The Combination of Sorafenib and Everolimus Abrogates mTORC1 and mTORC2 Upregulation in Osteosarcoma Preclinical Models

Ymera Pignochino1,2, Carmine Dell’Aglio1,2, Marco Basiricò1,2, Federica Capozzi1,2, Marco Soster3, Serena Marchiò3, Stefania Bruno4, Loretta Gammaitoni6, Dario Sangiolo2,6, Erica Torchiaro2,6, Lorenzo D’Ambrosio1,2, Franca Fagioli7, Stefano Ferrari8, Marco Alberghini8, Piero Picci8, Massimo Aglietta2,9, and Giovanni Grignani1

Abstract

Purpose: The multikinase inhibitor sorafenib displays antitumor activity in preclinical models of osteosarcoma. However, in sorafenib-treated patients with metastatic-relapsed osteosarcoma, disease stabilization and tumor shrinkage were short-lived and drug resistance occurred. We explored the sorafenib treatment escape mechanisms to overcome their drawbacks.

Experimental Design: Immunoprecipitation, Western blotting, and immunohistochemistry were used to analyze the mTOR pathway [mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)]. Cell viability, colony growth, and cell migration were evaluated in different osteosarcoma cell lines (MNNG-HOS, HOS, KHOS/NP, MG63, U-2OS, SISA-1, and SAOS-2) after scalar dose treatment with sorafenib (10–0.625 μmol/L), rapamycin-analog everolimus (100–6.25 nmol/L), and combinations of the two. Cell cycle, reactive oxygen species (ROS) production, and apoptosis were assessed by flow cytometry. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice injected with MNNG-HOS cells were used to determine antitumor and antimetastatic effects. Angiogenesis and vascularization were evaluated in vitro by exploiting endothelial branching morphogenesis assays and in vivo in xenografted mice and chorioallantoic membranes.

Results: After sorafenib treatment, mTORC1 signaling was reduced (downstream target P-S6), whereas mTORC2 was increased (phospho-mTOR Ser2481) in MNNG-HOS xenografts compared with vehicle-treated mice. Combining sorafenib with everolimus resulted in complete abrogation of both mTORC1 [through ROS-mediated AMP-activated kinase (AMPK) activation] and mTORC2 (through complex disassembly). The sorafenib/everolimus combination yielded: (i) enhanced antiproliferative and proapoptotic effects, (ii) impaired tumor growth, (iii) potentiated antiangiogenesis, and (iv) reduced migratory and metastatic potential.

Conclusion: mTORC2 activation is an escape mechanism from sorafenib treatment. When sorafenib is combined with everolimus, its antitumor activity is increased by complete inhibition of the mTOR pathway in the preclinical setting. Clin Cancer Res; 19(8); 2117–31. ©2013 AACR.

Introduction

Therapy for treatment of osteosarcoma is based on a multidisciplinary approach, encompassing aggressive polichemotherapy and radical surgery (1). Nevertheless, 30% of patients will relapse regardless of optimal therapy given, and this proportion is even higher for patients with metastatic disease at diagnosis (2, 3). In this context, targeted therapies have been investigated to improve these dismal results in patients with unresectable osteosarcoma (4, 5).

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Ymera Pignochino. Institute for Cancer Research at Candiolo, Fondazione del Piemonte per l’Oncologia, Strada Provinciale 142 Km 3.96, 10060 Candiolo, TO, Italy. Phone: 39-011-99-33-503; Fax: 39-011-99-33-290; E-mail: ymera.pignochino@ircc.it. doi: 10.1158/1078-0432.CCR-12-2293 ©2013 American Association for Cancer Research.
Osteosarcoma is the most common primary bone tumor in children and young adults. Despite improved prognosis following polichemotherapy introduction, the metastatic and relapsing forms continue to be fatal. Therapeutic alternatives are urgently needed. Sorafenib, a multikinase inhibitor, has displayed antitumor activity in osteosarcoma preclinical models but was only marginally active in sorafenib-treated patients with metastatic-relapsed osteosarcoma. In particular, disease stabilization and tumor shrinkage were short-lived and drug resistance occurred. In this article, we show that the mTORC2 upregulation observed in sorafenib-treated osteosarcoma may represent the escape mechanism from this targeted therapy. By combining sorafenib with the mTOR inhibitor everolimus, both mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) were fully blocked, enhancing antitumor, antimetastatic, and antiangiogenic activity.

The serine/threonine kinase mTOR is an integral component of phosphoinositide 3-kinase (PI3K)/AKT signaling and forms 2 alternative protein complexes. First is mTOR complex 1 (mTORC1), which is defined by the presence of Raptor; second is mTOR complex 2 (mTORC2), which is characterized by Rictor (12, 13). Activated in different ways, these complexes have distinct substrate specificity. As an environmental energy sensor, mTORC1 responds to amino acids, growth factors, and oxygen levels versus mTORC2, which is still ill defined but seems to be mainly regulated by growth factors and is active in cell size control, growth, and actin–cytoskeleton rearrangement (14). Highly regulated cross-talk exists between the 2 complexes: mTORC1 signals inhibit mTORC2 through the p70 ribosomal subunit S6 kinase (p70S6K)-mediated phosphorylation of Rictor (Thr1135; ref. 15) that leads to mTORC2 inhibition; conversely, mTORC2 regulates mTORC1 by activating AKT (13).

Activation of the mTOR pathway has been associated with tumor progression in several cancer models (13). Notably, mTOR and AKT have been linked to the metastatic behavior of osteosarcoma induced by ERM activation (16). Furthermore, high levels of mTOR and its transducer p70S6K have been consistently correlated with a poorer osteosarcoma prognosis (17). In light of the involvement of mTOR in osteosarcoma progression and metastatization, we investigated whether or not sorafenib treatment might affect the mTOR pathway, and if the specific inhibition of mTOR might raise the potential of sorafenib activity (18, 19).

We asked whether sorafenib treatment might affect the mTOR pathway, and if the specific inhibition of mTOR might raise the potential of sorafenib activity (18, 19). At least 2 mTOR inhibitors, namely, the analogs of rapamycin ridaforolimus and everolimus, have shown minimal activity in clinical and preclinical osteosarcoma therapy, respectively (20, 21). Rapamycin and its derivatives (rapalogs) acutely inhibit mTORC1 without directly affecting mTORC2 after low dose and short-term exposure; however, prolonged treatment has been shown to inhibit mTORC2 assembly and, as a result, its signaling capacity (22).

Herein, we show that single-agent sorafenib inhibits mTORC1 and induces mTORC2 activation in preclinical models of osteosarcoma. We show that this escape mechanism is completely blocked when combined with everolimus. The combination leads to both mTORC1 and mTORC2 inhibition resulting in enhanced antitumor, antiangiogenic, and antimetastatic effects in preclinical models of osteosarcoma.

Materials and Methods

Cell cultures, drugs, and reagents

Human osteosarcoma cell lines (MNNG-HOS, HOS, KHOS/NP, MG63, U-2 OS, S18A1, and SAOS-2) were purchased from the American Type Culture Collection and were cultured in RPMI-1640 (Invitrogen) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human umbilical vein endothelial cells (HUVEC) were prepared as described previously (23) and cultured on gelatin-coated culture dishes in M199 medium supplemented with 20% heat-inactivated bovine calf serum (BCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 5 U/L/mL heparin, 12 μg/mL bovine brain extract, and 200 mmol/L glutamine. Sorafenib and everolimus (Sequoia Research Product) stock solutions were prepared in dimethyl sulfoxide (DMSO), stored at 20°C, and diluted in fresh media immediately before use in the in vitro experiments. The butylated hydroxyanisole (BHA), puromycin, polybrene, and compound C used for culture preparation were all purchased from Sigma-Aldrich. For in vivo administration, pure powders of sorafenib tosylate and everolimus were dissolved in polyethylene glycol (PEG300) purchased by Sigma-Aldrich.

In vitro viability and colony-forming assays

For viability assays, osteosarcoma cell lines were seeded in 96-well plates (3,000 cells/well); after 24 hours, scalar
dilutions of sorafenib (from 10 to 0.625 μmol/L) and everolimus (from 100 to 6.25 nmol/L) were administered. Cell viability was evaluated 72 hours later with the Cell Titer-Glo Luminescent Kit (Promega Co.). Luminescent signals were detected using a GLOMAX 96 Microplate Luminometer (Promega). All experiments were repeated at least 3 times. Single cell-derived colonies were obtained by plating 250 cells per well on 12-well plates in complete RPMI-1640 medium that had been treated with scalar doses of sorafenib (from 5 to 1.25 μmol/L) and a single dose of everolimus (10 nmol/L), either alone or in combination, once colonies were established. The treatments and medium were replaced every 72 hours. After 10 to 15 days, colonies were stained with 0.1% crystal violet (Sigma-Aldrich) and their surface areas were quantified using the Pathway HT Bioimager System (BD Becton Dickinson).

**Immunoprecipitation assay and Western blotting**

To investigate whether or not mTOR phosphorylation had occurred in mTORC1 and/or mTORC2 complexes, immunoprecipitation assay and Western blot analysis were conducted. Osteosarcoma cell lines were treated with sorafenib (5 μmol/L) and everolimus (10 nmol/L), either alone or in combination, for 5 or 24 hours. These concentrations (calculating free drug) are relevant to free drug concentrations at steady state in humans (24, 25). For total protein analyses, cells (5–10 × 10^6) were lysed in boiling buffer (10% SDS, 0.5 mol/L Tris–HCl, pH 6.8) for 5 minutes. Samples were sonicated for 20 seconds and then centrifuged at 14,000 rpm for 30 minutes. Proteins were quantified using the BCA Protein Assay (Thermo Fisher Scientific) spectrophotometer (NanoDrop; Thermo Fisher Scientific). Total proteins were resolved on 4% to 15% SDS-PAGE and electro transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). For immunoprecipitation assays, total proteins were extracted on ice in lysis buffer (20 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 1% Triton, 10% glycerol, and 5 mmol/L EDTA), with a protease inhibitor cocktail (Sigma-Aldrich) and a phosphatase inhibitor cocktail (Sigma-Aldrich) and their surface areas were quantified using the Pathway HT Bioimager System (BD Becton Dickinson). Western blots were probed with antibodies to AMPKα (Ser236), vinculin (from Sigma-Aldrich). Specific signals were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized by extra-sensitive enhanced chemiluminescence reagents (LiteBlot TURBO; Euroclone). Luminescent signals were acquired by Bio-Rad ChemiDoc XRS system (Bio-Rad Laboratories).

**Generation of AMPKα knockdown stable KHOS and MNNG-HOS cells by lentiviral transduction**

Switching off gene expression by stable silencing of AMPKα was achieved using short hairpin RNAs (shRNA) transduced with Lentiviral Vectors. MISSION shRNAs for AMPKα and the control one were purchased from Sigma-Aldrich. Vector stocks were produced by transient transfection of 293T cells. Serial dilutions of freshly harvested conditioned medium were used to infect 10^5 cells in a 6-well plate in the presence of polybrene (8 μg/mL). The culture medium was replaced with fresh puromycin-containing medium (5.0 μg/mL) every 2 to 3 days to select resistant colonies. The expression level of AMPKα was detected by Western blot analyses in the puromycin-resistant colonies.

**Cell cycle, apoptosis, and ROS evaluation**

Effects of sorafenib and everolimus on the cell cycle were determined by evaluation of DNA content after propidium iodide (PI, Sigma-Aldrich) staining. Briefly, osteosarcoma cells were plated in complete culture medium and were either untreated or treated with sorafenib (2.5 or 5 μmol/L) and everolimus (10 nmol/L), as single agents and in combination. After a 24, 48, or 72 hours interval, the cells were first detached by a solution of trypsin–EDTA, then washed in cold PBS, and finally fixed for 24 hours in 70% ethanol at −20°C. Next, cells were incubated with PI staining solution (50 μg/mL PI, 100 μg/mL RNase in PBS) for 3 hours at 4°C in the dark, and then analyzed by Cyan ADP Flow cytometer (Beckman Coulter). Cell-cycle distribution was calculated using the Summit software v4.3 (Dako).

The effect of drug treatment on apoptosis was confirmed by annexin/PI staining. In this case, drug-treated cells were harvested, washed once with cold PBS, and twice with binding buffer (150 mmol/L NaCl2, 10 mmol/L CaCl2, and 10 mmol/L HEPES); this was followed by resuspension in binding buffer and incubation with APC-labeled Annexin V (eBioscience) and PI (0.5 μg/mL) for 15 minutes at room temperature in the dark. All samples were analyzed by Cyan ADP Flow cytometer using Summit v4.3 software. To test reactive oxygen species (ROS) production, cells were cultured in 6-well plates (Corning Incorporated) to 70% to 80% confluence, followed by drug or H2O2 treatment (as positive control) for 1 hour. Harvested cells were incubated for 30 minutes with 10 μmol/L carboxy-H2-DCFDA or 10 μmol/L MitoSOX Red Molecular Probes, washed twice with 1% bovine serum albumin in PBS. Flow cytometry was used for the analysis of fluorescence. In select experiments, apoptosis and ROS production were tested concomitantly by combining carboxy-H2-DCFDA with annexinV and PI staining in a unique 30-minute incubation.
Three-dimensional morphogenesis assay in Matrigel

To evaluate endothelial morphogenesis, 150 μL of growth factor-reduced Matrigel (BD Biosciences) was added per well to a 48-well plate and allowed to gelify for 1 hour at 37°C. HUVECs (20,000 cells) were suspended in M199 medium containing 10% FBS and seeded in the control medium or in the presence/absence of the drugs (5 μmol/L sorafenib, 10 nmol/L everolimus as single agents or in combination). Capillary tube formation was visualized after 5 hours, and the images were captured by the BD pathway HT Bioimager. Five microscopic fields per well were analyzed by ImageJ 1.44 software (open source: http://rsbweb.nih.gov/ij/index.html).

Wound healing assay

To evaluate cell migration, confluent monolayers of HUVECs or osteosarcoma cells were scratched with a pipette tip across the monolayer. Cells were washed with PBS and cultured in M199 medium (HUVEC) or RPMI-1640 (OS) supplemented with 2% FBS in the absence/presence of the drugs under investigation. Images were obtained using BD pathway HT Bioimager System at 0, 8, and 16 hours post-wounding. Cell migration was measured by ImageJ software, which calculated the difference in wound lengths after the different treatments.

Mice xenografts and experimental metastasis

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Charles River) were bred, maintained in sterile cage microinsulators, and handled under sterile conditions at the animal facility of the Institute for Cancer Research and Treatment (Candiolo, Italy) in accordance with and approved by the Institute’s Ethical Commission, the University of Torino Medical School (Candiolo, Italy), and the Italian Ministry of Health.

In 3 independent experiments, 30 female 4- to 6-week-old NOD/SCID mice were injected subcutaneously into the right flank with 10^6 MNNG-HOS cells in 50% growth factor-reduced BD Matrigel. Alternatively, 10^6 MNNG-HOS cells were injected into the tail vein to generate experimental metastasis models. When xenografts approximated a size of 100 mm^3, and after lung colony seeding (1 week from metastatic models). Tumor diameters were measured with a manual caliper, and volumes (V) were calculated according to the following equation, \[ V = \left( \frac{\text{largest diameter} \times \text{smallest diameter}}{2} \right)^2. \] 2. Thereafter, tumors and lungs were processed for their analysis.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays were used to evaluate the number of apoptotic cells in explanted tumors. The assays were conducted using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore). Briefly, paraffin-embedded tissue sections were processed by deparaffinization, hydration with graded ethanol, incubation with proteinase K, and endogenous peroxidase activity was blocked with a solution of 3% H₂O₂ in methanol. After treatment with an equilibration buffer, sections were successively incubated with working strength terminal deoxynucleotidyl transferase (TdT) enzyme, stop/wash buffer, anti-digoxigenin–conjugated HRP, and diamobenzidine. Slides were then prepared and counterstained with hematoxylin.

Immunostaining

Frozen tissues were cut into 10-μm sections and paraffin-embedded tissues were cut into 4-μm sections; both were mounted on SuperFrost Plus glass slides (Menzel-Gläser). Immunostaining was conducted according to standard protocols. Primary antibodies were purchased from Sigma-Aldrich (PCNA, dilution 1:200) and BD Pharmingen (CD31, dilution 1:100). Visible images were captured with a High-Performance IEEE 1394 FireWire Digital CCD Camera (QImaging), whereas fluorescent images were taken with a Leica TCS SP5 II confocal microscope. For signal quantification, 5 images/sample were prepared while parameters were held constant, and ImageJ software was used for their analysis.

Chick chorioallantoic membrane assay

We used the chick chorioallantoic membrane (CAM) model to investigate the antiangiogenic potential of combined sorafenib and everolimus in a robust in vivo assay. Fertilized chicken embryos were incubated for 3 days at 37°C. A small hole was created over the air sac at one end of the egg, and a second hole was open directly over the embryo. After 5 days, 1 × 10^6 KHOS/NP cells in 50% BD Matrigel were injected onto the CAM in the presence of 2.5 μmol/L sorafenib alone, 10 nmol/L everolimus alone, a combination of the 2, or the vehicle alone. After 7 days, CAMs and xenografts were collected and fixed with 4% paraformaldehyde for 24 hours at room temperature. The xenografts were measured with a manual caliper, and photographed with a QCam FAST1394 digital color camera (QImaging) connected to a stereomicroscope (model SZX9; Olympus). Paraffin-embedded samples were analyzed by conventional hematoxylin and eosin and Masson’s Trichrome staining (Bio-Optica).

Statistical analysis

With regard to in vitro experiments, differences between treatment groups were analyzed by the two-tailed Student t test. Means, SDs, and 95% confidence intervals were calculated using GraphPad Prism 5 (GraphPad Software, San Diego, CA).
Synergism (sorafenib + everolimus) and Calcusyn software (BIOSOFT). IC50 values and synergism (sorafenib + everolimus) were assessed through normalized isobologram and combination index (CI) by CalcuSyn software. All in vitro experiments were carried out at least 3 times. Also, in vivo experiments were compared using the two-tailed Student t test; when P values were less than 0.05, they were considered statistically significant.

Results
Sorafenib treatment affects mTOR pathway in OS preclinical models: mTORC1 is inhibited and mTORC2 is activated
We investigated the effects of sorafenib on mTOR pathway both in vitro (osteosarcoma cell lines: KHOS, MNNG-HOS, and U-2 OS) and in vivo (MNNG-HOS and U-2 OS xenografts in NOD/SCID mice). In vitro treatment with single-agent sorafenib caused the inhibition of mTORC1, visualized as a decreased Ser2448 phosphorylation of mTOR and both its downstream transducers 4E-BP1 (Ser65) and p70S6K (Thr389). As expected, the negative regulatory site on Rictor was dephosphorylated and the compensatory activation of mTORC2 was visualized by the increment in mTOR Ser2481 autophosphorylation (Fig. 1A). At the same time, we evaluated the activation of mTORC2 by Western blot analysis. We observed the increased phosphorylation of mTOR Ser2481 after 5 hours of sorafenib treatment (Fig. 1A). Similarly, in xenografts after 28 days of treatment with sorafenib compared with vehicle-treated mice, we observed a strong inhibition of mTORC1, as revealed by immunostaining and Western blot analysis of endogenous levels of phosphorylated S6 (Ser235), considered a functional readout of p70 S6 kinase activity (Fig. 1B). Contemporarily, we observed an increased mTOR Ser2481 phosphorylation (Fig. 1B). This effect could be attributable to mTORC2 activation as a drawback consequence of mTORC1 inhibition. The same results were observed in U-2 OS–derived xenografts (data not shown). These data show a dual effect of sorafenib on the mTOR signaling cascade, providing a biochemical explanation of a potential escape mechanism of osteosarcoma upon sorafenib treatment.

Sorafenib and everolimus combination completely inhibits mTORC1 and mTORC2
With the aim to prevent sorafenib-induced mTORC2 activation, we combined this drug with everolimus—a clinically available mTOR inhibitor. Single-agent everolimus at low dosage decreased mTORC1 activity (mTOR phosphorylation at Ser2448, and its downstream targets 4E-BP1 and p70S6K; Fig. 1C), and enhanced mTORC2 activity (release of negative regulation of Rictor at Thr1135, mTOR phosphorylation at Ser2481, and its downstream target Akt at Ser473; Fig. 1C).

Interestingly, the combination of everolimus and sorafenib led to complete inhibition of both mTORC1 (P-mTOR Ser2448, P-p70S6K, P-4E-BP1) and mTORC2 pathways (P-mTOR Ser2481, P-Akt Ser743), which indicated a strong synergism between the actions of these 2 drugs (Fig. 1C). Accordingly, p70S6K inactivation led to dephosphorylation of its target site, Thr1135, on Rictor after combined treatment. The observed mTORC2 inhibition could be attributed to mTORC2 complex disassembly, as revealed by coimmunoprecipitation experiments (Fig. 1C). Namely, mTOR coimmunoprecipitated with Rictor in vehicle and single agent–treated cells, but not in combined sorafenib/everolimus–treated cells.

In xenograft models, combined sorafenib–everolimus significantly reduced mTORC1 activity, as revealed by immunohistochemistry of P-S6 (Fig. 1D; P < 0.05). In addition, it prevented the drawback effect of mTORC2 activation induced by sorafenib alone, as shown by immunohistochemistry analysis of mTOR Ser2481 phosphorylation (Fig. 1D; P < 0.05).

Sorafenib and everolimus combination induces AMPK-mediated apoptosis in vitro
To explain the enhanced effect of combined sorafenib and everolimus on mTORC1 inhibition, we explored the cross-talk pathway linking ERK1/2 and mTORC1. Western blot analysis confirmed strong inhibition of ERK1/2 phosphorylation, specifically by sorafenib, either singly or in combination with everolimus (Fig. 2A). Because cross-talk between ERK1/2 and mTORC1 is mediated by the oncogene suppressor liver kinase B1 (LKB1) and the AMPK pathway (26, 27), we showed that the combination of sorafenib and everolimus strongly triggered AMPK activation without affecting LKB1 phosphorylation at its negative regulatory site Ser428 (Fig. 2A).

These data suggest that a different mechanism drives AMPK activation in osteosarcoma models. It is well known that AMPK can be activated either by increased levels of AMP or as a consequence of a ROS burst (25). We observed that sorafenib, either alone or in combination with everolimus, induced a significant production of ROS (Fig. 2B; P < 0.05) already detectable after 24 hours of treatment. This production caused an apoptotic response that reached maximum values after 48 and 72 hours (P < 0.05; Fig. 2C). Cell incubation with BHA, a ROS scavenger, protected osteosarcoma cells by combination-induced apoptosis (P < 0.05; Fig. 2D). AMPK is the mediator of apoptosis in this system; in fact, its selective inhibition, by compound C or stable downregulation with AMPK-specific shRNA, reduced the percentage of apoptosis among sorafenib-treated cells (P < 0.05; Fig. 2D).

Everolimus enhanced the antiproliferative and proapoptotic effects of sorafenib in vitro
We tested cell viability in 7 different human osteosarcoma cell lines (MNNG-HOS, HOS, KHOS/NP, MG63, U-2 OS, SJSA-1, and SAOS-2) after 72 hours of treatment with scalar doses of sorafenib (10–0.625 μmol/L) and everolimus (100–6.25 mmol/L). Sorafenib induced a dose-dependent antiproliferative effect with a typical sigmoid dose effect curve and IC50 values between 2 and 5 μmol/L for all tested cell lines (Fig. 3A and Table 1). At the highest dose
tested (100 nmol/L), everolimus reached 60% cell viability inhibition in 2 of 7 cell lines tested (Fig. 3A and Table 1); overall it induced 20% to 60% inhibition of cell viability. Combined treatment showed a synergistic antiproliferative effect with a combination index <1 in 5 of 7 cell lines (Table 1). In colony growth tests, a 10-day treatment with doses as low as 2.5 \mu mol/L sorafenib and 10 nmol/L everolimus in
combination resulted in significantly reduced colony growth (Fig. 3B). Investigation of cell-cycle perturbations were undertaken to further explain the effect of the 2 drugs and their combination on cell and colony growth. At 48 hours, we found that sorafenib alone or in combination with everolimus significantly reduced the percentage of proliferating cells (e.g., for KHOS cells, phase S and G2-M: 17.3% ± 2.9%) compared with everolimus alone (31.4% ± 4.5%) or to the untreated controls (41.4% ± 2.5%; P < 0.05). The synergism of combined sorafenib and everolimus was also revealed by a sharp increase in the sub-G0 (cells with fragmented DNA) fraction (control 2.3% ± 0.7% vs. everolimus 4.1% ± 1.2% vs. sorafenib 9.6% ± 1.9% vs. combination 27.6% ± 3.9%; P < 0.05; Fig. 3C).

Furthermore, the proapoptotic effect between sorafenib and everolimus was confirmed by PARP cleavage (Fig. 3E), the ultimate hallmark of caspase activation, and by double staining with Annexin V and PI (Fig 2B–D). Annexin V reveals the outer exposure of phosphatidylinserine (early event in apoptotic cells), whereas PI enter only in cells with permeabilized membrane (late event in apoptotic cells). The percentage of the sum of early and late apoptotic cells was 11.7% ± 2.2% (control) versus 20.8% ± 4.3% (everolimus) versus 58.5% ± 5.1% (sorafenib) versus 91.5% ± 6.3% (combination), P < 0.05.

**Sorafenib–everolimus combination exerts antitumor activity in vivo**

In the osteosarcoma xenograft model, a 28-day treatment with sorafenib (5 mg/kg/d), everolimus (1 mg/kg/d), and their combination reduced tumor growth when compared with vehicle-treated controls (34% ± 12%, 46% ± 9%, and 29% ± 8% of the controls, respectively; P < 0.05). A 10-fold dilution of the combined treatment was still effective, causing a mean tumor volume of 42% ± 9% of the controls. Each single agent decreased the percentage of proliferating (PCNA-positive) cells in tumor tissues, going down from 15.5% ± 0.9% (controls) to 12.5% ± 1.5% (sorafenib; P < 0.05) and 8.5% ± 0.6% (everolimus; P < 0.05). Combination of the 2 drugs further reduced the percentage of tumor proliferating cells to 3.7% ± 0.8% (P < 0.05; when compared with both untreated controls and single-agent treatments; Fig. 4A). The diluted combination was still able to significantly reduce this percentage to 7.7% ± 1.7% (P < 0.05, when compared with untreated controls and the everolimus-treated group). TUNEL assays showed that both sorafenib and everolimus as single agents produce a significant increase in apoptotic cells from 1.3% ± 0.5% (untreated control) to 6.4% ± 1.1% (sorafenib; P < 0.05) and 10.3% ± 1.2% (everolimus; P < 0.05). The combination of sorafenib and everolimus resulted in 16.2% ± 2.3% of TUNEL-positive cells (P < 0.05; when compared with untreated controls and single agents; Fig. 4B). The diluted combination was still able to increase the percentage of apoptotic cells in osteosarcoma xenografts (12.3% ± 1.6%; P < 0.05 when compared with controls and to sorafenib alone; Fig. 4B), showing that the combination of sorafenib and everolimus allows a consistent scale-down of the drug dosage to obtain a
comparable therapeutic activity in preclinical models of osteosarcoma.

**Everolimus enhances the antimetastatic effect of sorafenib in vitro and in vivo**

We also considered the effect of combined sorafenib and everolimus from the critical aspect of metastatization via cell migration behavior. *In vitro*, the wound healing assay revealed that everolimus (10 nmol/L) alone was incapable of blocking osteosarcoma cell migration after 24 hours, whereas sorafenib (2.5 μmol/L) did decrease cell motility. Of crucial importance, the combined treatment showed significantly more inhibition of osteosarcoma cell motility relative to the controls or the single agents (Supplementary Table S1).

To investigate the pharmacologic effect of sorafenib and everolimus as potential antimetastatic agents *in vivo*, we created a pseudo-metastatic model of osteosarcoma by injecting 10⁶ MNNG-HOS cells into the tail veins of NOD/SCID mice. This model confirmed the high metastatic potential of human osteosarcoma cells, a commonly experience in the clinical setting. We observed that mice treated for 21 days with sorafenib (5 mg/kg/d) or everolimus (1 mg/kg/d) displayed significantly fewer and smaller-sized lung metastases.
The combined drug treatment led to a significant inhibition of lung foci seeding and growth of osteosarcoma cells compared with either of the single agents (Fig. 4B and C). These data indicate that treatment with combined sorafenib and everolimus has potential as an antimetastatic regimen against osteosarcoma.

**Everolimus and sorafenib enhance antiangiogenic effects both in vitro and in vivo**

Both sorafenib and everolimus have been described as antiangiogenic drugs; however, our interest lays in whether or not they could generate an enhanced antiangiogenic effect when used in combination. First, we considered the formation of capillary tubes of HUVECs in 3D Matrigel assays, and observed that the combination of sorafenib (2.5 μmol/L) and everolimus (10 nmol/L) significantly inhibited capillary tube formation after 5 hours of exposure. In fact, cell treatment with the drug combination resulted in far fewer capillary branching points [26 ± 7/high-power field (HPF); *P* < 0.05] versus the untreated controls (86 ± 5/HPF) or the single agents (sorafenib, 71 ± 4/HPF; everolimus, 64 ± 3/HPF; Fig. 5A). We also evaluated any effect that
Figure 3. Sorafenib and everolimus in combination potentiates the antiproliferative and proapoptotic effects of the single drugs in osteosarcoma cells. A, dose-dependence curve of viability inhibition effect calculated after 72 hours treatment of U-2 OS cells with scalar doses of sorafenib, everolimus, and their combination. B, colony growth assay. After colony establishment, KHOS, MNNG-HOS, and U-2 OS cells were cultured for 10 days in the presence of 2.5 μmol/L sorafenib, 10 nmol/L everolimus, and their combination. They were then fixed and stained with crystal violet. C, average surface occupied by cell colonies, Y error bars = SD; *P < 0.05 versus control and single agents. D, cell-cycle analysis. Flow cytometry evaluation of DNA content (PI staining) of KHOS cells after 48 hours of treatment with 2.5 μmol/L sorafenib, 10 nmol/L everolimus, and their combination. E, Western blot analysis evaluation of PARP cleavage in KHOS cells after 24 hours of treatment with 2.5 μmol/L sorafenib, 10 nmol/L everolimus, and their combination. Vinculin staining is visualized as a loading control.
sorafenib and everolimus might impose on endothelial cell motility. Scratch assays on HUVEC monolayers showed that the sorafenib/everolimus combination significantly inhibited wound healing (79.3% ± 9.2% of the control) compared with either single agent (everolimus, 44.5% ± 8.4%; sorafenib, 55.5% ± 11.5%).

To elucidate the role of the combined drugs against tumor angiogenesis in vivo, we set up osteosarcoma xenografts in a CAM model. Under these conditions, the 2 drugs caused a significantly lower frequency of MNNG-HOS cell engraftment (5 of 25) and growth (mean tumor volume 14.01 ± 0.2 mm$^3$) relative to the controls (17 of 25 tumor engraftment; mean tumor volume 100.35 ± 3.2 mm$^3$) or single agents (sorafenib 9 of 25; mean tumor volume 22.05 ± 1.2 mm$^3$; everolimus 13 of 25; mean tumor volume 26.83 ± 2.3 mm$^3$; Fig. 5B). This effect was paralleled by a strong inhibition of neangiogenesis as evidenced by reduced numbers and dimensions of blood vessels nourishing the tumors, as highlighted by trichrome staining (Fig. 5C). Blood vessel numbers and dimensions were also investigated in mouse osteosarcoma xenografts by immunostaining for the endothelial marker CD31 (Fig. 5D). We observed reduced blood vessel numbers versus the controls (24 ± 7 CD31-positive blood vessels/HPF) and everolimus-treated group (18 ± 6) in both the sorafenib-treated group (7 ± 3 CD31-positive blood vessels/HPF) and the combination drug-treated group (5 ± 2). $P < 0.05$. These data show that the combination of sorafenib and everolimus increases the antiangiogenic effects of the single drugs both in vitro and in vivo.

**Discussion**

Unresectable metastatic osteosarcoma remains a problem because chemotherapy has proved unsuccessful in disease eradication. Several tyrosine kinase receptors (TKR), such as insulin-like growth factor-IR (IGF-IR), HER2, PDGFR, and VEGFR have each been implicated in osteosarcoma progression and death (28). Unfortunately, none of these receptors represents a “clinically-relevant” target, likely because the biologic forces driving osteosarcoma progression do not succumb to a single hit. Recently, we reported that the multikinase inhibitor sorafenib negatively impacted osteosarcoma growth (6), but that the effect was brief (11). Surely, these considerations, make a compelling case to explore drug combinations to enhance sorafenib activity in sorafenib-based regimens that can be effectively translated into therapeutic approaches.

In our search for an escape mechanism from sorafenib treatment, we showed that the drug affected the mTOR pathway. In preclinical models that were treated with sorafenib for long periods (28 days), mTOR autophosphorylation was increased. The mTORC1 pathway was inhibited, as shown by decreased phosphorylation in its downstream targets p70S6K and S6, which might be responsible for mTORC2 upregulation as p70S6K is also involved in the cross-talk between mTORC1 and mTORC2. In particular, p70S6K phosphorylates Rictor on Thr1135 and leads to mTORC2 inhibition (15). The release of Rictor inhibition might be responsible for upregulation of mTORC2 and, hence, increased mTOR autophosphorylation. We hypothesized that mTORC2 activation might represent a potential mechanism of escape from single-agent sorafenib treatment as mTORC2 has been implicated in growth factor signaling aside from cytoskeletal organization and tumor growth (14).

In an attempt to revert this phenomenon, we combined sorafenib with the rapamycin analog everolimus—a drug recently implicated in mTORC2 complex disassembly (29). We observed osteosarcoma models in which a combination of sorafenib and everolimus led to complete abrogation of both mTORC1 and mTORC2 signaling, and eventually to effective mTORC2 complex disassembly. To elucidate the molecular mechanism underlying sorafenib-induced mTOR pathway perturbations, we explored the cross-talk between the ERK1/2 pathway (the principal target of sorafenib inhibition) and mTOR. Data in the literature report that constitutively-activated ERK1/2 phosphorylate the oncosuppressor LKB1 at a negative regulatory site (Ser428; ref. 19), which leads to the release of AMPK-mediated mTORC2 activation (15). The release of Rictor inhibition might be responsible for upregulation of mTORC2 and, hence, increased mTOR autophosphorylation. We hypothesized that mTORC2 activation might represent a potential mechanism of escape from single-agent sorafenib treatment as mTORC2 has been implicated in growth factor signaling aside from cytoskeletal organization and tumor growth (14).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC$_{50}$ µmol/L sorafenib</th>
<th>IC$_{50}$ µmol/L everolimus</th>
<th>IC$_{50}$ µmol/L combination</th>
<th>CI ± est. SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG63</td>
<td>2.8</td>
<td>&gt;0.10</td>
<td>1.33 Sorafenib/0.013 everolimus</td>
<td>0.48 ± 0.13</td>
</tr>
<tr>
<td>U-2 OS</td>
<td>4.1</td>
<td>&gt;0.10</td>
<td>1.25 Sorafenib/0.012 everolimus</td>
<td>0.31 ± 0.07</td>
</tr>
<tr>
<td>HOS</td>
<td>4.8</td>
<td>&gt;0.10</td>
<td>2.27 Sorafenib/0.022 everolimus</td>
<td>0.50 ± 0.23</td>
</tr>
<tr>
<td>KHOS</td>
<td>2.4</td>
<td>&gt;0.10</td>
<td>1.50 Sorafenib/0.015 everolimus</td>
<td>0.66 ± 0.18</td>
</tr>
<tr>
<td>MNNG-HOS</td>
<td>4.6</td>
<td>0.093</td>
<td>3.21 Sorafenib/0.032 everolimus</td>
<td>1.05 ± 0.21</td>
</tr>
<tr>
<td>SAOS-2</td>
<td>3.9</td>
<td>0.079</td>
<td>1.35 Sorafenib/0.013 everolimus</td>
<td>0.51 ± 0.20</td>
</tr>
<tr>
<td>SJSA-1</td>
<td>5.0</td>
<td>&gt;0.10</td>
<td>3.66 Sorafenib/0.036 everolimus</td>
<td>1.03 ± 0.22</td>
</tr>
</tbody>
</table>

*CI calculated at IC$_{50}$ ± estimated SD calculated by CalcuSyn software based on Chou-Talalay method.
activated AMPK. Moreover, sorafenib and everolimus in combination sharply increased AMPK activation, whereas there was no observed LKB1 phosphorylation effect. These data suggested that an alternative mechanism was responsible for the observed mTOR perturbation. It is well known that AMPK is triggered not only during energy stress but also in the presence of oxidative damage (30). Our models showed that activation of AMPK might also be attributable to the ROS burst induced by sorafenib, either as a single agent or more so when combined with everolimus.

Also known is that sorafenib induces a massive stress of the endoplasmic reticulum (31), which can lead to calcium release and ROS production (32). ROS, in turn, are implicated in AMPK activation (31) and apoptosis induction (30). Consistently, we report that, when ROS concentration is reduced in osteosarcoma cells by a specific scavenger, the proapoptotic effect induced by combined sorafenib/everolimus treatment is prevented. Moreover, the specific AMPK downregulation (obtained with shRNA or compound C) counteracted sorafenib/everolimus-induced apoptosis, which confirmed the crucial role of AMPK activation in triggering apoptosis. This is, to our knowledge, the first formal demonstration that osteosarcoma cells treated with a combination of sorafenib and everolimus undergo ROS-induced, AMPK-mediated apoptosis. Worthy of note is the increased ROS production that may cause DNA damage and lead to such complex cellular responses as ATM and PP1 activation (32–34). This kinase and phosphatase might control cell growth and survival directly by mRNA translation and protein synthesis through the mTORC1-independent S6 regulation (32).

Consistent with the mechanism of action reported earlier, the effect of the combination of sorafenib and everolimus showed enhanced antiproliferative and proapoptotic activity against a panel of osteosarcoma cell lines. These results were even more pronounced after prolonged treatment, and inhibited growth in tumor cell colonies and xenografts. We also showed that these drugs interfere with 2 pivotal mechanisms involved in osteosarcoma invasion and metastasis, namely cell migration and angiogenesis. Indeed, mice treated with the 2-drug combination displayed a significant reduction in the number and size of
lung metastases following intravenous injection of human osteosarcoma cells.

The enhanced antimetastatic activity, along with the effects elicited in primary tumors, can also be attributed to tumor angiogenesis interference. It was shown \textit{in vitro} by reduced branching morphogenesis of HUVECs, and by diminished neovascularization of osteosarcoma tumors \textit{in vivo} onto CAMs and subcutaneously into immunodeficient mice. Indeed, sorafenib has been proven to inhibit VEGFRs and PDGFRs (35); everolimus has been shown to downregulate tumor cell VEGF production (36, 37). These results go beyond direct antiproliferative effects of sorafenib and everolimus on tumor cells; they put forth the idea that antiangiogenic effects have an important role and contribution in the final determination of antitumor activity \textit{in vivo}.

In summary, we have reported on observed mTOR pathway perturbations in sorafenib-treated osteosarcoma preclinical models, and have described a possible mechanism of escape in mTORC2 activation, as well as a potential point of sorafenib monotherapy failure. We showed that this negative effect could be overcome by treatment with sorafenib in combination with an mTOR inhibitor, such as everolimus. This drug combination leads to mTORC2 complex disassembly. We showed that this combination leads to potentiated antitumor, antiangiogenic, and antimetastatic effects. Finally, these results might suggest future testing of this combination therapy in the clinical setting in the treatment of patients with chemorefractory-advanced osteosarcoma.
Disclosure of Potential Conflicts of Interest

S. Ferrari has honoraria from Speakers Bureau of Takeda and Amgen. G. Grignani has an expert testimony from Bayer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: Y. Pignochino, F. Fagioli, P. Picci, M. Aglietta, G. Grignani


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Pignochino, C. Dell’Aglio, M. Basirico, F. Capozzi, M. Soster, L. Gammaitoni, L. D’Ambrosio, S. Ferrari, P. Picci, G. Grignani

Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): Y. Pignochino, C. Dell’Aglio, M. Basirico, F. Capozzi, M. Soster, S. Marchio, L. Gammaitoni, M. Alberghini, P. Picci, M. Aglietta, G. Grignani

Writing, review, and/or revision of the manuscript: Y. Pignochino, S. Marchiò, D. Sangiolo, L. D’Ambrosio, F. Fagioli, S. Ferrari, P. Picci, M. Aglietta, G. Grignani

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Grignani

Study supervision: F. Fagioli, P. Picci, M. Aglietta, G. Grignani

Grant Support

This work was supported by the Italian Association for Cancer Research (Associazione Italiana Ricerca Cancro) AIRC (grant IG2011:11515, and AIRC 5 per mille—9970), “Rete Oncologica del Piemonte e della Valle d’Aosta” (Development of an integrated platform for cryopreservation, banking, and generation of primary cell cultures from mesenchymal tumors; University of Torino-Progetti di Ateneo 2011 (grant Retino-ORTO11RTW). S. Marchio was granted by Italian Federation for Cancer Research (FIRC), Italian Association for Cancer Research, My First AIRC Grant (AIRC-MFAG), Banca d’Alba, Piedmont Region (Finalized Health

Figure 5. Sorafenib and everolimus in combination potentiates the antiangiogenic effect of the single drugs both in vitro and in vivo. A, capillary tube formation of HUVECs in 3D Matrigel in vehicle (control), everolimus (10 nmol/L), sorafenib (2.5 μmol/L), and their combination. B, CAM assays. 10^6 KHOS cells were injected with Matrigel onto the CAMs of fertilized chicken eggs, and incubated for 7 days in the presence of vehicle (control), everolimus (10 nmol/L), sorafenib (2.5 μmol/L), and combination. C, trichrome staining of KHOS cells subcutaneously implanted in immunodeficient mice treated with vehicle (control), sorafenib (5 mg/kg/d), everolimus (1 mg/kg/d), and combination-treated mice. White arrows indicate functional blood vessels (containing red blood cells). D, CD31 staining of MNNG-HOS cells subcutaneously implanted in immunodeficient mice treated with vehicle (control), sorafenib (5 mg/kg/d), everolimus (1 mg/kg/d), and combination.
Sorafenib and Everolimus Activity in Osteosarcoma Models

Research (Under 40), and Piedmont Foundation for Cancer Research (FPRC) Intramural Grant 5  1000 2008. C. Dell’Aglio was granted by “Gigi Ghirotti” foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

References
