Preclinical Development

Molecular Cancer Therapeutics

Sorafenib Enhances the Antitumor Effects of Chemoradiation Treatment by Downregulating ERCC-1 and XRCC-1 DNA Repair Proteins

Arti Yadav[1], Bhavna Kumar[1,2], Theodoros N. Teknos[1,2], and Pawan Kumar[1,2]

Abstract

Head and neck squamous cell carcinoma remains a challenging clinical problem because of the persisting high rate of local and distant failure due to the acquisition of chemo- and radioresistance. In this study, we examined if treatment with sorafenib, a potent inhibitor of Raf kinase and VEGF receptor, could reverse the resistant phenotype in tumor and tumor-associated endothelial cells, thereby enhancing the therapeutic efficacy of currently used chemoradiation treatment. We used both *in vitro* and *in vivo* models to test the efficacy of sorafenib either as a single agent or in combination with chemoradiation. Sorafenib, as a single agent, showed antitumor and angiogenesis properties, but the effects were more pronounced when used in combination with chemoradiation treatment. Sorafenib significantly enhanced the antiproliferative effects of chemoradiation treatment by downregulating DNA repair proteins (ERCC-1 and XRCC-1) in a dose-dependent manner. In addition, combination treatment significantly inhibited tumor cell colony formation, tumor cell migration, and tumor cell invasion. Combination treatment was also very effective in inhibiting VEGF-mediated angiogenesis *in vitro*. In a severe combined immunodeficient mouse xenograft model, combination treatment was very well tolerated and significantly inhibited tumor growth and tumor angiogenesis. Interestingly, following combination treatment, low-dose sorafenib treatment alone was highly effective as a maintenance regimen. Taken together, our results suggest a potentially novel strategy to use sorafenib to overcome chemo- and radioresistance in tumor and tumor-associated endothelial to enhance the effectiveness of the chemoradiation therapy. *Mol Cancer Ther; 10(7); 1241–51. ©2011 AACR.*

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer worldwide, and 5-year survival rate (<50%) is among the lowest of the major cancers (1, 2). Although advancements in the techniques for surgery, radiation, and chemotherapy have increased the local control of HNSCC, the overall survival rates have not improved significantly over the last 3 decades. This poor outcome becomes even worse (20%; 5-year survival rate) for advanced stage HNSCC patients whose tumors are not amenable for surgery (3). Concurrent chemoradiation regimen, generally used for the treatment of advanced cases, is often associated with acute toxicity (4). In addition, the response rate is still poor and overall survival being measured in months (5). Therefore, it is imperative that new therapeutic strategies are developed to increase the long-term survival of these patients as well as decrease the adverse effects associated with chemoradiation.

To develop tumor specific therapies, recent research efforts have attempted to exploit the biological differences that may exist between normal and malignant cells. One such therapeutic target for advanced head and neck tumors is epidermal growth factor receptor (EGFR), which is overexpressed in more than 80% of patient tumors as compared with normal mucosa (6). In addition, EGFR expression directly correlates with decreased survival in HNSCC patients (7–9). EGFR predominantly mediates its survival and proliferative function via the activation of Ras–Raf–MAPK (mitogen-activated protein kinase) and PI3K/Akt signaling pathways (10). HNSCC tumors are also shown to overexpress VEGF and this overexpression has been associated with lymph node metastasis and poor survival (11, 12). We have recently shown that VEGF induces chemo- and radioresistance in endothelial cells by upregulating Bcl-2 protein (13). Moreover, Bcl-2 protects endothelial and tumor cells against radiation-induced apoptosis by upregulating the expression of survivin via the Raf–MAP/ERK kinase–ERK (extracellular signal regulated kinase) pathway (14). MAPK pathway is also involved in the upregulation

Authors' Affiliations: [1]The Ohio State University Comprehensive Cancer Center; and [2]Department of Otolaryngology-Head and Neck Surgery, The Ohio State University, Columbus, Ohio

Corresponding Author: Pawan Kumar, Department of Otolaryngology-Head and Neck Surgery, The Ohio State University, 420, W. 12th Avenue, Room no. 464, Columbus, OH 43210. Phone: 614-292-2496; Fax: 614-688-4761; E-mail: pawan.kumar@osumc.edu

doi: 10.1158/1535-7163.MCT-11-0004

of DNA repair proteins particularly ERCC-1 and XRCC-1 (15), and a number of recent studies have highlighted the role of these repair proteins in the acquisition of chemo- and radioresistance (16–18). ERCC-1 plays a key role in nucleotide excision repair by forming a complex with xeroderma pigmentosum complementation group F and this complex is required for the excision of damaged DNA (19). Similarly, XRCC-1 protein plays an important role in the repair of ionizing radiation-mediated double strand DNA breaks, single strand breaks, or recombination repair (20, 21). Therefore, we hypothesize that targeting of Raf kinase in head and neck cancer may enhance the therapeutic efficacy of chemoradiation by modulating the expression of DNA repair proteins and inhibiting the acquisition of chemo- and radioresistance by tumor and tumor-associated endothelial cells.

Sorafenib is an oral multikinase inhibitor (22, 23) currently being used in clinics to treat patients with advanced renal cell carcinoma (RCC), hepatocellular carcinoma (HCC), and thyroid cancer (24–26). Initially, sorafenib was identified as a potent inhibitor of Raf serine/threonine kinase isoforms *in vitro*. Sorafenib since then has been shown to have potent inhibitory effects on other Raf isoforms as well (27, 28). In addition to targeting Raf kinase, sorafenib also inhibits proangiogenic mediators including VEGF receptor 1 (VEGFR-1), VEGFR-2, VEGFR-3, and platelet-derived growth factor receptor-β (27). In a phase II clinical trial for patients with HNSCC, sorafenib was well tolerated but showed only modest anticancer activity (29). However, recent studies have shown that the anticancer activity of sorafenib is significantly enhanced when combined with chemotherapy or signal transduction inhibitors in advanced HCC and gastric cancer patients (30, 31). Therefore, full clinical activity of sorafenib may be achieved by combining it with chemoradiation or other signal transduction inhibitors.

In this study, we used both *in vitro* and *in vivo* models to investigate if treatment with sorafenib could enhance the antitumor and antiangiogenesis effects of chemoradiation in HNSCC. Taken together, our results show that sorafenib can be successfully combined with low-dose chemoradiation regimen to potentiate its antitumor and antiangiogenesis activities. Our study provides a scientific rationale to evaluate this or a similar combination strategy for clinical trials.

Materials and Methods

Cell culture and reagents

Primary human dermal microvascular endothelial cells were purchased from Lonza and were characterized by immunofluorescent staining for von Willebrand's antigen (positive) and smooth muscle α-actin (negative). Endothelial cells were maintained in endothelial cell basal medium-2 containing 5% FBS and growth supplements. CAL27 was obtained from American Type Culture Collection and UM-SCC-74A was obtained from Dr. Thomas E. Carey, University of Michigan, Ann Arbor, Michigan. Both these HNSCC cell lines were authenticated by genotyping and maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS. ERCC-1 antibody for Western blotting (clone FL297) was purchased from Santa Cruz Biotechnology, and ERCC-1 antibody for immunohistochemistry was purchased from Lab Vision. pAkt, pERK1/2, tubulin, and XRCC-1 antibodies were purchased from Cell Signaling Technology.

Transfection with short interfering RNA

Tumor cells were transfected with short interfering RNA (siRNA) for ERCC-1 or XRCC-1 or ERCC-1 and XRCC-1 together by using siGENOME SMART pool siRNAs from Dharmacon according to the manufacturer's instructions. Seventy-two hours posttransfection, cells were either used for proliferation experiments or whole cell lysates were prepared for Western blotting.

Cell proliferation assay

HDMEC, CAL27, and UM-SCC-74A cells were treated with different concentrations of sorafenib (sorafenib tosylate; LC Laboratories), cisplatin (Sigma), or radiation. For combination treatment, cells were treated with sorafenib (5 μmol/L), cisplatin (2 μmol/L), and radiation (7.5 Gy) with a gap of 1 hour in between each. After 72 hours, cell proliferation was assessed by using an MTT assay kit (Roche Diagnostics). The percentage cell growth inhibition for each group was calculated by adjusting the control group to 100%.

Tumor cell colony formation assay

Colony formation assay was conducted in 35 mm culture petri dishes as described previously (32). Tumor cells were treated with sorafenib (5 μmol/L), cisplatin (2 μmol/L), or radiation (7.5 Gy) alone or in combination. After 14 days of culture, colonies were stained with crystal violet (0.005%) for 1 hour and counted by using Nikon Eclipse Ti microscope with DS-Fi1 camera at ×40 magnification.

Western blot analysis

Whole cell lysates were separated by 4% to 12% NuPAGE Bis-Tris gels (Invitrogen) and transferred onto polyvinylidene difluoride membranes Nonspecific binding was blocked by incubating the blots with 3% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. The blots were then incubated with primary antibody in TBST + 3% BSA at 4°C overnight. After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:10,000) or with donkey anti-rabbit IgG (1:10,000) for 1 hour at room temperature. An ECL-plus detection system (Amersham Life Sciences) was used to detect specific protein bands. Protein loading in all the experiments was normalized by stripping the blots and then re-probing with antitubulin antibody. Alpha Innotech imaging software was used to quantify Western blot bands.

Tumor and endothelial cell motility assay

Cell motility assay was conducted in 6-well plates. A fine scratch in the form of groove was made by using a sterile pipette tip in about 90% confluent cells. Cells were then treated with sorafenib (5 μmol/L), cisplatin (2 μmol/L), or radiation (7.5 Gy) alone or in combination. The migration of cells was monitored microscopically by using Nikon Eclipse Ti microscope with DS-Fi1 camera.

Tumor cell invasion assays

Tumor cell invasion assay was carried out on Matrigel coated 24-well plate inserts (8 μmol/L pore size; BD Biosciences) as described previously (33). The number of cells that had invaded through the Matrigel was counted in 5 random high power fields.

Matrigel *in vitro* endothelial tube formation assay

Endothelial cell tube formation was carried out on Matrigel coated chamber slides as described previously (34). Each assay was photographed (Nikon Eclipse Ti microscope with DS-Fi1 camera) at ×40 magnification, and total area occupied by endothelial cell-derived tubes in each chamber was calculated by using software (NIS-Elements-Basic Research; Nikon) and expressed as an angiogenic score.

SCID mouse flank xenograft model

Six- to 8-week-old severe combined immunodeficient (SCID) mice (NCI) were used in all the *in vivo* experiments (33). Tumor cells (UM-SCC-74A or CAL27; 1×10^6) and endothelial cells (1×10^6) were mixed with 100 μL of Matrigel and injected in the flanks of SCID mice. After 8 days, mice were stratified into different groups (5 mice per group), so that the mean tumor volume in each group was comparable. At days 8, 11, 14, 17, 21, 24, and 28, animals were treated with sorafenib (10 mg/kg) and cisplatin (2 mg/kg) via intraperitoneal injections. One day following sorafenib and/or cisplatin treatment, on days 9, 12, 15, 18, 22, 25, and 29, flank tumors were treated with radiation (3 Gy), whereas the rest of body was shielded from irradiation by a lead shield. Tumor volume measurements [volume (mm^3) = $L \times W^2/2$ (L, length in mm; W, width in mm)] began on day 6 and continued twice a week until the end of the study. After 36 days, primary tumors were carefully removed and analyzed for tumor angiogenesis.

For sorafenib maintenance dose study, at day 36, 10 animals from combination treatment group were stratified into 2 groups (5 mice per group). One group was treated with sorafenib (10 mg/kg) twice a week and the other group was untreated control. Tumor volume was measured as described above.

Immunohistochemistry for angiogenesis, ERCC-1, and XRCC-1

Tumor sections were stained for angiogenesis (Von Willebrand factor), ERCC-1, and XRCC-1 as described previously (34). Microvessel density was calculated by counting 5 random high power fields (×200). Percentage positive cells for ERCC-1 or XRCC-1 were calculated by quantifying ERCC-1 and XRCC-1 positive cells, and the total number of cells (positive and negative) present in 5 randomly selected high power fields (×400) of each tumor samples.

Statistical analysis

Data from all the experiments are expressed as mean ± SEM. Statistical differences were determined by 2-way ANOVA and Student's *t*-test. $P < 0.05$ was considered significant.

Results

Sorafenib induces a dose-dependent inhibition of tumor and endothelial cell proliferation

Head and neck tumor cell lines (UM-SCC-74A and CAL27) and endothelial cells (HDMEC) showed similar sensitivities to sorafenib treatment (Fig. 1B). Sorafenib treatment for 72 hours resulted in 10%, 23%, 54%, 91%, and 95% growth inhibition for UM-SCC-74A; 7%, 18%, 51%, 93%, and 95% growth inhibition for CAL27 cells; and 2%, 18%, 49%, 86%, and 87% growth inhibition for HDMEC at 1, 2.5, 5, 10, and 20 μmol/L, respectively (Fig. 1B). In contrast, UM-SCC-74A cell line was highly resistant to both cisplatin (CDDP) and radiation treatment, whereas CAL27 cell line was quite sensitive to both cisplatin and radiation treatment (Fig. 1C and D).

Combination of low doses of sorafenib, cisplatin, and radiation significantly inhibits endothelial cell and tumor cell proliferation

Combination treatment significantly inhibited cell growth (70%, 81%, and 75% for UM-SCC-74A, CAL27, and HDMEC, respectively; Fig. 1E–G). This cell growth inhibition by combination treatment was significantly higher than treatment with sorafenib alone (43%, 42%, and 46% for UM-SCC-74A, CAL27, and HDMEC, respectively), cisplatin alone (13%, 47%, and 28% for UM-SCC-74A, CAL27, and HDMEC, respectively), or radiation alone (28%, 45%, and 41% for UM-SCC-74A, CAL27, and HDMEC, respectively) or in dual combinations of sorafenib + cisplatin (45%, 57%, and 54% for UM-SCC-74A, CAL27, and HDMEC, respectively), sorafenib + radiation (49%, 59%, and 53% for UM-SCC-74A, CAL27, and HDMEC, respectively) or cisplatin + radiation (41%, 43%, and 56% for UM-SCC-74A, CAL27, and HDMEC, respectively).

Combination treatment significantly inhibits UM-SCC-74A and CAL27 tumor cell colony formation in soft agar assay

We next investigated the effect of combination treatment on tumor cell colony formation. Similar to growth inhibition assay, combination treatment with sorafenib, cisplatin, and radiation induced significant inhibition of tumor cell colony formation in both the cell lines (93% and 95% for UM-SCC-74A and CAL27, respectively;

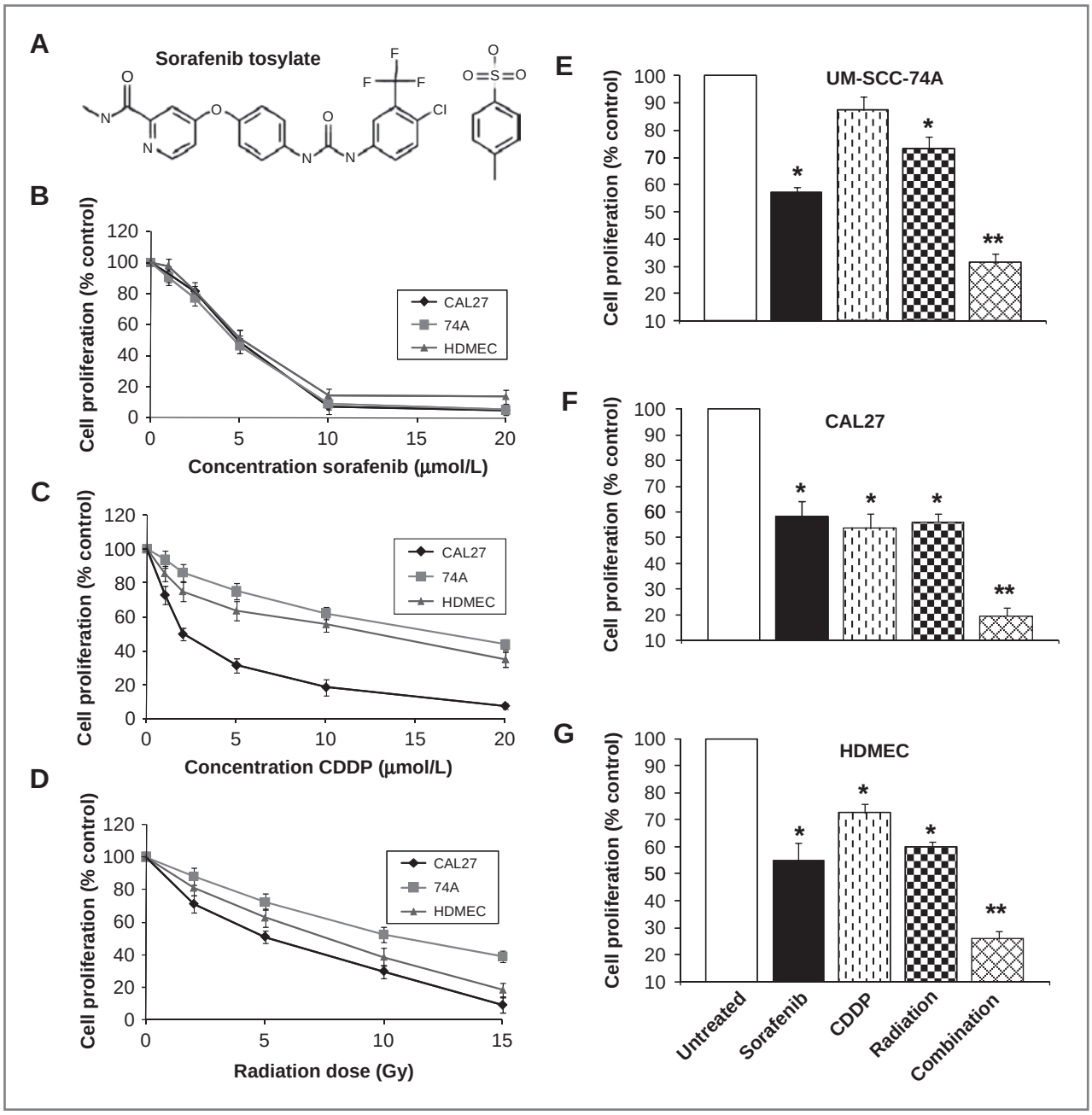

Figure 1. Sorafenib enhances the antiproliferative effects of chemoradiation treatment. A, chemical structure of sorafenib tosylate. B–D, UM-SCC-74A, CAL27, or HDMECs were treated with different concentrations of sorafenib, CDDP, or radiation. E–G, cells were treated with sorafenib, cisplatin, or radiation alone or in combination. After 72 hours, cell proliferation was assessed by MTT assay. The percentage cell growth inhibition for each group was calculated by adjusting the control group to 100%. *, represents a significant difference ($P < 0.05$) as compared with the untreated control group; **, represents a significant difference ($P < 0.05$) as compared with the single or double treatment groups.

Fig. 2). Tumor cell colony formation inhibition by combination treatment was significantly higher than sorafenib alone (28% and 26% for UM-SCC-74A and CAL27, respectively), cisplatin alone (14% and 24% for UM-SCC-74A and CAL27, respectively), or radiation treatment alone (24% and 21% for UM-SCC-74A and CAL27, respectively) or in double combinations of sorafenib + cisplatin (43% and 46% for UM-SCC-74A and CAL27, respectively), sorafenib + radiation (54% and 47% for UM-SCC-74A and CAL27, respectively), or cisplatin + radiation (46% and 51% for UM-SCC-74A and CAL27, respectively). In addition, the

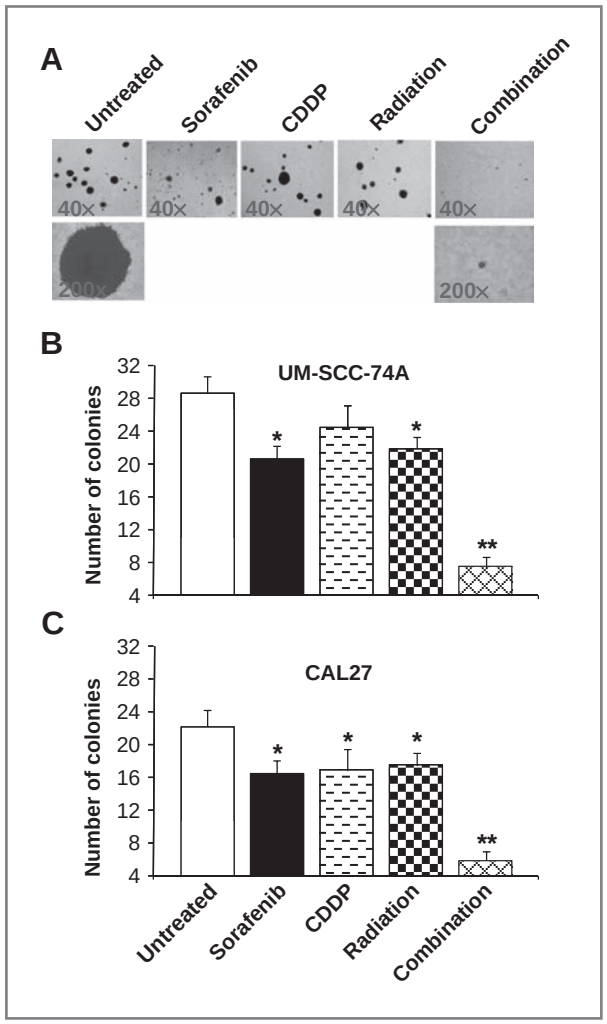

Figure 2. Combination treatment significantly inhibits tumor cell colony formation. Tumor cell colony formation assay was conducted in low melting point agarose in 35 mm culture petri dishes. Tumor cells UM-SCC-74A (A and B) or CAL27 (C) was treated with sorafenib (5 μmol/L), cisplatin (2 μmol/L), or radiation (7.5 Gy) alone or in combination and cultured at 37°C. After 14 days, colonies were stained with crystal violet (0.005%) for 1 hour and counted. *, represents a significant difference ($P < 0.05$) as compared with no treatment group; **, represents a significant difference ($P < 0.05$) as compared with single or double treatment groups.

size of colonies in combination treatment group was significantly smaller than no treatment, single treatment, or double treatment groups (Fig. 2A).

Combination treatment markedly reduces endothelial cell and tumor cell motility

Combination treatment effect on tumor (UM-SCC-74A and CAL27) and endothelial cell (HDMEC) motility was examined by scratch assay. Combination treatment significantly inhibited tumor and endothelial cell migration as compared with sorafenib, cisplatin, or radiation alone or in double combinations (Fig. 3A–D). Similar migration inhibitory effect of combination treatment was observed in the second tumor cell line (CAL27; data not shown).

Combination treatment significantly inhibits tumor cell invasion in Matrigel invasion assay

We used Matrigel invasion assay to examine the effect of sorafenib combination treatment on tumor cell invasiveness. Sorafenib (5 μmol/L), cisplatin (2 μmol/L), or radiation (7.5 Gy) alone showed 39%, 32%, and 27% inhibition of tumor cell (UM-SCC-74A) invasion through matrigel (Fig. 3C and D). Sorafenib in combination with cisplatin and radiation was highly effective in inhibiting tumor cell invasion (92%).

Combination treatment significantly inhibits tumor growth *in vivo*

The effect of combination treatment on tumor growth *in vivo* was examined by using SCID mouse model for tumor growth and tumor angiogenesis (33). We carried out 2 sets of experiments for tumor growth studies. In the first set of experiments, we investigated the effect of a combination therapy on tumor growth and tumor angiogenesis. Animals bearing UM-SCC-74A tumors treated with sorafenib, cisplatin, or radiation alone showed 26%, 20%, and 32% decrease in tumor size (day 36), whereas animals bearing CAL27 tumors showed 27%, 22%, and 36% decrease in tumor size, respectively (Fig. 4A and B). Combination treatment, with 2 agents together, more than doubled the inhibition of tumor growth as compared with each of these agents given alone [(sorafenib + cisplatin: 55% and 62% for UM-SCC-74A and CAL27, respectively), (sorafenib + radiation: 64% and 63% for UM-SCC-74A and CAL27, respectively), (cisplatin + radiation: 48% and 64% for UM-SCC-74A and CAL27, respectively)]. Triple combination treatment with sorafenib, cisplatin, and radiation resulted in greater than 90% reduction in tumor size (91% for UM-SCC-74A and 93% for CAL27). This reduction in tumor size was significantly greater than treatment with single agent or dual agents. In addition, the combination treatment did not cause any animal mortality or induce significant decrease in body weight.

In the second set of experiments, we investigated if sorafenib treatment could be used as maintenance therapy after the completion of combination treatment. In this study, animals undergoing combination treatment were randomized into 2 groups on day 36. One group received maintenance sorafenib treatment (combination-maintenance) and other group was untreated control (combination-untreated). Sorafenib as a single agent maintenance therapy was very effective, and it completely prevented tumor recurrence in CAL27 (Fig. 4D) and significantly inhibited UM-SCC-74A tumor growth (63% inhibition at day 48; Fig. 4C).

Combination treatment significantly inhibits tumor angiogenesis

Tumor samples (UM-SCC-74A) from animals treated with sorafenib, cisplatin, or radiation alone showed 27%, 16%, and 19% decrease in tumor vessel density (Fig. 5A and B). Combination treatment with sorafenib, cisplatin, and irradiation together was most effective by inhibiting

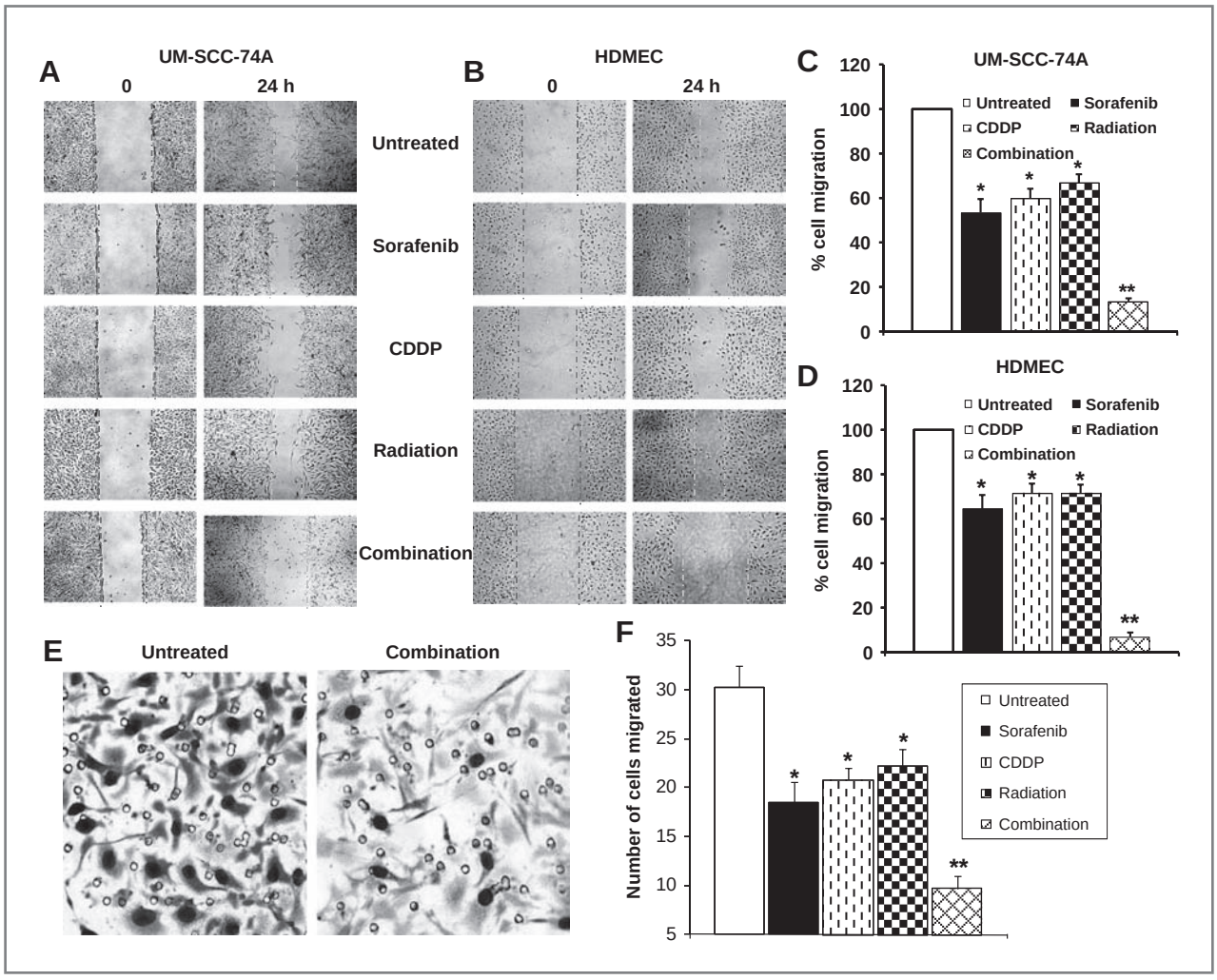

Figure 3. Combination treatment significantly inhibits tumor and endothelial cell motility and tumor cell invasion. A and B, tumor cell and endothelial cell motility was examined by scratch assay. Dotted lines in the left panels mark the edges at the start of experiments, and dotted lines in the right panels mark the edges at the end of experiments. C and D, each assay was photographed and distances between the migrating cell edges were quantified and percentage cell migration calculated. E and F, tumor cell invasion was examined by Matrigel invasion assay. The number of tumor cells that had invaded through the Matrigel was counted in 5 high power fields. *, represents a significant difference ($P < 0.05$) as compared with untreated group; **, represents a significant difference ($P < 0.05$) as compared with single or double treatment groups.

more than 90% of tumor angiogenesis (Fig. 5A and B). Similar decrease in blood vessel density was observed in CAL27 tumors (Fig. 5B).

We next examined if sorafenib combination treatment mediates its antiangiogenesis effects by inhibiting VEGF-mediated angiogenesis. VEGF treatment of endothelial cells significantly enhanced the tube formation on growth factor reduced Matrigel (Fig. 5C and D). Low-dose combination of sorafenib (5 μmol/L), cisplatin (2 μmol/L), and radiation (7.5 Gy) completely inhibited VEGF-mediated tube formation (Fig. 5C and D), whereas sorafenib, cisplatin, and radiation treatment alone showed 33%, 30%, and 36% inhibition of endothelial cell tube formation, respectively (Fig. 5C and D). VEGF predominantly mediates angiogenesis via the activation of PI3K/Akt and MAPK signaling cascade (27), and combination treatment significantly inhibited Akt and ERK1/2 activation (81% and 71%, respectively; Fig. 5E).

Sorafenib enhances tumor cell chemoradiation sensitization by downregulating ERCC-1 and XRCC-1

Tumor cells often develop chemo- and radioresistance by overexpressing DNA repair proteins, particularly ERCC-1 and XRCC-1 (16, 18). MAPK pathway is one of the key pathways involved in the upregulation of ERCC-1 and XRCC-1 (15). We therefore examined if sorafenib enhanced the antitumor effects of chemoradiation by downregulating these DNA repair proteins. Indeed, sorafenib inhibited ERK1/2 phosphorylation and downregulated the expression of ERCC-1 and XRCC-1 in a dose-dependent manner (Fig. 6A). In addition,

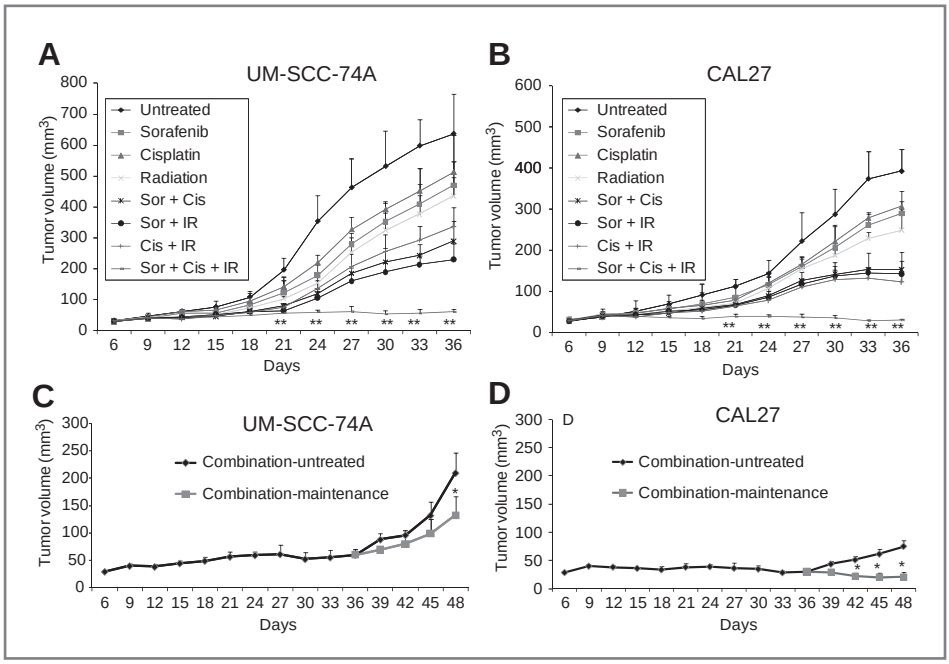

Figure 4. Combination treatment significantly inhibits tumor growth. A and B, tumor bearing animals were treated with sorafenib (10 mg/kg), cisplatin (2 mg/kg), or radiation (3 Gy) alone or in combination as described in Materials and Methods. C and D, for sorafenib maintenance dose study, at day 36, 10 animals from combination treatment group were stratified into 2 groups (5 mice per group). One group was treated with sorafenib (combination-maintenance; 10 mg/kg) twice a week, and the other group was untreated control (combination-untreated). *, represents a significant difference ($P < 0.05$) as compared with untreated group; **, represents a significant difference ($P < 0.05$) as compared with single or double treatment groups.

combination treatment significantly decreased the expression of both ERCC-1 and XRCC-1 (Fig. 6B).

We next stained tumor samples from our *in vivo* study to examine the effect of combination treatment on the expression of ERCC-1 and XRCC-1. Radiation treatment alone significantly increased ERCC-1 and XRCC-1 expression *in vivo* (7-fractionated radiation treatments over 21 days), whereas it did not significantly alter ERCC-1 and XRCC-1 levels *in vitro* (single radiation treatment and cell lysates prepared after 24 hours). These results suggest that tumor cells may require repeated exposure to radiation (fractionated doses) to upregulate ERCC-1 and XRCC-1 expression. As observed in our *in vitro* experiments, combination treatment with sorafenib, cisplatin, and radiation significantly reduced ERCC-1 (82%) and XRCC-1 (89%) expression (Fig. 6C and D).

To further investigate the roles of ERCC-1 and XRCC-1 in protection against chemoradiation treatment, we selectively knocked down ERCC-1 or XRCC-1 or both of them together in tumor cells. Western blot analysis showed a complete knockdown of ERCC-1 and more than 80% knockdown of XRCC-1 in both the cell lines (Fig. 6E; data shown for UM-SCC-74A). Knockdown of ERCC-1 or XRCC-1 alone showed about 50% decrease in cell proliferation when treated with low doses of cisplatin (2 μmol/L) and radiation (7.5 Gy), whereas knocking down of both ERCC-1 and XRCC-1 together decreased more than 80% of cell proliferation (Fig. 6F).

Discussion

The chemoradiation regimen is one of the most commonly used treatments for many head and neck cancer patients. However, this intense therapeutic regimen often results in significant toxicity leading to decreased quality of life. Therefore, there is an urgent need to develop combination treatment regimens that can improve the therapeutic efficacy of chemoradiation while minimizing the toxic side effects. However, a major challenge for developing combination treatments is the identification of specific target molecule(s) that can provide the highest degree of synergistic antitumor activity with traditional therapies. One such target molecule for head and neck cancer is Raf kinase. Majority of head and neck tumors (>80%) overexpress EGFR, and increased EGFR expression is directly correlated with worse prognosis, including advanced stage, poorly differentiated tumors, and poor survival (6–9). EGFR expression in HNSCC also correlate positively with the acquisition of radioresistance (35). Raf-MAPK is one of the key pathways EGFR uses to mediate its biological effects. RAF-MAPK pathway also plays an important role in mediating radio- and chemoresistance in tumor-associated endothelial cells (14). Therefore, we hypothesize that targeting of Raf kinases in advanced head and neck tumors could reverse the resistant phenotype in tumor cells as well as tumor-associated endothelial cells, thereby enhancing the therapeutic efficacy of standard chemoradiation.

To test this hypothesis, we have carried out combination treatment studies *in vitro* as well as *in vivo*, by using our SCID mouse model. We selected sorafenib, a multikinase inhibitor, for this study because it is a potent antitumor agent as well as it is equally effective against tumor stroma components particularly tumor-associated endothelial cells (27). In addition, sorafenib has been successfully used in clinics for the treatment of advanced RCC, HCC, and thyroid cancer (25, 26, 36). We selected 2 HNSCC cell lines (CAL27 and UM-SCC-74A) based on

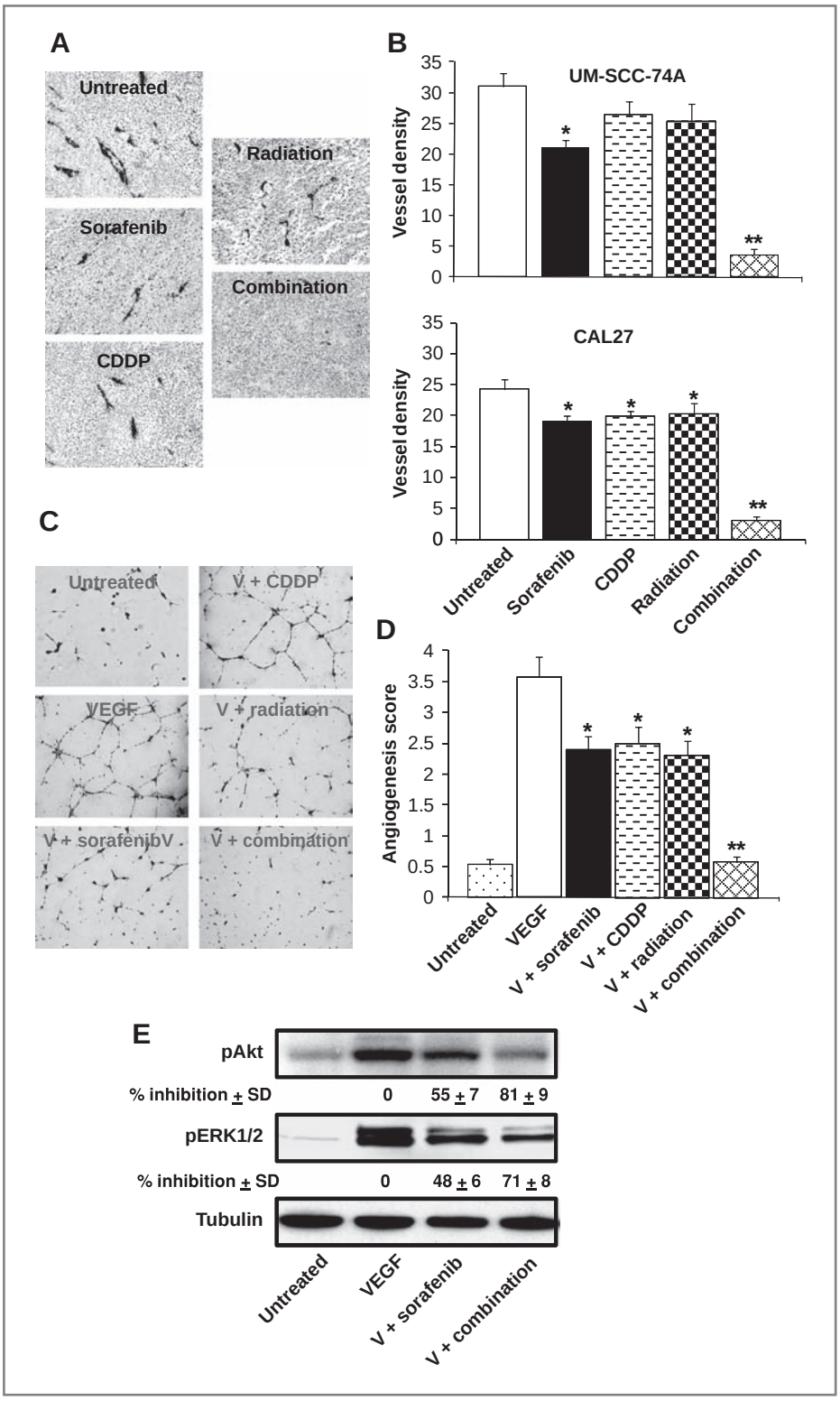

Figure 5. Combination treatment significantly inhibits tumor angiogenesis and VEGF-mediated endothelial cell tube formation. A, representative photomicrographs of tumor blood vessel staining for untreated, sorafenib, CDDP, or radiation alone or combination groups for UM-SCC-74A tumors. B, microvessel density in the tumor samples was calculated by counting 5 random fields (×200) and expressed as vessel density ± SE. C, representative photomicrographs of *in vitro* tube formation assay for untreated, VEGF, VEGF + sorafenib (V + sorafenib), VEGF + cisplatin (V + CDDP), VEGF + radiation (V + radiation), and VEFG + combination treatment (V + combination). D, quantitative data for tube formation expressed as angiogenic score ± SE from 3 independent experiments. *, represents a significant difference ($P < 0.05$) as compared with VEGF group; **, represents a significant difference ($P < 0.05$) as compared with VEGF or single treatment groups. E, endothelial cells were treated with sorafenib (5 μmol/L) or combination treatment (sorafenib + cisplatin + radiation) for 1 hour and then treated with VEGF for 30 minutes. Whole cell lysates from each group were Western blotted and probed for pAkt and pERK1/2. Equal sample loading was verified by stripping the blots and re-probing with antitubulin antibody. Band density of each protein was normalized with tubulin and expressed as % inhibition ± SE as compared with controls from 3 independent experiments.

their chemo- and radiation sensitivities for our *in vitro* and *in vivo* work. CAL27 is a relatively sensitive cell line to chemo- and radiation treatment. In contrast, UM-SCC-74A is highly resistant to both chemo- and radiation treatment. In addition, UM-SCC-74A contains wild-type p53, whereas CAL27 has mutant p53 gene. Interestingly, both these cell lines were equally sensitive to sorafenib treatment regardless of p53 mutational status, thereby

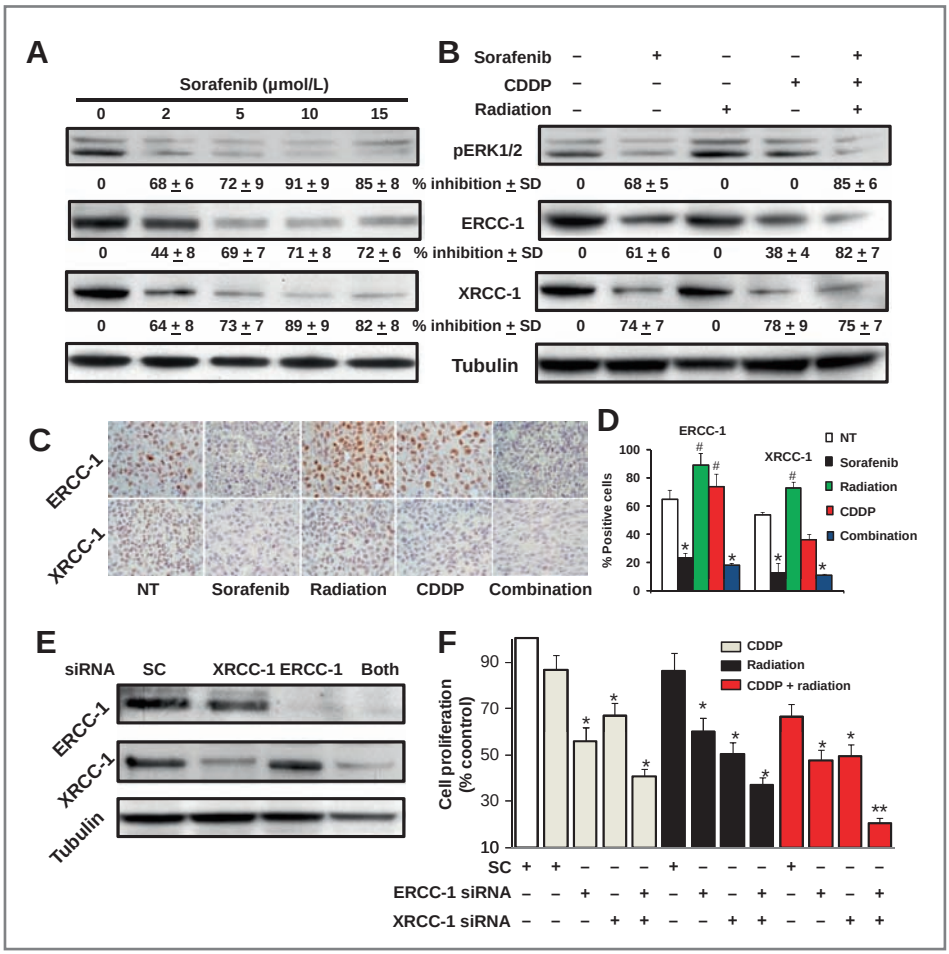

Figure 6. Sorafenib enhances chemoradiation-mediated antitumor effects by downregulating DNA repair proteins ERCC-1 and XRCC-1. A, UM-SCC-74A cells were treated with different concentrations of sorafenib for 24 hours. B, UM-SCC-74A cells were treated with sorafenib (5 μmol/L), cisplatin (2 μmol/L), or radiation (7.5 Gy) alone or in combination for 24 hours. Equal sample loading was verified by stripping the blots and re-probing with antitubulin antibody. Band density of each protein was normalized with tubulin and expressed as % inhibition ± SE as compared with controls from 3 independent experiments. C, paraffin-embedded UM-SCC-74A tumor samples were stained for ERCC-1 and XRCC-1 and representative photomicrographs (×400) of untreated (NT), sorafenib, radiation or CDDP alone or combination groups. D, ERCC-1 and XRCC-1 positive cells were quantified in 5 high power fields (×400) of each tumor samples and percentage of positive cells calculated. *, represents a significant decrease ($P < 0.05$); #, represents a significant increase ($P < 0.05$) as compared with the untreated control group. E, ERCC-1, XRCC-1, or both (ERCC-1 and XRCC-1) proteins were knocked down in UM-SCC-74A cells by respective siRNA(s). Whole cell lysates from each experiment were separated by using 4% to 12% NuPAGE Bis-Tris gels and probed for ERCC-1 and XRCC-1. Equal sample loading was verified by stripping the blots and re-probing with antitubulin antibody. F, cell proliferation was measured in UM-SCC-74A cells after knocking down ERCC-1, XRCC-1, or both and treating with cisplatin (2 μmol/L), radiation (7.5 Gy), or both (cisplatin and radiation) for 72 hours. The percentage cell growth inhibition for each group was calculated by adjusting the scramble control group to 100%. *, represents a significant difference ($P < 0.05$) as compared with the scramble control group; **, represents a significant difference ($P < 0.05$) as compared with the scramble control or single treatment groups.

suggesting that sorafenib mediates its antitumor effects independent of p53 status. This is important as more than 50% of head and neck tumors have mutant p53, and some of the targeted inhibitors selectively inhibit cell growth in cancer cells with wild-type p53 only (37, 38).

In the combination treatment studies, we selected doses of sorafenib (5 μmol/L), cisplatin (2 μmol/L), and radiation (7.5 Gy) that by themselves showed about 50% growth inhibition in chemoradiation sensitive tumor cell line (CAL27). The rationale for selecting these low doses was to have the greatest possible synergistic antitumor effect without the serious side effects that are often associated with the maximum tolerated doses of radiation and chemotherapy. Indeed, pretreatment with sorafenib significantly enhanced chemoradiation-mediated inhibition of tumor cell proliferation and colony formation. A number of studies have showed that tumor cells acquire chemo- and radioresistance by increasing the expression of antiapoptotic proteins and/or DNA repair proteins (16, 18). Efficient DNA repair in the cancer cells is an important mechanism of therapeutic resistance (39), and inhibition of DNA repair pathway would make tumor cells more sensitive to DNA damaging agents like chemotherapy and radiation treatment. In this study, we

have shown for the first time that sorafenib can downregulate the expression of DNA repair proteins ERCC-1 and XRCC-1 in a dose-dependent manner. In addition, combination treatment was equally effective in inhibiting tumor cell invasion. Sorafenib may be decreasing tumor cell invasion by inhibiting matrix metalloproteinase production by blocking the Raf-MAPK pathway (40) which has been shown to induce the production of matrix metalloproteinases (41). Sorafenib in combination treatment also significantly inhibited VEGF-mediated endothelial cells tube formation (*in vitro* angiogenesis assay). This could be because of the direct inhibition of VEGFR2 and VEGFR3 signaling by sorafenib (27). Endothelial cell tube formation is a complex process that involves endothelial cell migration and/or proliferation. Our results suggest that combination treatment predominantly affects endothelial cell tube formation by inhibiting endothelial cell motility as combination treatment showed a similar inhibition of endothelial cell tube formation (91%) and endothelial cell migration (92%), whereas the combination treatment effect on endothelial cell proliferation was significantly less (45%; data not shown) at 24 hours posttreatment.

To determine if the observed *in vitro* synergy between sorafenib, cisplatin, and ionizing radiation extends to the *in vivo* setting, we used a SCID mouse model to study the effect of combination treatment on tumor growth and tumor angiogenesis. Low doses of sorafenib (10 mg/kg), cisplatin (2 mg/kg), and radiation treatment (3 Gy; fractionated dose) exhibited even more pronounced antitumor effects. This marked inhibition of tumor growth by combination therapy particularly in chemo- and radioresistant cell line could be because of sorafenib-mediated inhibition of tumor proliferation via the Raf-MAPK pathway (40), downregulation of DNA repair proteins, and antiapoptotic Mcl-1 protein (42) as well as reduction in the formation of new blood vessels by inhibiting VEGF signaling (27) as observed in tube formation assay. Sorafenib's potent antiangiogenesis effects may also be responsible for significant inhibition of tumor growth when sorafenib was used as a maintenance therapy. This combination treatment was very well tolerated in the animals. It did not cause any animal mortality or induced significant weight loss or induced any major systemic toxicity such as dry scaly skin or respiratory distress which has been reported in animals treated with high doses of chemoradiation treatment or other small molecular weight inhibitors (43).

In conclusion, we have showed that sorafenib significantly enhances the therapeutic efficacy of chemoradiation by inhibiting tumor and endothelial cell survival, tumor cell invasiveness, and angiogenesis. These results suggest a potentially novel strategy to enhance the therapeutic efficacy of chemoradiation for head and neck cancers. Moreover, this strategy of using a combination of low doses of sorafenib, cisplatin, and radiation has the potential of significantly decreasing side effects associated with the concurrent chemoradiation treatment although maintaining their therapeutic efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

NIH/NCI-CA133250 (P. Kumar) and Joan's fund research grant (B. Kumar and P. Kumar).

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Received January 3, 2011; revised April 13, 2011; accepted April 19, 2011; published OnlineFirst May 6, 2011.

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