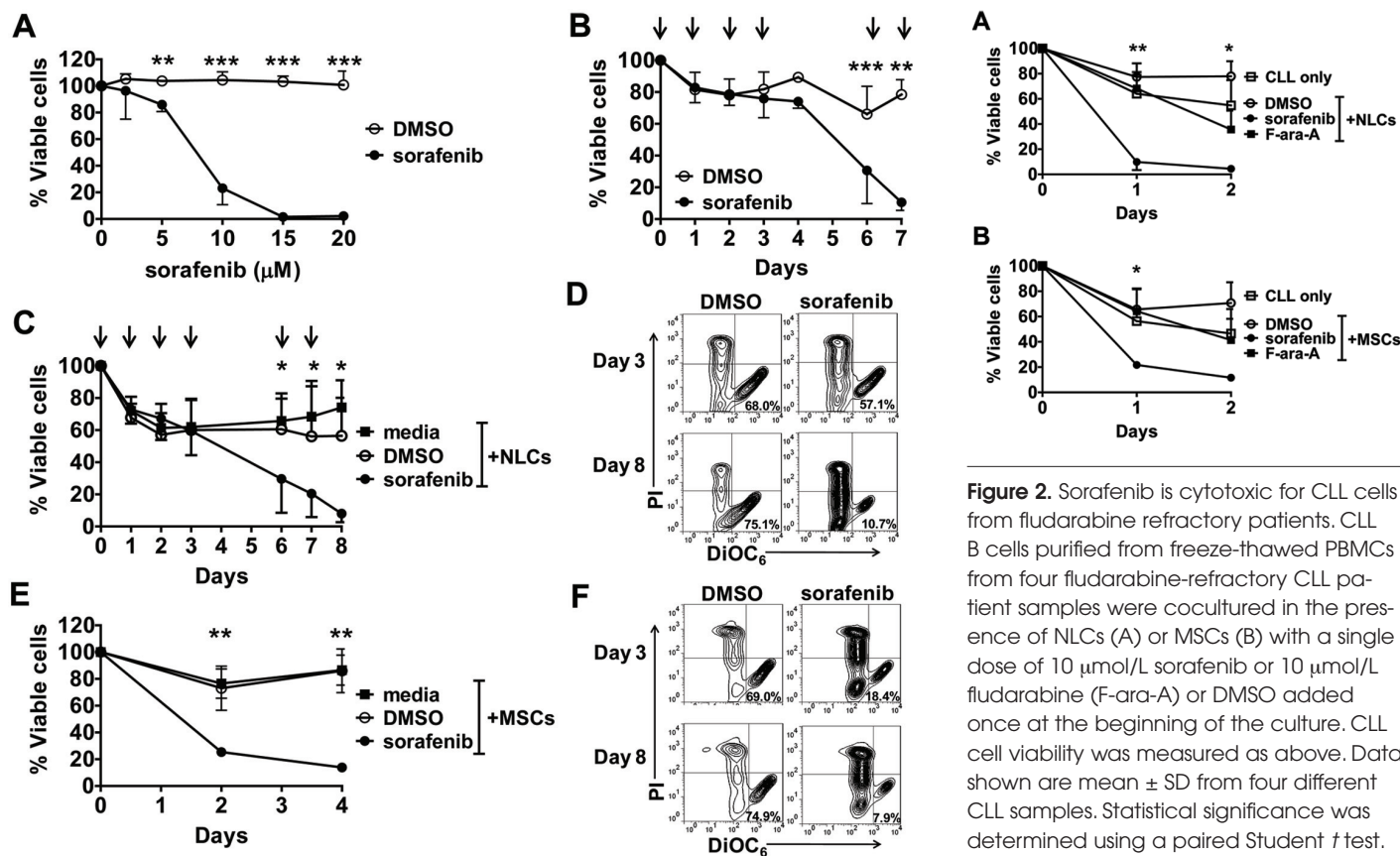
Figure 1. Sorafenib causes apoptosis of fresh and viably freeze-thawed CLL cells, in the presence of cells from the microenvironment. (A) CLL PBMCs were isolated from blood freshly drawn from CLL patients and exposed immediately after isolation to increasing doses of sorafenib. CLL cells were harvested after 48 h, stained with $DiOC_6$/PI and analyzed by flow cytometry to determine viability. The fraction of viable cells, identified as PI-negative and $DiOC_6$ bright cells, are represented, relative to d 0 (100%). Data shown are means ± SD from five different CLL samples. (B) Freshly isolated CLL PBMCs were exposed to 1 μmol/L sorafenib or equivalent volume of DMSO as vehicle control on d 0, 1, 2, 3, 6, 7 and 8, as indicated by the arrows. Cell viability was measured as above. Data shown are mean ± SD from eight different CLL samples. Statistically significant difference: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (paired Student *t* test). (C) CLL B cells were purified from freeze-thawed PBMCs and cultured in the presence of NLCs. Sorafenib (1 μmol/L) or DMSO was added to the cocultures on d 0, 1, 2, 3, 6 and 7, as indicated by the arrows. CLL cell viability was measured as above. Data shown are mean ± SD from four CLL samples. (D) Representative flow cytometry data for (C) is shown for one patient sample in the presence of sorafenib or DMSO control and NLCs (d 3 and 8). (E) CLL B cells purified from freeze-thawed PBMCs were cultured in the presence of MSCs and a single dose of sorafenib (10 μmol/L) or the equivalent volume of DMSO was added at d 0. CLL cell viability was measured and analyzed as above. Data shown are from two CLL samples cocultured with MSCs derived from two different CLL patients, for a total of four independent experiments (mean ± SD). (F) Representative flow cytometry data for (E) is shown for one patient sample in the presence of sorafenib or DMSO control and MSCs (d 2 and 4).

Figure 2. Sorafenib is cytotoxic for CLL cells from fludarabine refractory patients. CLL B cells purified from freeze-thawed PBMCs from four fludarabine-refractory CLL patient samples were cocultured in the presence of NLCs (A) or MSCs (B) with a single dose of 10 μmol/L sorafenib or 10 μmol/L fludarabine (F-ara-A) or DMSO added once at the beginning of the culture. CLL cell viability was measured as above. Data shown are mean ± SD from four different CLL samples. Statistical significance was determined using a paired Student *t* test.

Measurement of Cell Viability

Purified CLL cells were cultured at 1×10^6 cells/mL in 24-well plates (BD) under various conditions. Determination of CLL cell viability was on the basis of the analysis of mitochondrial transmembrane potential ($\Delta\Psi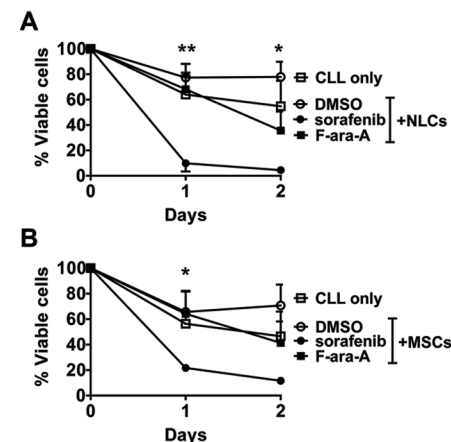_m$) using 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) (Invitrogen) and cell membrane permeability to propidium iodide (PI) (Sigma). For viability assays, 100 μL of the cell culture was collected at the indicated day and transferred to polypropylene tubes containing 100 μL of 40 μmol/L $DiOC_6$ and 10 μg/mL PI in culture media. The cells were then incubated at 37°C for 15 min and analyzed within 30 min by flow cytometry using a FACSCalibur (Becton Dickinson). Fluorescence was recorded at 525 nm for $DiOC_6$ and at 600 nm for PI. Data were analyzed using the FlowJo 7.2.2 software (Tree Star). The percentage of viable cells was determined by gating on PI-negative and $DiOC_6$ bright cells. When CLL cells were collected from cocultures in the presence of NLCs or MSCs, there were typically <5% supportive cells followed along with CLL cells. These supportive cells were excluded from the analysis on the basis of their significantly larger size using forward and side scatter analysis.

Immunoblot

For the evaluation of signal transducer and activator of transcription 3 (STAT3) expression levels after sorafenib exposure in the presence of MSCs, the culture media were replaced 1 d before the experiment on MSCs. On the day of the experiment, CLL cells were first serum-starved for 2 h in RPMI, followed by a pretreatment of 30 min with 10 µmol/L sorafenib or DMSO. At that point, CLL cells were spun down, and the cell pellet was resuspended in 24-h MSC-conditioned media, to which 10 µmol/L sorafenib or DMSO was added, and CLL cells were cocultured with MSCs for another 30 min. At that point, the CLL cells were collected for protein extraction as described below.

For the study of prosurvival proteins and modulation of the RAF/MEK/ERK pathway by sorafenib, CLL cells were exposed to 30 nmol/L CXCL12 (prepared as described by Messmer *et al.* [7]), NLCs or MSCs without prior starvation, and at the time of coculture, 10 µmol/L sorafenib or DMSO was added for 24 h. CLL cells were collected and the adherent NLCs or MSCs were left behind in the wells, as confirmed by bright-field microscopy. CLL cells were lysed for 20 min on ice with radioimmunoprecipitation assay lysis buffer (10 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mmol/L ethylenediaminetetraacetic acid supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, Halt phosphatase inhibitor [Thermo Fisher Scientific, Rockford, IL, USA], 1 mmol/L sodium vanadate, 1 mmol/L sodium fluoride and protease inhibitor cocktail [Roche Applied Science, Indianapolis, IN, USA]). Protein concentration was determined using the detergent compatible (DC) protein assay (Bio-Rad, Hercules, CA, USA). The lysates were snap-frozen and stored at –80ºC. Equal amounts of protein lysates (~20 or 30 µg) were separated by gel electrophoresis using a NuPAGE Novex 4–12% Bis-Tris Midi Gel (Invitrogen) and transferred to polyvinylidene fluoride membranes (Bio-

Rad). Membranes were washed with 1× Tris-buffered saline tween-20 (TBST), blocked for 1 h at room temperature in 5% milk/TBST and probed overnight for phospho-B-RAF (Ser445), B-RAF, Bcl-X$_L$, Bcl-2 interacting mediator of cell death (Bim), phospho-C-RAF (Ser338), C-RAF, phospho-p44/p42 (ERK1/2; Thr202/Tyr204), myeloid cell leukemia sequence 1 (Mcl-1), phospho-STAT3 (Tyr705), STAT3, β-actin or GAPDH, using antibodies from Cell Signaling Technology (Danvers, MA, USA), for Bcl-2 using an antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and for poly(ADP-ribose) polymerase (PARP) using an antibody from BD Biosciences. The next day, membranes were washed with 1× TBST and incubated with goat-anti-rabbit or anti-mouse horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) diluted to 1:12,000 to 1:15,000 in 5% milk/TBST for 1 h at room temperature. Antibodies were detected either using an enhanced chemiluminescence (ECL) detection kit (GE Healthcare) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). For densitometry analysis, the intensity of each band was determined using the free National Institutes of Health ImageJ software (http://rsbweb.nih.gov/ij), divided by the intensity of control protein (as indicated in the figure legends).

Statistical Analysis

Data are represented as means ± SD. Data were analyzed for statistical significance using the paired Student *t* test. *P* values <0.05 were considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001).

RESULTS

Sorafenib Causes Apoptosis of CLL Cells in the Presence of NLCs and MSCs

We previously observed that sorafenib interrupts CXCL12-induced activation of MEK and ERK in CLL cells and causes

cell death (7). CXCL12 is produced by the cellular microenvironment and functions as a survival signal for CLL cells (8,15). Here we evaluated the cytotoxic effect and the interrupted signaling pathways by sorafenib in a more complex microenvironment of CLL cells cocultured with MSCs and NLCs, which have been shown to protect CLL cells from spontaneous and chemotherapy-induced apoptosis (4,6,16). We first examined to what extent sorafenib is cytotoxic for freshly isolated CLL cells. A dose-dependent reduction in CLL cell viability was observed after exposure to sorafenib (Figure 1A), similarly to what we have previously reported with freeze-thawed CLL cells (7). Furthermore, repeated *in vitro* dosing of 1 µmol/L sorafenib to freshly isolated CLL cells strongly and significantly reduced CLL cell viability to 31 ± 21% (day 6) and 11 ± 5% (day 7) in the absence of support cells (Figure 1B; mean ± SD). Since data on fresh and freeze-thawed CLL cells showed no difference, all subsequent experiments were conducted using viably freeze-thawed CLL cells. In the presence of NLCs, the repeated addition of 1 µmol/L sorafenib also potently induced CLL cell apoptosis, leading to a fraction of viable CLL cells of 30 ± 21% (day 6), 20 ± 15% (day 7) and 8 ± 5% (day 8) (Figure 1C; mean ± SD, Figure 1D: representative flow cytometry data). For additional rigor to test sorafenib cytotoxicity, we also evaluated its effect in CLL cells cocultured with a different type of support cells derived from bone marrow of CLL patients: MSCs. Like NLCs, MSCs can protect CLL cells from spontaneous apoptosis *in vitro* (17,18) and are thought to closely mimic the microenvironment that CLL cells encounter *in vivo* in the marrow (19). As observed with NLCs, a single dose of 10 µmol/L sorafenib was cytotoxic for CLL cells, even in the presence of MSCs (Figures 1E, F). CLL cell viability rapidly and significantly declined in the presence of sorafenib, to 25 ± 3% (day 2) and 14 ± 3% (day 4) (Figure 1E, mean ± SD, Figure 1F: representative flow cytometry data).

CLL Cells from Fludarabine-Refractory Patients Are Sensitive to Sorafenib-Mediated Cytotoxicity, Even in the Presence of the Protective Microenvironment

The frontline therapy for CLL is the purine analog fludarabine (2). However, a fraction of CLL patients will become refractory to fludarabine and have only limited treatment options (14). Thus, we evaluated the cytotoxicity of sorafenib on CLL cells isolated from fludarabine-refractory patients. CLL cells from patients designated fludarabine refractory (see Materials and Methods) were exposed to fludarabine, sorafenib or a combination of both in the presence of NLCs or MSCs. Remarkably, the viability of CLL cells strongly declined after exposure to sorafenib after just 1 d, both in the presence of NLCs (Figure 2A) and MSCs (Figure 2B), whereas fludarabine had no significant impact on CLL cell survival at that time point. Sorafenib exposure reduced the fraction of viable CLL cells to $10 \pm 4\%$ (day 1) and $4 \pm 2\%$ (day 2) in the presence of NLCs (Figure 2A) and to $22 \pm 3\%$ (day 1) and $12 \pm 3\%$ (day 2) in the presence of MSCs (mean $\pm$ SD) (Figure 2B). Although fludarabine induced significant death in CLL cells after 2 d, it was to a lesser extent than sorafenib. These results suggest that sorafenib could be a possible second-line therapy for fludarabine-refractory patients.

Sorafenib-Mediated Apoptosis in CLL Cells is Caspase-Dependent

Sorafenib-mediated CLL cell death was marked by the loss of mitochondrial transmembrane potential (20) ($\Delta\psi_m$), as measured using $DiOC_6$ staining by flow cytometry (see Materials and Methods) in both the presence of NLCs and MSCs (Figure 3), indicating induction of apoptosis. To further corroborate that sorafenib induces CLL cell death via apoptosis, the cleavage of PARP was assessed (21). In line with the loss of the mitochondrial transmembrane potential, PARP cleavage was detected in CLL cells
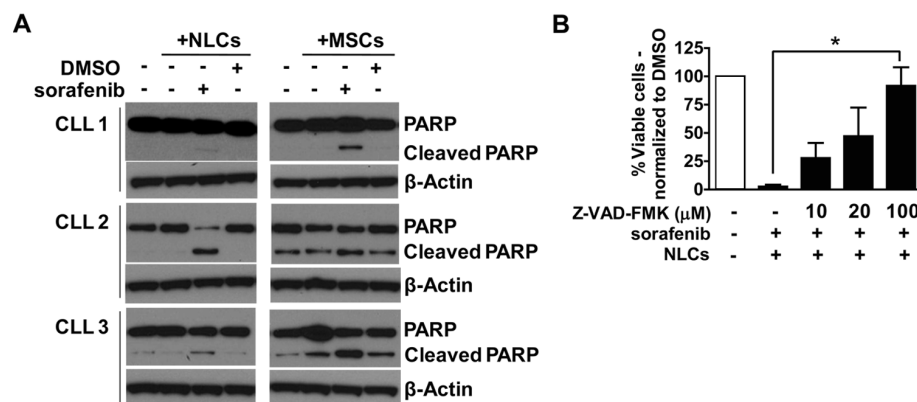

Figure 3. Sorafenib-mediated apoptosis in CLL cells is caspase-dependent. (A) CLL B cells purified from freeze-thawed PBMCs were cocultured with NLCs or MSCs for 24 h in the presence of 10 µmol/L sorafenib or DMSO, and lysates were analyzed for the cleaved fragment of PARP and β-actin as loading control by Western blot. (B) CLL B cells purified from freeze-thawed PBMCs were seeded on NLCs and incubated for 30 min with an increasing amount of the pan-caspase inhibitor Z-VAD-FMK, or the equivalent volume of DMSO. Subsequently, sorafenib (10 µmol/L) or DMSO was added, and the cocultures were incubated for 18 h, at which point the CLL cells were collected for viability measurement as in Figure 2. Results are represented relative to d 0 (100%). Data shown are mean ± SD from three different CLL samples. Statistical significance was determined using a paired Student *t* test.

from all patients tested after a 24-h exposure to sorafenib, both in the presence of NLCs or MSCs (Figure 3A), further supporting the induction of CLL cell apoptosis by sorafenib in conditions mimicking the CLL microenvironment *in vitro*. PARP cleavage in cells undergoing apoptosis can be attributed to the protease activity of the caspases (21). To investigate the involvement of caspases in sorafenib-mediated apoptosis in CLL, we used the pan-caspase inhibitor Z-VAD-FMK. Pretreatment of CLL cells with the caspase inhibitor rescued CLL cells from sorafenib-induced apoptosis (Figure 3B), suggesting that sorafenib induced caspase-dependent apoptosis in CLL cells.

Sorafenib Decreases Mcl-1 Expression in CLL Cells in the Presence of NLCs and MSCs

To investigate the underlying mechanism of sorafenib-mediated apoptosis in CLL cells, we assessed the expression levels of pro- and antiapoptotic proteins focusing on Bcl-2 family members, known to play a critical role in the regu-

lation of apoptosis (22). The effect of sorafenib on CLL cells was investigated in the context of CLL cells cultured alone, stimulated with CXCL12, cocultured with NLCs and MSCs or stimulated with MSC conditioned media (Figure 4). After 24 h of exposure to sorafenib, CLL cells when cultured alone showed decreased expression of the prosurvival protein Mcl-1 and in the proapoptotic Bcl-2 family member Bim, whereas no changes were observed in the levels of Bcl-2 and Bcl-X_L (Figure 4A). In CLL cells stimulated with CXCL12, sorafenib caused a decrease in Bcl-X_L levels and an increase in the cleaved, proapoptotic fragment of Bcl-2 (23) in addition to a decline in the levels of Mcl-1 and Bim (Figure 4B). When CLL cells were cocultured with NLCs or MSCs, sorafenib only caused a decline in Mcl-1 levels, whereas levels of Bcl-2 and Bcl-X_L remained constant, and a modest reduction in Bim levels was only observed in the presence of NLCs (Figure 4C). Sorafenib also consistently affected Mcl-1 protein levels in CLL cells stimulated with MSC-conditioned media
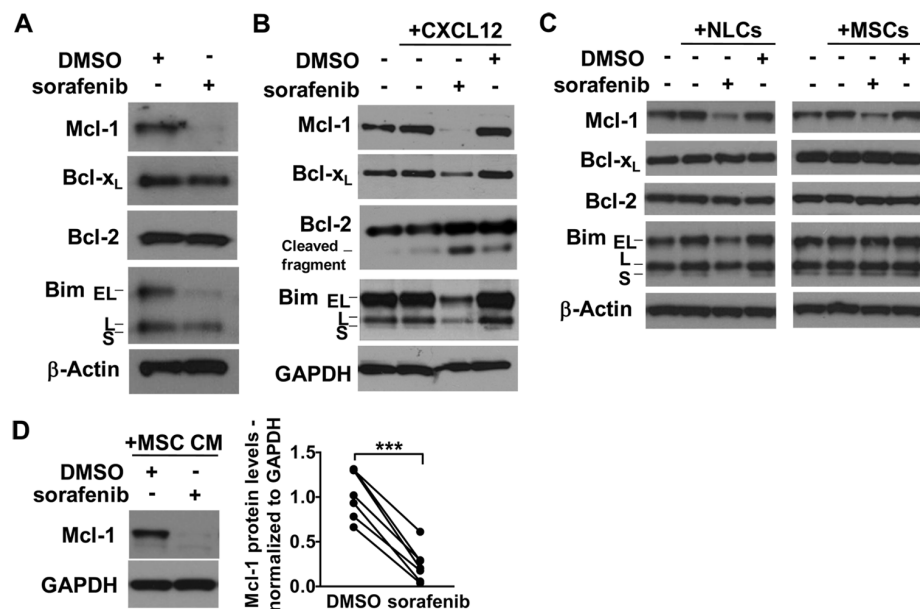
Figure 4. Sorafenib induces a downmodulation of the prosurvival molecule Mcl-1 in CLL cells cultured alone or in the presence of CXCL12, NLCs and MSCs. CLL B cells purified from freeze-thawed PBMCs were either cultured alone (A), in the presence of 30 nmol/L CXCL12 (B), or in the presence of NLCs or MSCs (C) and exposed to 10 μmol/L sorafenib or DMSO for 24 h. CLL cell lysates were analyzed for the expression of Mcl-1, Bcl-X$_L$, Bcl-2, Bim and β-actin. Mcl-1 downregulation was observed in five of six CLL samples and Bim downregulation in four of six CLL samples, and unchanged levels of Bcl-2 and Bcl-xL were observed in six of six CLL samples. Data from one representative of six CLL samples is shown in (A), and one representative CLL sample out of three is shown in (B) and (C). (D) CLL cells purified from freeze-thawed PBMCs were incubated in MSC-conditioned media in the presence of sorafenib (10 μmol/L) or DMSO for 24 h, at which point the cells were harvested, lysed and analyzed by Western blot for the expression of Mcl-1 and GAPDH as loading and transfer control. Data from one representative CLL samples out of seven is shown on the left and densitometry analysis quantifying the modulation of Mcl-1 expression after sorafenib exposure is shown on the right for all seven patients. Mcl-1 protein expression levels are shown normalized to GAPDH.

(Figure 4D). The change in Mcl-1 protein levels was further quantified by densitometry analysis and showed a statistically significant decline in all patients tested after sorafenib exposure (Figure 4D; n = 7). Since Mcl-1 reduction was a common phenomenon in all culture conditions, these results show that sorafenib-induced apoptosis is associated with a reduction of Mcl-1, which is known to play a critical role in CLL cell survival (24).

Sorafenib Reduced the Levels of Active B-RAF, C-RAF and ERK in CLL Cells

Sorafenib was initially discovered for its ability to inhibit the kinase activity of RAF (25) by binding to its inactive form and sequestering it into inactive complexes (26), and its cytotoxic activity is thought to be mediated at least in part by its impact on RAF activity. Thus, we investigated the effect of sorafenib on the levels of the activated phosphorylated forms of B- and C-RAF, which are the most active isoforms involved in the activation/phosphorylation of ERK (27) in the context of the cellular microenvironment. A sorafenib-mediated reduction in phospho-B-RAF, phospho-C-RAF and phospho-ERK levels was observed in CLL cells cocultured with NLCs and MSCs (Figure 5). Because sorafenib does not directly inhibit ERK phosphoryla-

tion (28), these results indicate that sorafenib can actively inhibit RAF as well as its downstream effectors in CLL cells in the presence of a supportive cellular microenvironment.

Collectively, these results suggest that sorafenib induces apoptosis of CLL cells as a result of Mcl-1 downregulation. To further investigate if the inhibition of the RAF/MEK/ERK pathway can lead to Mcl-1 downregulation in CLL cells, we tested whether inhibition of MEK leads to downregulation of Mcl-1 using the MEK inhibitor PD98059. Exposure of CXCL12-stimulated CLL cells to PD98059 induced apoptosis (not shown), which was accompanied by a downregulation of Mcl-1 (Figure 6A). Furthermore, exposure of CLL cells to PD98059 and to the RAF inhibitor GW5074 in the presence of MSCs also caused apoptosis and resulted in a downregulation of Mcl-1 protein levels (Figure 6B). These results demonstrate that in CLL cells, the RAF/MEK/ERK pathway can regulate Mcl-1 expression levels and that this axis is critical for CLL viability. It further suggests that sorafenib might cause apoptosis of CLL cells through the inhibition of the RAF/MEK/ERK pathway and Mcl-1 downregulation.

CLL Cells Are Not Sensitive to Inhibition of KIT, PDGFR and FLT3 but Are Sensitive to Inhibition of RAF and VEGFR

Sorafenib is a multikinase inhibitor, targeting not only RAF, but also platelet-derived growth factor receptor (PDGFR), KIT, FMS-like tyrosine kinase 3 (FLT3) and vascular endothelial growth factor receptor (VEGFR) (28). To identify which other targets of sorafenib are critical for CLL cell viability, and therefore might contribute to sorafenib-mediated cytotoxicity, we compared a set of inhibitors sharing overlapping targets with sorafenib for their ability to abrogate CLL cell viability (Figure 7A). We tested KG5, a kinase inhibitor of RAF signaling through B-RAF and C-RAF in addition to PDGFR α and β, FLT3 and KIT (29), and KG1, a kinase inhibitor that targets all of
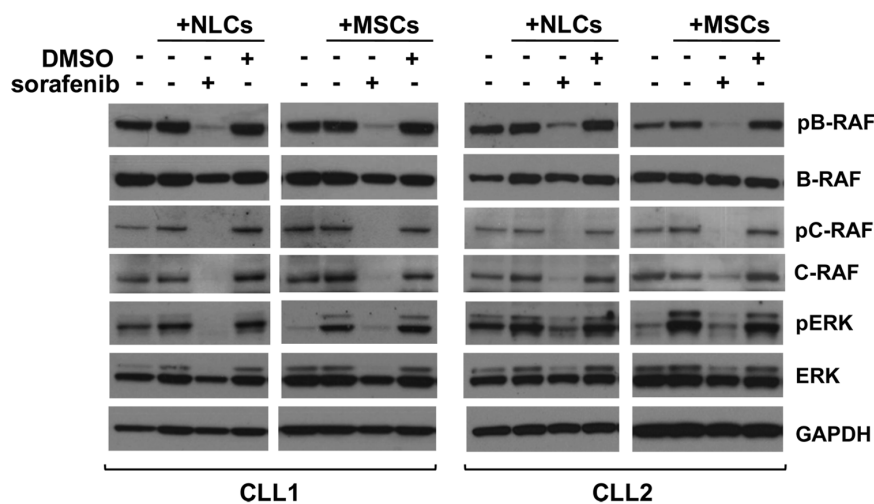
Figure 5. Sorafenib downregulates the levels of active B-RAF, C-RAF and ERK in CLL cells in the presence of NLCs or MSCs. CLL cells purified from frozen PBMCs were seeded on NLCs or MSCs and exposed to 10 μmol/L sorafenib or DMSO control for 24 h. CLL cells were analyzed by Western blot for the expression of the indicated proteins. Data from two representative CLL samples out of three are shown.
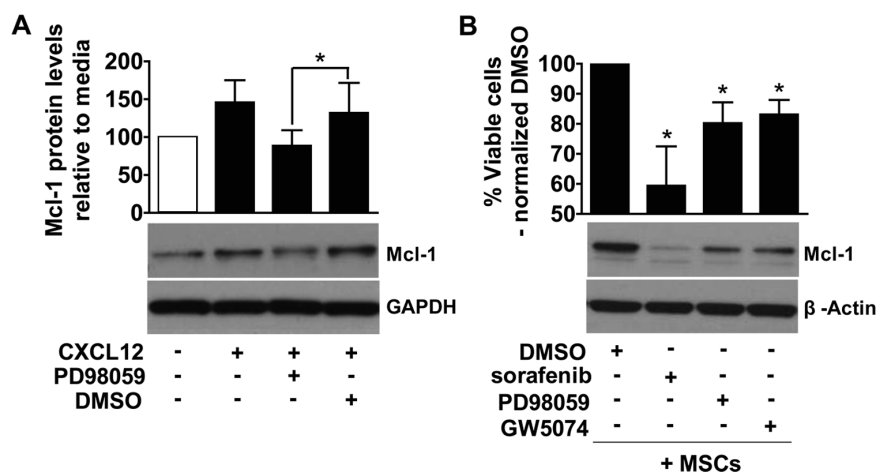

Figure 6. The inhibition of the RAF/MEK pathway in CLL cells causes downregulation of Mcl-1 protein. (A) CLL cells purified from frozen PBMCs were cultured in the presence of 30 nmol/L CXCL12 and 50 μmol/L PD98059 or the equivalent DMSO control for 17 h and analyzed by Western blot for the expression of Mcl-1 and GAPDH. Western blot data from one representative CLL sample out of four is shown. Densitometry analysis was performed for each patient sample to quantitatively evaluate the levels of Mcl-1 expression, which is depicted as a ratio of Mcl-1 expression over GAPDH, normalized to media control. Data shown are means ± SD from four different CLL samples. *Statistically significant difference: $P < 0.05$ (paired Student t test). (B) CLL cells purified from frozen PBMCs were cocultured with MSCs for 18 h in the presence of 10 μmol/L sorafenib, 50 μmol/L PD98059, 10 μmol/L GW5074 or DMSO as control. Viability was assessed by flow cytometry using PI-DiOC6 staining. Data shown are mean ± SD for three patients. *Statistically significant difference: $P < 0.05$ (paired Student t test). At the same time as viability was assessed, cells were collected and lysed for Western blot analysis, as described in Materials and Methods. Representative results of the Mcl-1 and β-actin Western blot are shown for one out of three patients.

these kinases except B- and C-RAF (29) and compared their activity on CLL cell viability. In CLL cells cultured alone (Figure 7B) or in the presence of MSCs (Figures 7C, D), KG5 but not KG1 induced CLL cell apoptosis. This suggests that KIT, PDGFR and FLT3 are unlikely to play a critical role in CLL cell viability and thus unlikely to be involved in sorafenib-mediated apoptosis of CLL cells. Vatalanib, which targets KIT, PDGFR and VEGFR, caused apoptosis of CLL cells (Figures 7E, F), most likely through its activity on VEGFR, since data obtained with KG1 show that KIT and PDGFR did not appear critical for CLL viability. Because in CLL cells, VEGFR signaling does not activate the ERK or AKT but does activate the transcription factor STAT3 (30), we used STAT3 activation as a surrogate indicator for VEGFR signaling. Our results show that STAT3 is activated in CLL cells in the presence of MSCs and that it can be downregulated by sorafenib (Figure 8). These results show the involvement of VEGFR in CLL cell viability and that signaling pathways downstream of VEGFR can be modulated by sorafenib in CLL cells.

Overall, in comparison to the other inhibitors, sorafenib was still the most potent drug-inducing CLL cell apoptosis. Because the inhibition of KIT, PDGFR and FLT3 did not affect CLL cell survival, our results suggest that RAF and VEGFR are the most likely active targets of sorafenib in CLL.

DISCUSSION

Abundant evidence shows that CLL cells can be rescued by accessory cells from spontaneous and drug-induced apoptosis (16,31,32) and protect CLL cells from fludarabine-induced apoptosis *in vitro* (4). Thus, it is essential to evaluate potential therapeutics in CLL accessory cell cocultures. Here, we show preclinical data reporting the sensitivity of CLL cells to sorafenib-mediated cytotoxicity when cocultured in the presence of accessory cells, suggesting that sorafenib might be a potent new therapeutic for CLL. Sorafenib has
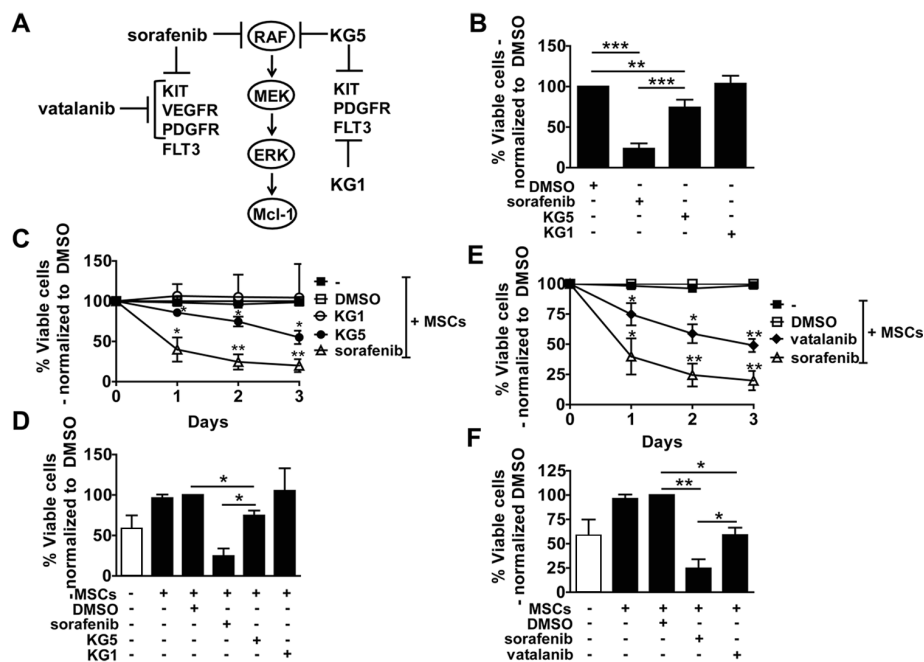
Figure 7. CLL cells are not sensitive to inhibition of KIT, PDGFR and FLT3 but are sensitive to inhibition of RAF and VEGFR. (A) Summary of sorafenib, KG1, KG5 and vatalanib targets. (B) CLL B cells purified from frozen PBMCs were cultured in the absence of support cells and exposed to sorafenib, KG1 or KG5 compound (all at 10 μmol/L) or DMSO, added once on d 0. After 48 h, CLL cells were analyzed for viability as in Figure 2. Data shown are means ± SD from six different CLL samples. (C, D) CLL cells purified from frozen PBMCs were cocultured in the presence of MSCs with a single dose of sorafenib, KG1, KG5 (all at 10 μmol/L) or DMSO added once at the beginning of the culture. CLL cell viability was determined as above. (C) Results are represented relative to DMSO as means ± SD from three different CLL samples. (D) The 48-h time point of the time course is presented as a bar graph, along with the statistical analysis comparing the inhibitors with one another using a paired Student *t* test. (E, F) Purified CLL cells were cocultured in the presence of MSCs with a single dose of sorafenib, vatalanib (both at 10 μmol/L) or DMSO added once at d 0. Viability was measured as above. (E) Results are represented relative to DMSO, as means ± SD from three different CLL samples. (F) The 48-h time point of the time course is presented as a bar graph, along with the statistical analysis comparing the inhibitors with one another using a paired Student *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Figure 8. Sorafenib downregulates the activation of STAT3 in CLL cells. (A) CLL cells purified from frozen PBMCs were preincubated with sorafenib (10 μmol/L) or DMSO for 30 min and then seeded on MSCs, as described in Materials and Methods, for 30 min, at which point the cells were harvested, lysed and analyzed by Western blot for the expression of pSTAT3, STAT3 and β-actin as loading and transfer control. Data from one representative CLL samples out of five are shown. (B) Densitometry analysis quantifying the modulation of pSTAT3 expression after sorafenib exposure. The ratio of pSTAT3 over STAT3 protein expression are presented for each of the five CLL patients tested in (A). **Statistically significant difference: $P < 0.01$ (paired Student *t* test).

a reported elimination half-life of 20.0–27.4 h (33). When given twice daily at 400 mg, the maximum plasma concentration reaches 8.5 μmol/L after 28 d (33), 9.7 μmol/L after 7 d (34) and 9.9 μmol/L after 6 h (35). We show that a single dose of 10 μmol/L sorafenib, a level achievable *in vivo*, dramatically induces caspase-dependent apoptosis in CLL cells in the presence of NLCs and MSCs. Moreover, sorafenib effectively induced apoptosis of CLL cells isolated from fludarabine-refractory patients even when cocultured with NLCs and

MSCs, further suggesting its potential for clinical use as second-line therapeutic strategy.

Sorafenib-induced cytotoxicity of CLL cells appears to be mediated by its impact on the antiapoptotic Bcl-2 family member protein Mcl-1, which was downregulated in all CLL samples cultured alone and in the presence of accessory cells. Mcl-1 plays an important role in CLL cell survival, since silencing of Mcl-1, but not that of Bcl-X$_L$ or XIAP (X-linked inhibitor of apoptosis), reduced CLL cell viability (24,31). Consistent with our observations,
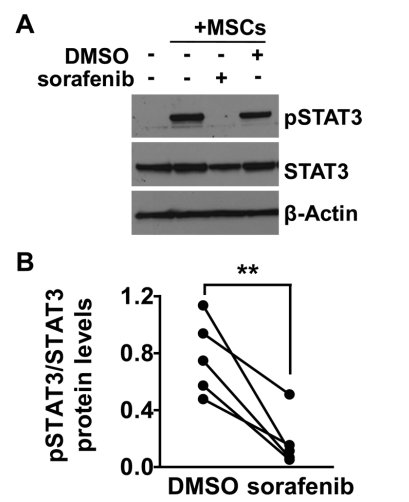
sorafenib has been shown to induce apoptosis along with a reduction of Mcl-1 protein levels in leukemia cell lines (10) and in CLL cells cultured in absence of the microenvironment (36). In addition, the enforced expression of Mcl-1 in cell lines reduced sorafenib-mediated apoptosis (10), supporting the notion that Mcl-1 downmodulation is likely contributing to sorafenib-mediated cytotoxicity in primary CLL.

In other cancers, sorafenib-induced apoptosis has been shown to involve the downregulation of Mcl-1 via RAF/MEK/ERK-dependent as well as -independent pathways depending on the tumor type (37). We found that

sorafenib reduced the activation of B-RAF and C-RAF as well as its downstream mediator ERK in CLL cells cultured in the presence of NLCs or MSCs. Because sorafenib was shown *in vitro* not to be a direct inhibitor of the activity of MEK and ERK (28), it strongly suggests that the impact of sorafenib on ERK is related to the inhibition of its upstream mediators B- and C-RAF. Several lines of evidence demonstrate a link between ERK and Mcl-1 expression in CLL cells. We showed that treatment of CLL cells with the MEK inhibitor PD98059 inhibited CXCL12-included Mcl-1 upregulation, showing that MEK signaling contributes to Mcl-1 expression in CLL cells. Our results also show that the same strategy to inhibit MEK in CLL cells led to a downregulation of Mcl-1, even in the presence of MSCs, further supporting the regulatory role of the RAF/MEK/ERK pathway on Mcl-1 expression. Comparable observations were made in melanoma cells, where inhibition of MEK using PD98059 also caused downregulation of Mcl-1 (38). In addition, it has been shown that ERK activation can lead to Mcl-1 phosphorylation, which in turn increases its stability (39). Moreover, we show that the RAF inhibitor GW5074 reduced Mcl-1 expression and viability in CLL cells in the presence of the microenvironment, further supporting the functional link between RAF, Mcl-1 expression and viability of CLL cells. Thus, we reasoned that the impact of sorafenib on RAF activity contributes to Mcl-1 downregulation and consequently CLL cell death.

Because sorafenib is a multikinase inhibitor, we evaluated which of its targets in addition to RAF (VEGFR, PDGFR, KIT, FLT3) are critical for CLL cell viability using a set of kinase inhibitors. KG5 is a kinase inhibitor of RAF, PDGFR α and β, FLT3 and KIT (29), whereas KG1 targets PDGFR α and β, FLT3 and KIT but not RAF (29). Vatalanib targets KIT, PDGFR and VEGFR (40). Our results show that sorafenib, KG5 and vatalanib induced apoptosis of CLL cells, but KG1 failed to do so. Because the common targets of the active drugs are RAF and VEGFR, these results suggest that PDGFR, FLT3 and KIT are unlikely to be not critical for CLL cell viability and that sorafenib most likely causes CLL cell apoptosis via its inhibition of RAF and VEGFR. Although the role of VEGFR in CLL remains controversial, the majority of the evidence points toward the involvement of VEGFR to CLL cell viability. Huber *et al.* (36) have shown that bevacizumab, a monoclonal antibody against VEGF, did not induce apoptosis in CLL cells, and immunoprecipitation of VEGFR in CLL cells treated with sorafenib showed no effects on phosphotyrosine. However, Lee *et al.* (41) showed that inhibition of VEGFR signaling in CLL cells decreases Mcl-1 levels and induces cell death when 10-fold higher levels of bevacizumab were used, supporting the role of autocrine VEGF in CLL survival. In line with these findings, VEGFR signaling was demonstrated to support CLL cell survival through the upregulation of XIAP and Mcl-1 (30). In CLL, VEGFR signaling is not mediated through the activation of ERK or AKT (30), but through the activation of STAT3, which physically associates to VEGFR and translocate to the nucleus after activation (41). The blockade of VEGFR signaling in CLL using monoclonal antibodies or specific receptor tyrosine kinase inhibitor (SU11657) was shown to inhibit STAT3 activation and to induce apoptosis, marked by Mcl-1 downregulation (41). Comparable findings were also recently reported regarding the effect of vatalanib on CLL cells *in vitro*, which was shown to directly reduce the activation of VEFGR, to induce CLL cell apoptosis and to modulate Mcl-1 expression levels in a dose-dependent manner (42). Overall, these studies indicate that VEGFR signaling plays a role in CLL cell survival, which involves the activation of STAT3 and Mcl-1. We show that sorafenib downregulates STAT3 as well as Mcl-1 and have demonstrated a functional link between RAF and Mcl-1 expression in CLL cells, suggesting that sorafenib downregulated Mcl-1 expression by interfering with the VEGFR/STAT3 and the RAF/MEK/ERK pathways.

On the basis of the reported *in vivo* pharmacokinetic data and safety profile in the treatment of solid tumors (33), sorafenib represents a promising therapeutic agent for CLL. The dramatic reduction in CLL cell viability at 10 μmol/L and lower concentrations of sorafenib *in vitro*, even in the presence of a supportive microenvironment, substantiates its use in CLL. The sensitivity of CLL cells derived from fludarabine-refractory patients further suggests that sorafenib could represent a viable option as a second-line therapy for CLL. A phase I/II clinical trial is being initiated to assess the effect of sorafenib in CLL patients.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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