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Mechanism of enhancement of radiation-induced cytotoxicity by sorafenib in colorectal cancer

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Sorafenib, an orally available multikinase inhibitor, combined with radiation has shown potential as an anticancer treatment in an *in vitro and in vivo* colon cancer model. In this study, we investigated the mechanism of enhancement of radiation-induced cytotoxicity by sorafenib in colorectal cancer. The effects of sorafenib on radiation-induced cytotoxicity of DLD-1 and HT-29 were evaluated via clonogenic assay. The impact of sorafenib on radiation-induced cell cycle kinetics and on apoptosis was analyzed using flow cytometry. Cyclin B1 was examined by western blot. As a measure of DNA damage after treatment, γ-H2AX foci and nuclear fragmentation were determined as a function of time after irradiation plus sorafenib combination. Tumor growth delay was used to evaluate the effects of sorafenib on *in vivo* radiation-induced cytotoxicity. Exposure of each cell line to sorafenib combined with irradiation resulted in an increased radiation-induced cytotoxicity with dose enhancement factors at a surviving fraction of 0.37 ranging from 1.13 to 1.76. Sorafenib strengthened radiation-induced accumulation of tumor cells in the G2-M phase with attenuated expression of cyclin B1, but had no effect on radiation-induced apoptosis. Exposure to sorafenib and radiation resulted in a greater number of remaining γ-H2AX foci and fragmented nuclei than radiation alone. *In vivo* tumor xenograft study confirmed that administration of sorafenib results in significant tumor growth inhibition when combined with radiation. These results indicate that sorafenib enhances radiation-induced cytotoxicity in colorectal cancer and suggest that the mechanism is associated with delaying repair of radiation-induced DNA damage and down-regulation of cyclin B1.

Keywords: radiation; sorafenib; colorectal cancer; DNA damage; cell cycle

INTRODUCTION

Radical surgery has been the mainstay of management of colorectal cancer. In postoperative adjuvant settings, radiotherapy with chemotherapy was previously believed to reduce local failure and distant metastasis, thereby improving the clinical outcomes of patients with high risk factors [1–3]. Unlike in the US, European investigators have continuously favored a short course preoperative radiotherapy that has been shown to improve local control in clinical studies [4–6]. The German Rectal Cancer Trial Study Group demonstrated that preoperative chemoradiotherapy (CRT), as compared with postoperative CRT, improved local control and was associated with reduced toxicity, but did not improve overall survival [7]. Therefore, the standard adjuvant treatment was shifted from postoperative CRT to preoperative CRT in mid- and distal rectal cancers. Pathologic treatment response or downstaging after neoadjuvant CRT seems to have a significant clinical implication that pathologic response after preoperative CRT may be closely related to prognoses [8, 9]. Therefore, an enhanced radiation response is necessary for better pathologic response after preoperative CRT. A promising strategy is to use molecular targeted agents.

Sorafenib is an orally available multikinase inhibitor that targets kinases of wild-type B-Raf, mutant V559EB-Raf, and C-Raf, blocking tumor growth. Furthermore, sorafenib shows potent inhibition of receptor tyrosine kinases involved in angiogenesis, including human vascular endothelial growth factor receptors-2 and -3, as well as the platelet-derived growth factor receptor-β [10]. Sorafenib has been shown to inhibit the proliferation of a variety of human cancer cell lines and xenograft models. A series of clinical studies tested the antineoplastic potency of sorafenib in cancer patients. Therein, sorafenib prolonged progression-free and overall survival in phase III clinical studies of patients with metastatic renal cell carcinoma or advanced heaptocellular carcinoma, respectively [11, 12]. Moreover, preliminary data from a series of combination studies with sorafenib and a variety of anti-cancer agents for various solid tumor entities have been published [13]. Previous studies have shown sorafenib inhibited *in vivo* tumor growth combined with radiation, but did not suggest the mechanism *in vitro* [14, 15]. Subsequently, in this study we attempted to investigate the mechanism of enhancement of radiation-induced cytotoxicity by sorafenib using *in vitro* and *in vivo* colorectal cancer models.

MATERIALS AND METHODS

Cell cultures and reagents

DLD-1 and HT-29 cells originated from colorectal adenocarcinomas were maintained in minimum essential medium and Rosewell Park Memorial Institute media supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere, and the media were replaced every 3–4 days. Sorafenib was provided by Bayer Pharmaceutical Corporation (West Haven, CT, USA).

Clonogenic assay for radiation survival experiment

Log-phase cells were trypsinized, plated in triplicate per data point into 25-cm^2 cell culture flasks, and then permitted to attach overnight. In the radiation survival experiment, the cells were irradiated with graded doses of X-rays. Tumor cells were irradiated with PRIMART (Siemens, Berlin, Germany) with 6 megavoltage and a dose rate of 0.3 Gy/min. Immediately after irradiation, the cells were exposed to a mock (DMSO) or to sorafenib for 72 h and then maintained in drug-free medium for 10 days to allow for the formation of colonies and then stained with 0.5% crystal violet in absolute methanol. The colonies were counted with a cutoff value of 50 viable cells. We then calculated the dose enhancement ratio (DER) as the dose (Gy) for the radiation alone divided by the dose for radiation plus sorafenib (normalized for drug toxicity) at a surviving fraction of 37% (D_1).

Detection of cell cycle changes and apoptosis via flow cytometry

The cells were exposed to single dose of X-rays and then exposed to the appropriate concentrations of sorafenib or vehicle. After additional 16, 24 and 48 h of incubation, the cells were collected, fixed with 75% ethanol, and then incubated with propidium iodide (PI) and RNase A. The number of cells at each cell cycle was evaluated using the FACSCalibur system (Becton Dickinson, San Jose, CA, USA). To evaluate apoptotic cells, both adherent and non-adherent cells were harvested at 12, 24 and 36 h after each treatment. The experiments were performed using the Annexin V-FITC Apoptosis Detection kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's manual.

Immunofluorescent staining for γ-H2AX and measurement of nuclear fragmentation

Cells were grown and treated in chamber slides. At specified timings, the cells were fixed in 4% paraformaldehyde and incubated overnight with anti-γ-H2AX antibody (Abcam, Cambridge, UK). Cells again were incubated in the dark with an FITC-labeled secondary antibody for 1 h, incubated in the dark with 4,6-diamidino-2-phenylindole (DAPI), and cover slips were mounted with an antifade solution. Detection of fluorescence and acquisition of images were done with a Zeiss LSM 510 Meta confocal fluorescent microscope. For each treatment condition, γ-H2AX foci were determined in at least 100 cells. To visualize nuclear fragmentation, cells were fixed with 4% paraformaldehyde, and stained with DAPI followed by staining with α-tubulin. A single field containing 300 cells was selected at random for each treatment and photographed with epifluorescence. Nuclear fragmentation was defined as the presence of two or more distinct nuclear lobes within a single cell.

Western blotting

The cells were lysed in radioimmunoprecipitation assay buffer, and protein concentrations were determined. Proteins were denatured and fractionated on polyacrylamide gels and then transferred to polyvinylidene difluoride membranes after electrophoresis. After blocking, the filters were incubated overnight in primary antibodies (Cyclin B1, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The filters were incubated with a horseradish peroxidase-conjugated anti-mouse immunoglobulin as a secondary antibody for 1 h at a 1:5,000 dilution. After three additional washes, the filters were developed with an enhanced chemiluminescence system and scanned.

Tumor xenograft model and tumor growth delay

Five- to six-week-old nude mice were used in these studies. They were housed in a specific pathogen-free (SPF) barrier area at the Division of Laboratory Animal Medicine,

College of Medicine, Yonsei University. The temperature (22°C) and humidity (55%) were controlled constantly. Water (RO water) and food (PMI) were supplied. The care and use of laboratory animals in this study were based on the Guidelines and Regulations for the Use and Care of Animals at Yonsei University College of Medicine. DLD-1 cells were injected subcutaneously into the right hind leg. Irradiation was performed using a linear accelerator with animals restrained in a custom jig. To obtain tumor growth curves, perpendicular diameter measurements of each tumor were made every 2 days with digital calipers, and volumes were calculated using the formula $(L \times W \times W)/2$. Tumors were followed until the tumors of the radiation plus sorafenib group reached a mean size of 2000 mm^3. Growth delay (GD) was calculated as the time for treated tumors to reach an average volume of 2000 mm^3 minus the time for control tumors to reach 2000 mm^3, with $t = 0$ defined as the first day of treatment. EF was then determined as follows: Enhancing factor (EF) = $(GD_{Sorafenib+RT} - GD_{Sorafenib})/GD_{RT}$.

Statistical analysis

Data were expressed as means ± standard error of mean (SEM) and then analyzed with regard to statistical significance using two-tailed Student's t-tests. *P*-values < 0.05 were considered to be significant.

RESULTS

To determine the effects of sorafenib on radiation-induced toxicity, clonogenic survival analysis was performed using DLD-1 and HT-29 cell lines. In these studies, we used a sorafenib concentration of 4 μmol/l, corresponding to an inhibitory concentration of 25% at a 72 h exposure to sorafenib. To assess and characterize the radiation-enhancing effects of sorafenib, the cells were exposed to graded doses of radiation either with or without various concentrations of sorafenib for 72 h. These cells were all then permitted to form colonies in drug-free medium. The drug was administered immediately after radiation treatment and dose-enhancing ratio values were determined to be 1.76 and 1.13 at D_1, respectively (Fig. 1).

The effects of sorafenib on inhibition of cell cycle progression and modulation of interactions with radiation were evaluated via flow cytometry in control (mock-treated), sorafenib monotherapy, radiation alone, and the combination of radiation and sorafenib-treated groups. While radiation induced accumulation of cells in the G2-M phase, sorafenib showed no arrest in any specific cell phase. Sorafenib treatment combined with radiation in the DLD-1 and HT-29 cells resulted in significantly increased and sustained rates of G2-M phase cells, compared with that observed as a result of radiotherapy alone in each cell line (Fig. 2).

We further evaluated whether mechanisms of interaction between sorafenib and radiation were involved in cell killing mediated by apoptosis in HT-29 and DLD-1 cells. Cells were harvested after 12, 24 and 36 h of exposure to vehicle, sorafenib (4 μM), radiation (6 Gy) or combination treatment. Apoptosis was measured with flow cytometry using Annexin V and PI staining. Apoptotic induction rates after radiation and sorafenib combination treatment showed no apparent synergistic increase in any of the cells, when compared with the values observed after administration of each treatment separately in both cell lines (Table 1).

We investigated the activity of cyclin B1 by western blot analysis in DLD-1 and HT-29 cell lines. Cells were harvested after 12 and 24 h of exposure to sorafenib, radiation or a combination thereof. While radiation induced expression of cyclin B1, sorafenib inhibited cyclin B1 expression

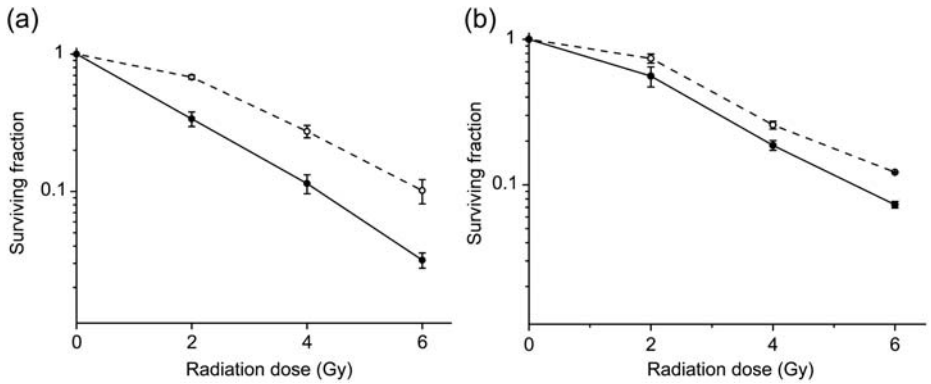

Fig. 1. Clonogenic survival curves for radiation plus sorafenib treatment in DLD-1 (**a**) and HT-29 (**b**) cells. White circles, radiation alone; black circles, radiation plus sorafenib (4 uM) treatment. DERs were 1.76 and 1.13 at 0.37. Bars, SEM of three independent experiments.

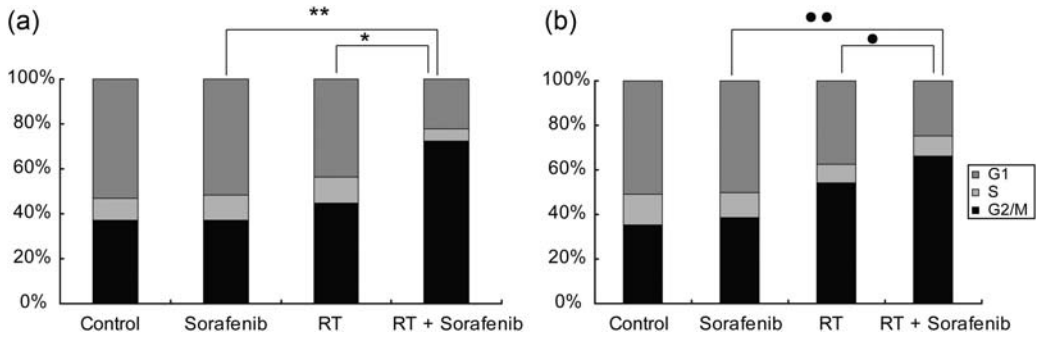

Fig. 2. Impact of sorafenib and radiation on cell cycle phase distribution. Radiation (6 Gy) and sorafenib (4 uM) treatment were significantly higher compared with other treatment groups at 48 h (*, $P = 0.037$; **, $P = 0.016$) in DLD-1 (**a**) and HT-29 cells (**b**) (one black circle, $P = 0.004$; two black circles, $P = 0.02$). Columns, mean values of triplicate samples.

Table 1: Measurements of apoptotic fraction were measured after Annexin V and PI staining using flow cytometry in DLD-1 (**A**) and HT-29 (**B**)

	Control	**Sorafenib 4 uM**	**Radiation 6 Gy**	**Sorafenib 4 uM + Radiation 6 Gy**
(A) DLD-1				
12 h	1.53	2.57	1.08	0.80
24 h	2.08	4.28	3.03	3.79
36 h	0.63	1.13	1.29	1.40
(B) HT-29				
12 h	1.40	4.01	1.11	1.57
24 h	1.54	2.16	1.74	2.16
36 h	0.76	1.14	0.57	1.12

No interval changes were observed among treatment groups until 36 h in both cells. The data represent the average value of three independent experiments.

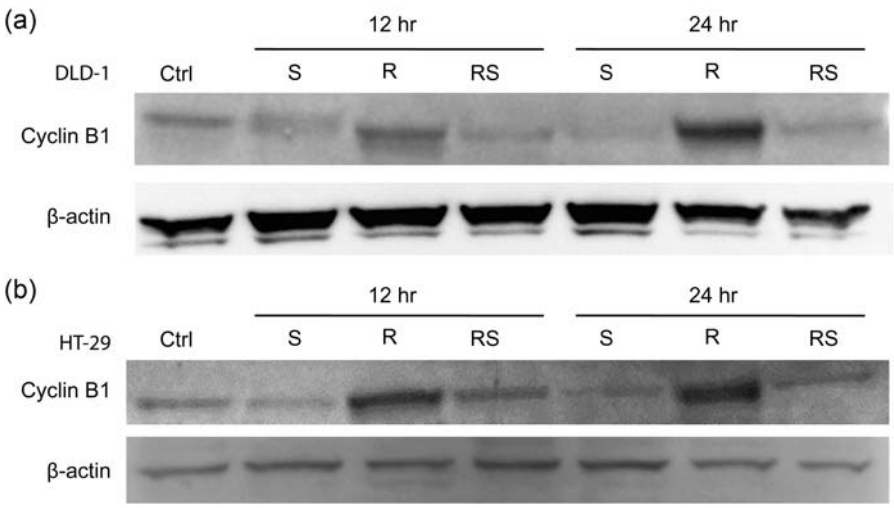

Fig. 3. Expression of cyclin B1 by sorafenib (4 uM) and radiation (6 Gy) in DLD-1 (**a**) and HT-29 (**b**) cell lines by western blot analysis. Ctrl, control; S, sorafenib; R, radiation; RS, combination.

at all time points. Sorafenib also attenuated expression when combined with radiation (Fig. 3).

As a measure of radiation-induced DNA damage, we evaluated the induction of nuclear foci of γ-H2AX, which has been established as a sensitive indicator of DNA double strand breaks (DSBs), with the dispersion of foci corresponding to DSB repair [17]. Immunofluorescent stains for γ-H2AX foci were determined at 0, 6 and 24 h after radiation. Both cell lines showed significant γ-H2AX production at 6 h post-radiation regardless of sorafenib

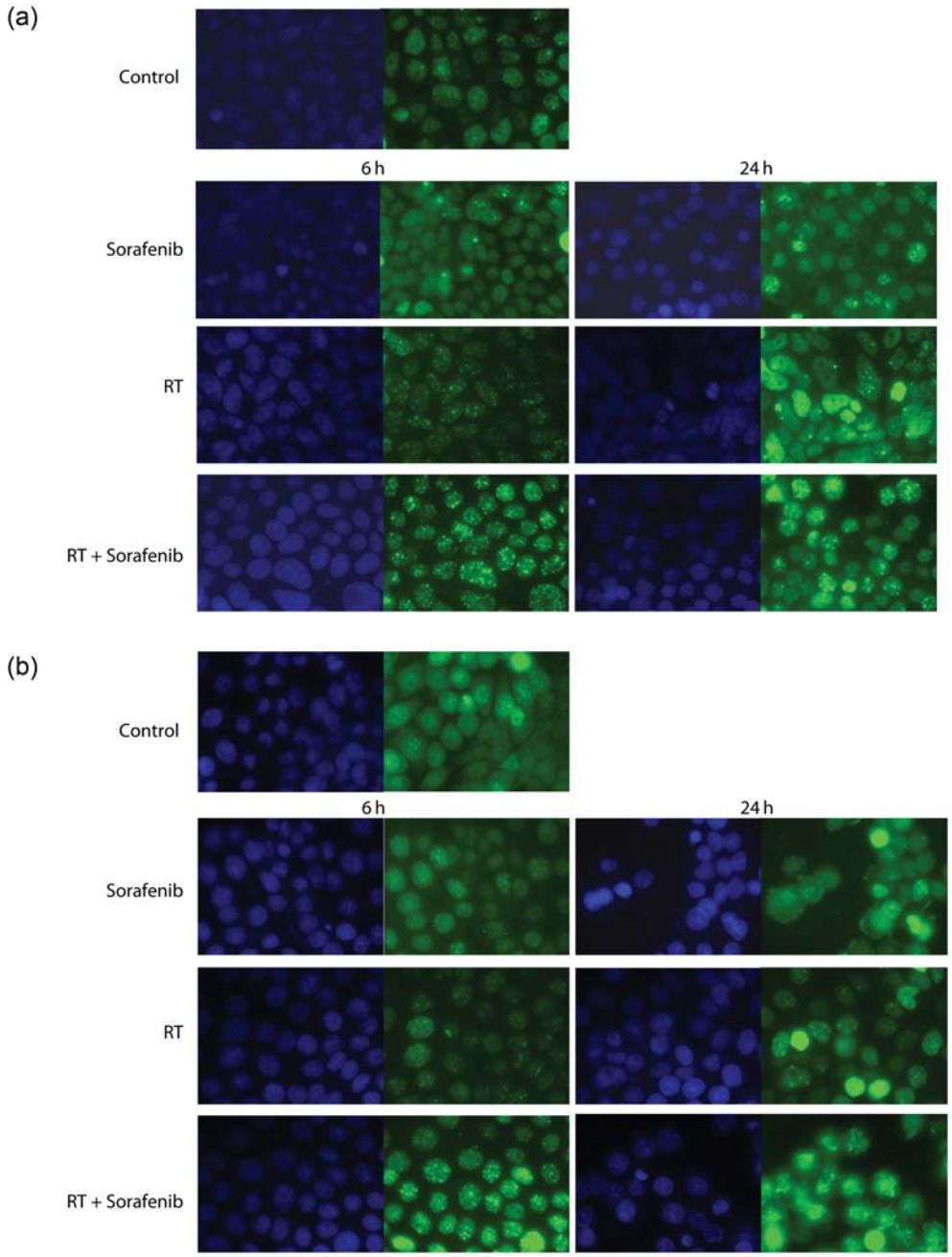

Fig. 4. Confocal microscopy for detection of γ-H2AX foci. DLD-1 (**a**) and HT-29 (**b**) cells on coverslips were treated with 2 Gy ± sorafenib, and then confocal microscopy was performed to detect γ-H2AX foci (green colored image, right-side) as well as nucleus (blue colored image, left-side). Measurement of average fluorescence intensity of γ-H2AX foci per cell in DLD-1 (**c**) and HT-29 (**d**) cells. The combination group resulted in a greater number of remaining γ-H2AX foci than radiation alone at 24 h with statistical significances in DLD-1 (*, $P < 0.05$), and in HT-29 (black circle, $P < 0.05$). The plotted data represent the average value and SEM of three independent experiments.

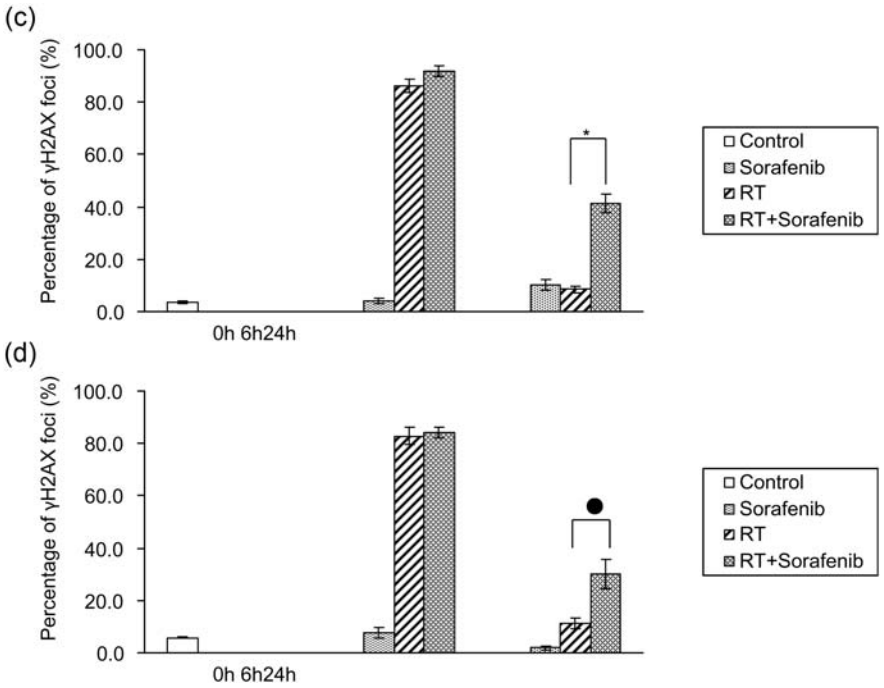

Fig. 4. (Continued)

treatment. However, significant differences of clearance γ-H2AX were observed between radiation and combination at 24 h post-radiation (Fig. 4). The combination arm delayed the clearance of γ-H2AX, suggesting that sorafenib maintains DNA damage and increases radio-sensitivity.

To measure the proportion of cells that underwent the aberrant mitotic process, we counted the number of cells showing nuclear fragmentation according to sorafenib and/or radiation treatment. The percentage increased up to 72 h after radiation. In cells receiving the combination treatment, a significantly greater number of cells showing nuclear fragmentation were detected at 48 and 72 h, compared with radiation alone. Therefore, these data suggest that the radiation-induced cytotoxicity by sorafenib results in an increase in the number of cells undergoing aberrant mitosis (Fig. 5).

Tumors were generated at the hind legs of nude mice. When tumors grew to a mean volume of 166 mm^3, mice were randomized into four groups: mock alone, sorafenib alone (60 mg/kg by p.o. gavage), irradiation (7.5 Gy/3 fraction/3 consecutive days) alone, and sorafenib plus radiation. Each experimental group contained eight to ten mice. In our study, significant tumor growth delay was confirmed between the sorafenib plus radiation group and all the other groups in the tumor xenograft model. Sorafenib treatment alone delayed tumor growth as did sorafenib plus radiation treatment for the first 5 days, but thereafter tumors rapidly grew and caught up to the control group on Day 12. Fractionated radiation treatment with 7.5 Gy produced a 2-day GD. In contrast, the combined treatment of 60 mg/kg sorafenib and 7.5-Gy fractionated radiation produced a GD of 7 days. This resulted in an EF of 3.5, indicating more than just an additive effect for the combination treatment (Fig. 6).

DISCUSSION

In this study, treatment with sorafenib enhanced the cytotoxic effect of radiation. As found in previous reports, post-radiation sorafenib treatment was more lethal compared with pre-radiation treatment in colon cancer cell lines. Sorafenib treatment with radiation exhibited significant tumor growth delay in an *in vivo* system [14, 15]. The mechanism by which radiation-enhancement occurred, however, appeared to be somewhat more complex than predicted in previous studies.

The accumulation of the cells in the G2-M phase may partially explain the higher susceptibility to radiation-induced DNA damage, reflected in the increase in both nuclear fragmentations and delayed clearance of γ-H2AX, compared with cells treated with radiation alone. Because of DNA repair inhibition by sorafenib and subsequent chromosomal abnormalities as they go into mitotic stage, this could explain some of the enhanced radiation-induced cytotoxicity observed with sorafenib post-incubation. Likewise, Sorafenib results in downregulation of cyclin B1 after radiation leading to an increase in the percentage of cells in the G2-M phase of the cell cycle.

The phosphorylation of γ-H2AX in DSBs is regarded as an early response to various DNA damage including

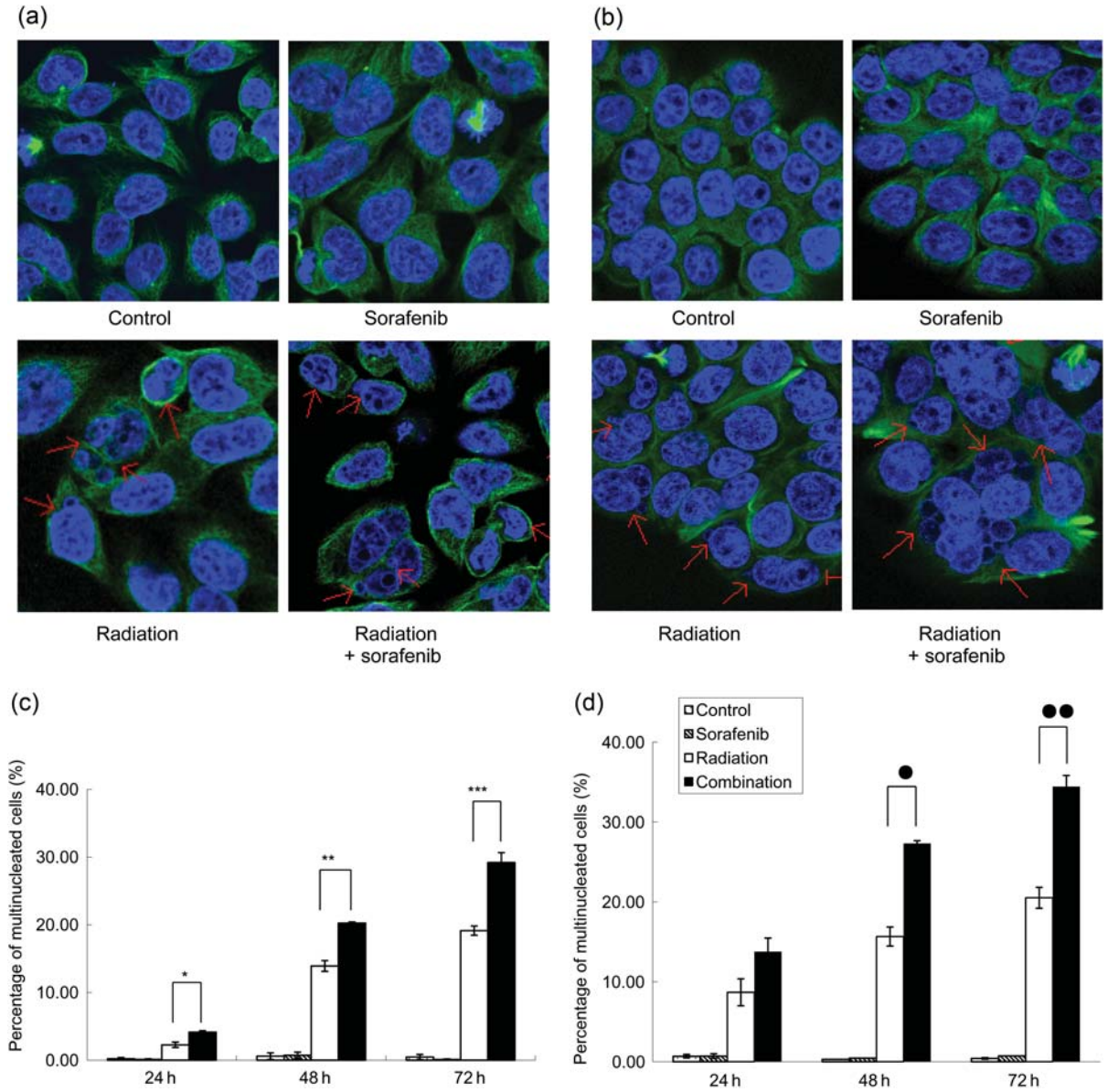

Fig. 5. Influence of sorafenib on radiation-induced nuclear fragmentation. DLD-1 (**a**) and HT-29 (**b**) cell lines growing in chamber slides were assigned to control, sorafenib (4 uM), radiation (6 Gy) and combination groups and fixed at 24 h, 48 h and 72 h. Cells were fixed with 4% paraformaldehyde, and stained with DAPI followed by staining with α-tubulin. Images were taken with a Zeiss LSM 510 Meta confocal microscope. Columns, mean; bars, SE. Nuclear fragmentation was defined as the presence of two or more distinct lobes within a single cell. *, **, ***, one black circle, two black circles, $P < 0.05$ according to Student's t-test.

radiation, even though it is not necessary for the initial activation of DNA damage repair or signal transduction. While γ-H2AX foci induction is found several minutes after DNA damage, the inactivation of γ-H2AX foci by phosphatases occurs over hours to days and reflects the activity of DNA repair proteins following DSB [16]. Several investigators have demonstrated the correlation between the persistence of γ-H2AX and radio-sensitivity in a wide range of cell lines. Combined treatment with radiation and radio-sensitizing agents shows effective cytotoxicity and appears to affect the kinetics of γ-H2AX clearance after radiation. However, there are conflicting data on the kinetics thereof. Several studies suggested that suppression of phosphorylation of γ-H2AX foci by agents can sensitize tumors to radiation in *in vitro* and *in vivo* tumor models [17–19]. The actions of agents are supposed to either control γ-H2AX directly or result in γ-H2AX foci inactivation after radiation by interfering with upstream kinase activity. In contrast, one report suggested that overexpression of γ-H2AX foci appeared to sensitize glioma cell lines to radiation, possibly,

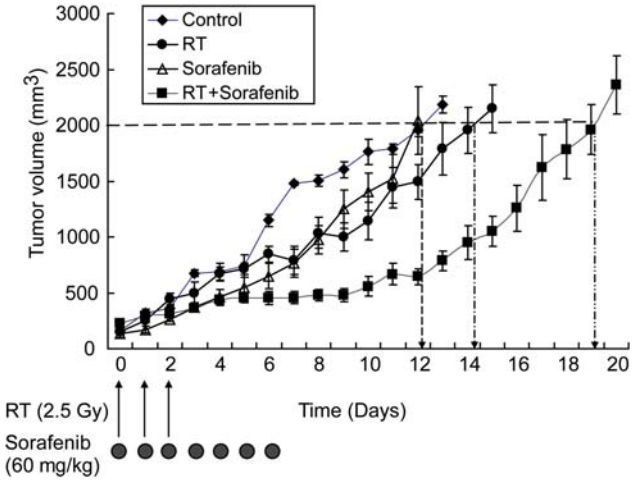

Fig. 6. The combined effect of fractionated radiation and sorafenib on tumor growth delay of DLD-1 human tumor xenografts in nude mice. Day 0 was defined as the first day of treatment. Tumors were treated with vehicle or 60 mg/kg sorafenib on Days 0–6. Radiation fractions (2.5 Gy) were given starting at Day 0 for 2 consecutive days. Bars, ± SEM from six to eight mice. EF was 3.5 on DLD-1 tumors.

by contributing to G2-M arrest [20]. Our data showed delayed clearance of γ-H2AX foci in combination treatment compared with radiation alone at 24 h. The addition of sorafenib to radiation seems to be associated with delaying repair of radiation-induced DSB.

Interestingly, sorafenib-treated cells after radiation failed to undergo the mitotic phase. The block of sorafenib-mediated cell cycle progression, hence, seems to be due to failure of the cells to undergo the transition from G2 to M phase. The failure of the cells to escape from the G2-M phase seems to be related to the sorafenib-mediated down-regulation of cyclin B1 levels. When combined with radiation, sorafenib reinforces radiation-induced mitotic arrest by attenuating cyclin B1. The ability of sorafenib to selectively impair the accumulation of cyclin B1 could underlie its potent radiation-enhancing activity. Exposure of radiation-arrested cells to sorafenib can hence enhance nuclear fragmentation and aberrant mitosis. Although much remains to be clarified regarding the action of sorafenib on the cell cycle, the ability of sorafenib to reinforce radiation-induced mitotic arrest has highlighted a potent and novel antiproliferative activity that may have significant implications for the targeting of cancerous cells and enhancing radiation response.

In conclusion, exposure of each cell line to sorafenib combined with radiation resulted in an increase in radioresponse. Sorafenib combined with radiation-induced accumulation of tumor cells in the G2-M phase and also showed a greater number of remaining γ-H2AX foci and greater nuclear fragmentation, representing aberrant mitosis, than radiation treatment alone. Sorafenib-mediated down-regulation of cyclin B1 levels contributed to radiation-induced cell cycle arrest and DNA damage. Tumor xenograft study confirmed that administration of sorafenib results in significant tumor growth inhibition when combined with radiation. Sorafenib may be promising as an agent for enhancing radiation response in colorectal cancer. Therefore, clinical trials are necessary to test the efficacy of radiation with sorafenib.

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