Sorafenib in combination with ionizing radiation has a greater anti-tumour activity in a breast cancer model

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High expression of vascular endothelial growth factor (VEGF) in patients with breast cancer has been associated with a poor prognosis, indicating that VEGF could be linked to the efficacy of chemotherapy and radiotherapy. It has also been suggested that radiation resistance is partly due to tumour cell production of angiogenic cytokines, particularly VEGF receptor (VEGFR). This evidence indicates that inhibition of VEGFR might enhance the radiation response. Sorafenib tosylate (Bay 54-9085) is an oral, small-molecule multikinase inhibitor of several targets including RAF/MEK/ERK MAP kinase signalling, VEGFR-2, VEGFR-3 and platelet-derived growth factor receptor-beta. Sorafenib has shown clinical efficacy in treating solid tumours such as renal cell and hepatocellular carcinomas. However, strategies are yet to be identified to prolong and maximize the anticancer effect of this multikinase inhibitor. The objective of this study was to determine whether a combination of Sorafenib and radiation will enhance the treatment response *in vitro* and *in vivo*. Radio-modulating effect of Sorafenib was assessed by performing clonogenic assays. In addition, cell cycle analyses as well as annexin-V apoptosis assays were performed 24 and 48 h after treatment, respectively. To confirm our in-vitro results, tumour growth delay assays were performed. Our results showed a strong and supra-additive antitumour effect of radiation combined with Sorafenib *in vitro* (dose enhancement factor of 1.76). The combined therapy demonstrated a strong and significant G2/M cell cycle arrest (combined treatment vs. irradiated alone: $P<0.0008$). Moreover, annexin-V staining showed a significant increase in the level of apoptosis (combined treatment vs. irradiated alone: $P<0.0004$). Study of the syngeneic model demonstrated the superior potency of the Sorafenib combined with radiotherapy. Our results demonstrate that higher antitumour activity can be achieved when radiation and Sorafenib are combined. *Anti-Cancer Drugs* 23:525–533 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Radiation is a mainstay of nonsurgical cancer treatment. Approximately two-thirds of cancer patients receive radiation therapy. During the last decades, radiation therapy has advanced mainly due to technological improvements in radiotherapy planning and delivery methods; however, efforts made towards understanding the biological parameters that affect the overall therapeutic outcome have not achieved the same success. Thus, radiotherapy is delivered without considering the potential differences within and between the tumours. Although an understanding of the biological basis could have a significant impact on clinical radiation oncology, this knowledge could also be exploited to develop new treatment protocols and perhaps novel combined therapies.

Radiotherapy is relatively well tolerated by patients and has been successful in local tumour control [1,2]. However, the overall rate of patient survival improves when radiation therapy is combined with chemotherapy [3]. Notably, secondary cancers, skeletal complications, radiation-induced heart disease and lung disease are the common side effects of radiation therapy [4–6]. Therefore, due to the toxicity of radiation, considerable focus has been placed on improving its cancer cell specificity. This includes the effort to develop agents that sensitize cancer cells to radiation or protect normal cells from damage induced by radiation [2,6,7].

Over the last decade, the combination of ionizing radiation with chemotherapy has led to marked improvement in local control, organ preservation and survival for locally advanced solid tumours. However, this strategy is limited by the toxicity resulting from each respective treatment and their combination. Therefore, targeting tumour-specific defects should provide an advantage over conventional therapy in which the major drawback is normal tissue toxicity [8].

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Sorafenib tosylate (Nexavar, Bay 54-9085) is an oral, small-molecule multikinase inhibitor of several targets including vascular endothelial growth factor receptor-2 (VEGFR-2), VEGFR-3 and platelet-derived growth factor receptor-beta, RAF-1 and BRAF [9]. Sorafenib has shown clinical activity against metastatic renal cell carcinoma and is considered to be a standard second-line therapy for patients with metastatic renal cell carcinoma [10,11]. Moreover, combination therapy with Sorafenib in phase I and II clinical trials has shown some promising results in melanoma patients [12]. Importantly, Sorafenib has been shown to significantly increase the overall survival rate of patients (nearly 3 months) with advanced hepatocellular carcinoma (HCC) [13]. Patients with HCC in hepatitis B-endemic areas may also benefit from single-agent Sorafenib treatment as a phase II clinical trial has shown fairly good efficacy and acceptable tolerability in these patients [14]. However, the effect of Sorafenib is temporary and the continuous dose for Sorafenib is needed to inhibit tumour growth for longer [15].

Retrospective analysis of patients with breast cancer has shown an unfavourable prognosis in patients with high expression levels of VEGF [16,17]. This indicates that VEGF could be associated with the efficacy of chemotherapy and radiotherapy. It has also been shown that radiation resistance is partly due to tumour cell production of angiogenic cytokines, particularly VEGF, which protects endothelial cells through survival pathways [18,19]. Moreover, it has been shown that VEGF inhibition combined with radiation enhances radiation control of bone destruction and the pain associated with cancer progression in bone metastases [20].

Here, we studied the efficacy of Sorafenib combined with radiation and determined whether this treatment modality could enhance tumour growth inhibition.

Materials and methods

Reagents

The cell culture reagents were obtained from Gibco, Invitrogen (Burlington, Ontario, Canada). Foetal bovine serum was purchased from Wisent Inc., (St Bruno, Quebec, Canada). Propidium iodide (PI) was obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Sorafenib tosylate (Bay 54-9085) was provided by Bayer Pharmaceutical Corp. (West Haven, Connecticut, USA) and was reconstituted in dimethyl sulfoxide for in-vitro use and in ethanol/Cremephore L (Sigma-Aldrich) (50 : 50) for in-vivo use at 4 × concentration. The 4 × solution of Sorafenib was freshly prepared every day. The final dosing solution was prepared by diluting the 4 × solution to 1 × in sterile water (Gibco, Invitrogen) every day before its administration to the animals. The concentrations of dimethyl sulfoxide were maintained lower than 0.2% in all in-vitro experiments.

Cell culture

The highly metastatic mouse mammary cancer cell line, 4T1, was a generous gift from Dr Fred Miller, Karmanos Cancer Institute, Wayne State University, Michigan, USA. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, penicillin–streptomycin 1% and kept at 37°C in 95% air/5% CO_2.

Irradiation

Irradiation for in-vitro and in-vivo experiments was carried out at room temperature using a Theratron T-780 ^{60}Co irradiator (MDS Nordion, Kanata, Ontario, Canada). The dose delivered in each experimental set-up used in this work was verified by the radiochromic film dosimetry protocol developed by Tomic *et al.* [21].

Colony-forming assay

Cells were plated at specific cell numbers in six well plates. At a 0 Gy radiation dose, 100 cells per well were used and for each subsequent radiation dose (2, 4, 6 and 8 Gy), 200, 400, 800 and 1600 cells were seeded, respectively. They were treated with Sorafenib alone (5 and 7.5 μmol/l for 2 h) and in combination with radiation (2, 4, 6 and 8 Gy) [22]. After 6–8 days of incubation, the colonies were fixed and stained with methylene blue. Only colonies containing more than 50 cells were counted. The plating efficiency was calculated by dividing the number of colonies formed in the untreated control plates by the number of cells plated. Survival fractions were calculated by counting the number of colonies for each specific radiation dose and dividing by the number of cells seeded at the same dose multiplied by plating efficiency. In order to plot the survival curve, the survival fractions were normalized according to the controls (nonirradiated). Radiosensitivity was measured by determining the dose enhancement factor, which is the ratio of the radiation doses at a survival fraction of 0.1 or 0.01 of non-drug-treated cells to drug-treated cells [23,24].

Flow cytometry analysis

Cell cycle analysis

Cells were treated with Sorafenib (5 and 7.5 μmol/l) and were irradiated as described. The cells were harvested and washed 24 h after treatment, after which they were fixed with ethanol, labelled with PI and analysed by flow cytometry (BD Biosciences, San Jose, California, USA). Cell cycle distribution was analysed using the Mod-Fit LT software package (Verity Software House, Topsham, Maine, USA).

Analysis of apoptosis by annexin-V binding

Cells were treated with Sorafenib (5 and 7.5 μmol/l) and were irradiated to a dose of 4 Gy. They were harvested and washed with PBS 1 × (Gibco, Invitrogen) at 48 h after treatment. They were labelled with annexin V–FITC and PI according to the manufacturer's protocol (TACS apoptosis kit; R&D Systems, Minneapolis, Minnesota,

USA). Cells were analysed by flow cytometry (BD Bioscience) and characterized as follows: cells appearing at the lower left quadrant of the dot plot were considered viable. Those observed at the lower right quadrant were identified as early apoptotic. The late apoptotic and necrotic cells appeared at the upper right and the upper left quadrants, respectively.

Western blot analysis

The 4T1 cells were incubated in two sets of six well plates with serum-free media for 18 h and were subsequently exposed to the 5 and 20 μmol/l of Sorafenib for 2 h and 4 Gy of radiation. To test whether Sorafenib is still effective in the case of overactivation of receptor tyrosine kinases (RTKs) such as VEGFR, one set of the plates was subsequently treated with 25 ng/ml of VEGF for 20 min and cells were harvested within 1 h, after which the whole cell lysates were prepared. Fifty micrograms of protein was loaded onto Bis-Tris gradient gels (Invitrogen, Carlsbad, California, USA). Western blot analysis was performed using antibodies for p-Erk 1,2 and Erk 1,2 as well as tubulin (Cell signaling Technology Inc., Beverly, Massachusetts, USA).

In-vivo tumour model

Six to eight-week-old female BALB/c mice (Charles River Laboratories, Montreal, Canada) were used in this study. Mice were caged in groups of five or less. 4T1 tumour cells (2×10^6 cells) were injected subcutaneously into the right hind leg. All protocols were approved by the McGill University Animal Care Committee following the guidelines of the Canadian Council on Animal Care.

Tumour growth delay assay

When tumours reached a mean volume of 144 mm^3, mice were randomized into four groups: vehicle, Sorafenib alone, irradiation (15 Gy) alone, and Sorafenib plus irradiation [25]. A single dose of Sorafenib (60 mg/kg) was administered by gavage daily for 7 days [9,26]. The drug was administered 6 h before local tumour irradiation (15 Gy) on day 3 (schedule A). In case of schedule B, radiation was delivered 24 h before the start of drug treatment. To obtain tumour growth curves, perpendicular diameter measurements of each tumour were made every 2–3 days with digital callipers, and volumes were calculated using the formula $(L \times W^2)/2$. Tumours were followed until the mean tumour volume reached $\sim$2400 mm^3, after which the animals were sacrificed. The relative tumour volume was calculated by dividing each individual animal's tumour volume by the mean tumour volume of the same group. Each experimental group included six to eight mice.

Statistical analysis

The effects of various treatments in all experiments were compared using a two-tailed *t*-test (GraphPad prism 5; GraphPad software Inc., California, USA). Differences with a *P* value less than 0.05 were considered statistically significant. The data presented are means and SEM from multiple independent experiments.

Results

Sorafenib increases the sensitivity of 4T1 cells to radiation *in vitro*

To evaluate whether Sorafenib has an effect on the ability of cancer cells to form colonies *in vitro*, clonogenic assays were performed. As shown in Fig. 1, the dose enhancement factor was as high as 1.39 and 1.76 when Sorafenib was combined with radiation at 5 and 7.5 μmol/l concentrations, respectively. To test whether this effect is schedule-dependent, three schedules were used: (a) Sorafenib 24 h before radiation, (b) Sorafenib concurrent with radiation and (c) radiation 24 h before Sorafenib administration. Interestingly, pretreatment with Sorafenib (schedule A) and the concurrent schedule (schedule B) seemed to be more effective *in vitro* (Fig. 2a and b).

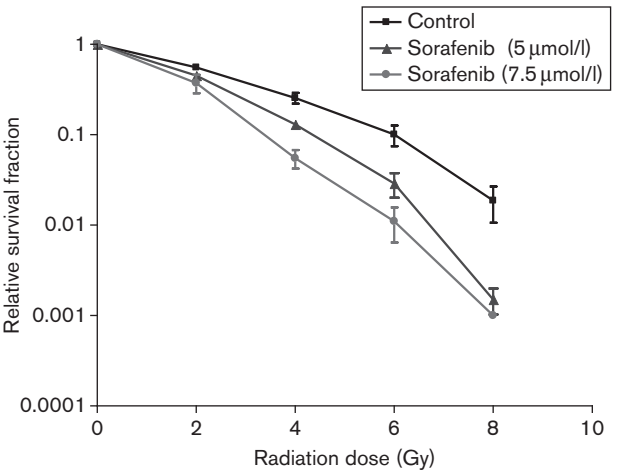
Sorafenib combined with radiation induces G2/M arrest

To assess the effect of Sorafenib in combination with radiation on cell cycle progression, cell cycle analysis was performed. As shown in Fig. 3, Sorafenib in combination with radiation had a significant and strong effect on cell cycle arrest at G2/M. Consequently, the G1 and S population was significantly decreased.

Combination of Sorafenib and radiation enhances the level of apoptosis

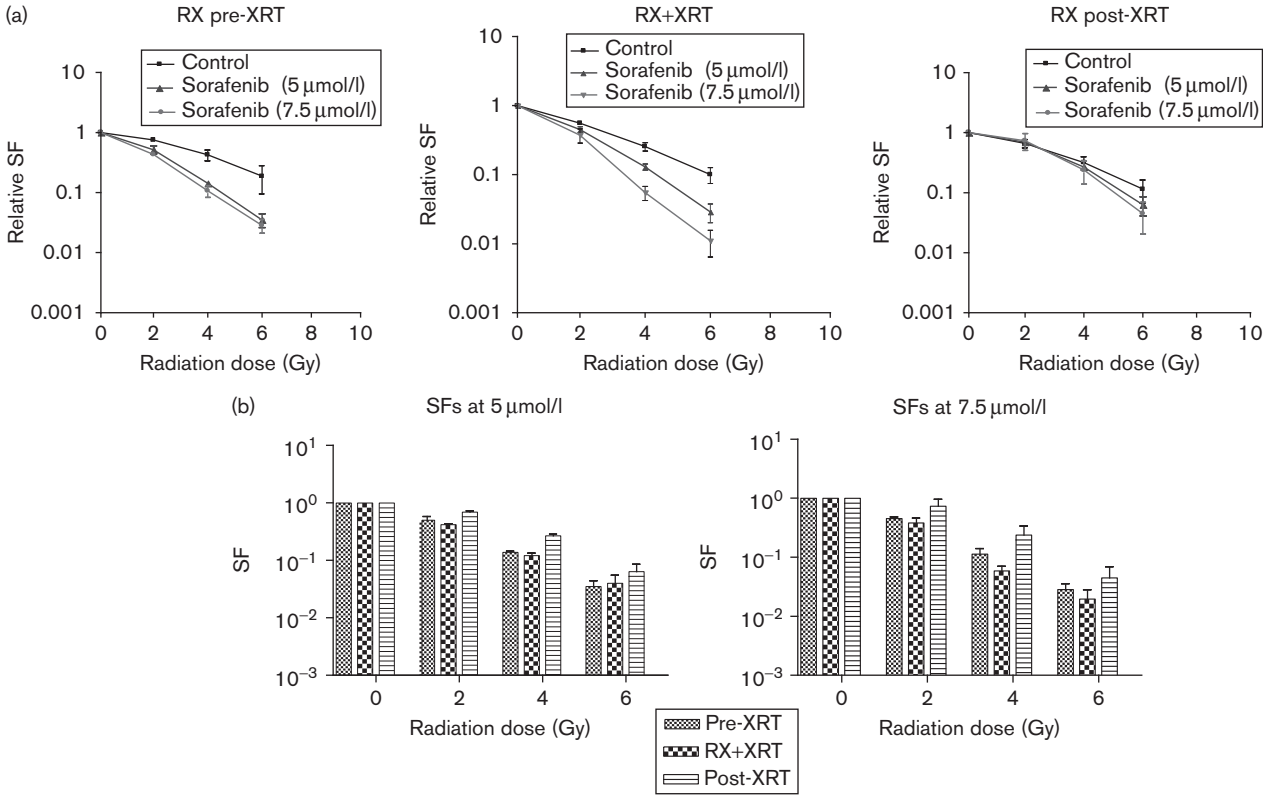
In order to determine whether the multi-inhibitory activity of Sorafenib would induce high levels of apoptosis when combined with radiation, an annexin-V binding

Fig. 1



Analysis of cell response to the combination of Sorafenib and radiation using a clonogenic assay. Cells were treated with Sorafenib (5 and 7.5 μmol/l) with or without radiation (4 Gy). Data represent means and SEM from three independent experiments.

Fig. 2



(a) Different schedules of combination (Sorafenib before radiation, Sorafenib concurrent with Sorafenib and Sorafenib 24 h after radiation) were tested on 4T1 cells. (b) Comparison of survival fractions (SFs) at the different combination schedules at specific Sorafenib doses. RX: drug (Sorafenib); XRT: radiation.

assay was performed with cells exposed to radiation and Sorafenib alone or in combination. As shown in Fig. 4, Sorafenib alone induced apoptosis at levels of up to 15 and 33% at 5 and 7.5 µmol/l, respectively, whereas apoptosis induced by radiation alone was approximately 10% of the total analysed cells. When Sorafenib was combined with 4 Gy of radiation, the level of apoptosis reached 30 and 40% at 5 and 7.5 µmol/l concentrations.

Sorafenib with/out radiation inhibits phosphorylation of Erk1/2 downstream of receptor tyrosine kinases

To confirm the inhibitory activity of Sorafenib in our breast cancer model, a western blot analysis was performed. As Sorafenib is a multikinase inhibitor of several RTKs, we evaluated the phosphorylation of Erk 1/2 as an indicator of activation of the downstream pathway. As in Fig. 5, Sorafenib at 5 µmol/l completely inhibits the activation of Erk1/2 downstream of RTKs irrespective of radiation treatment.

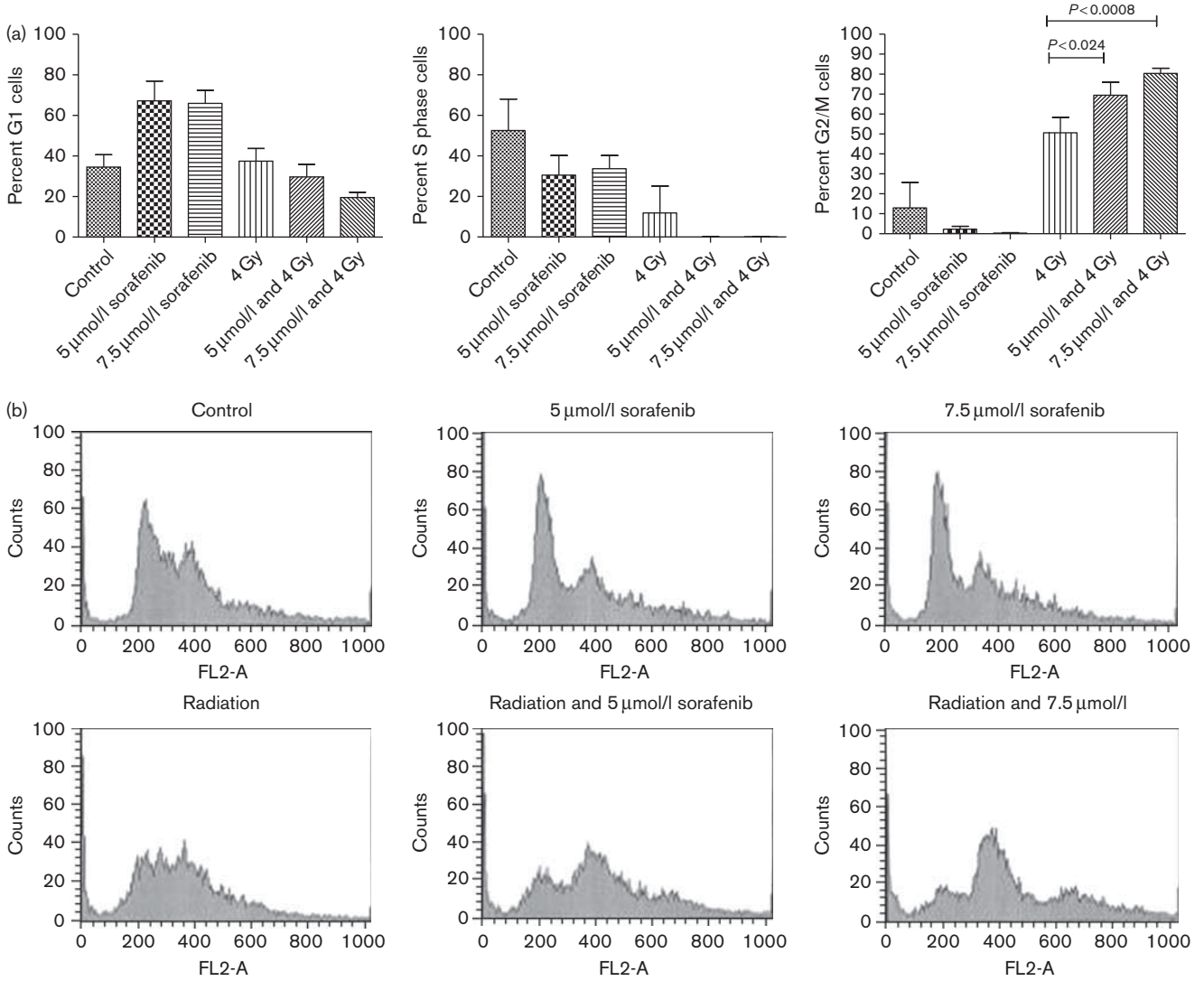
Sorafenib increases the tumour growth delay caused by radiation as an early response

In order to evaluate and validate our in-vitro results, we performed an in-vivo experiment with 4T1 mouse mammary cancer cells. As shown in Fig. 6a and b, the in-vivo results suggest that, in tumours treated with the combination therapy, Sorafenib increases the delay in tumour growth caused by radiation by almost 7 days. Moreover, Sorafenib combined with ionizing radiation has significantly more antitumour effect against 4T1 tumours than Sorafenib alone in BALB/c mice.

Sorafenib was as potent as the combined treatment only until the end of the drug treatment (day 6). Soon after the end of the drug treatment (day 10), tumours started to grow (Sorafenib-treated group vs. combination group: $P = 0.0406$). On day 17 (Fig. 6a), the tumour volume of the Sorafenib-treated group was significantly larger compared with the irradiated or the combined treated tumours (Sorafenib vs. combination: $P = 0.0002$). The same pattern was observed when radiation was delivered 24 h before the start of Sorafenib treatment.

In schedule A (when radiation was delivered concurrently with Sorafenib treatment), the tumour growth delay was increased from 4.2 days in the control group to 11 and 10.5 days in Sorafenib alone or radiation alone, respectively. The growth delay, in the case of the combination of Sorafenib and radiation, was increased to 18 days.

Fig. 3



Cell cycle analysis of 4T1 cells following exposure to Sorafenib or radiation and the corresponding combination. (a) Cell distribution in G1, S and G2M. Cells were treated with Sorafenib (5 and 7.5 µmol/l) alone and in combination with radiation (4 Gy) and cell cycle analysed by flow cytometry 24 h later. Data represent means and SEM from three independent experiments. (b) A representative histogram showing the G2M arrest in combined treatment.

Similarly, in schedule B, when radiation was delivered 24 h before Sorafenib treatment, the growth delay was increased from 6.5 days in the control group to 13 and 14 days in Sorafenib alone and radiation alone and 20.5 days in mice treated with both modalities.

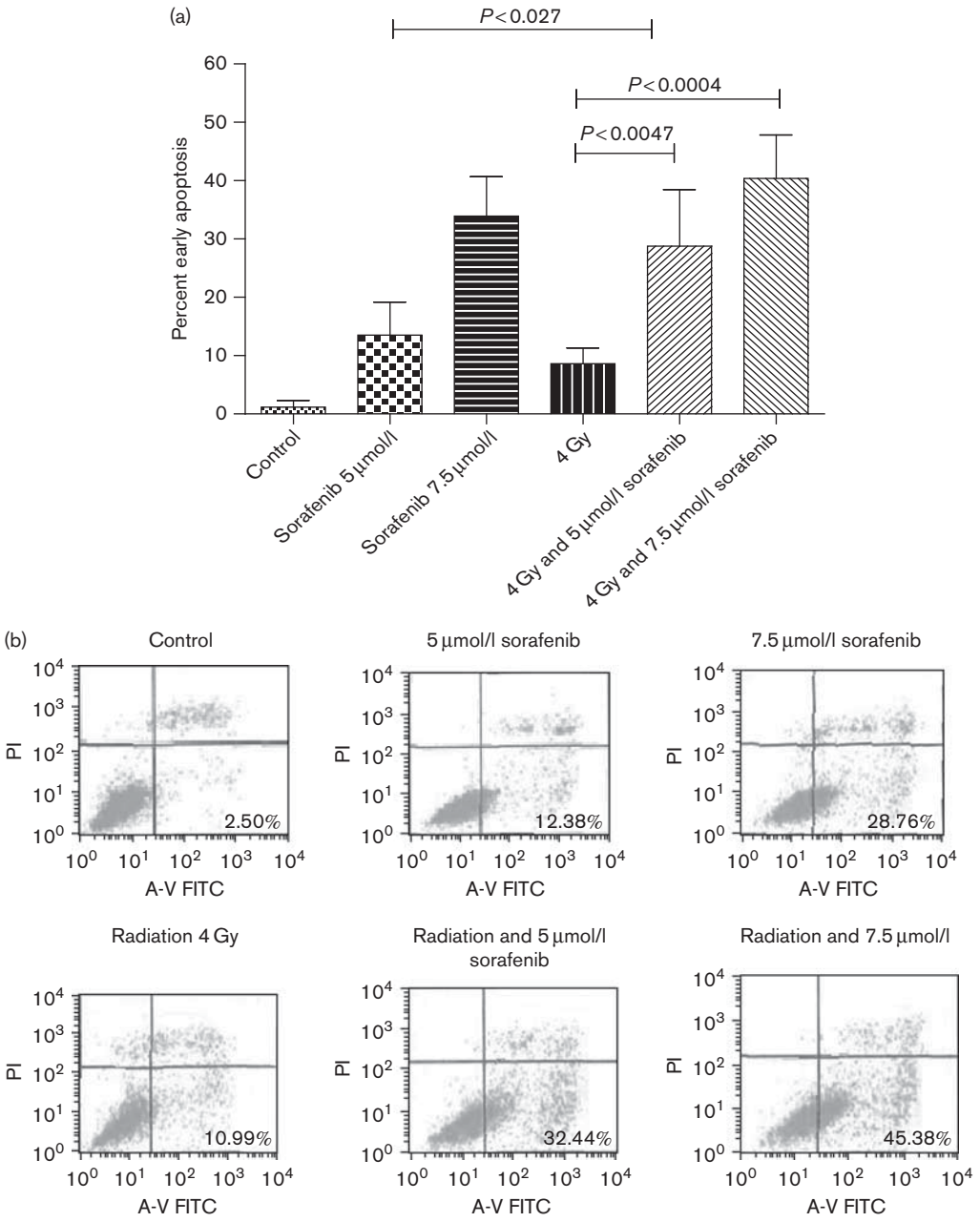
No significant loss of body weight resulted from any of the treatments and the treatments were well tolerated by the end of the experiment (Fig. 6c and d).

Discussion

In this study, we demonstrated that Sorafenib induces a greater antitumour activity when it is combined with radiation in 4T1 cells, both *in vitro* and *in vivo*. 4T1 cells are highly metastatic cancer cells and are considered to be a suitable model to study the effect of antiangiogenesis agents *in vitro* and *in vivo* [27–30].

The increased antitumour activity of Sorafenib combined with radiation in 4T1 cells can be partially explained by the significant cell cycle arrest we observed at G2/M. Cancer cells show more sensitivity to ionizing radiation at the G2/M and G1 whereas cells residing in the S stage of the cell cycle are less radiosensitive [31]. As was shown by our result, there was a significant decrease in the S-phase population, which could explain the higher potency of the combined treatment.

Fig. 4

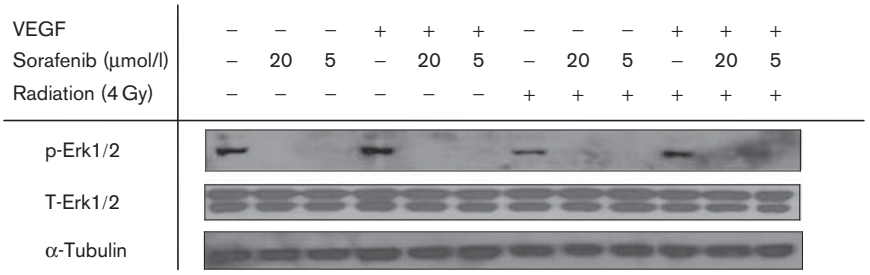


Analysis of apoptosis induced by Sorafenib or radiation and the corresponding combination in 4T1. (a) Cells were treated with Sorafenib (5 and 7.5 µmol/l) alone and in combination with radiation (4 Gy) and were harvested at 48 h after treatment. Presented data are means and SEM of multiple independent experiments. (b) Representatives dot-plot data showing the effect of Sorafenib or/and radiation on the level of apoptosis. PI, propidium iodide.

The increased efficacy of Sorafenib in combination with radiation could also be due to the augmented apoptosis level in 4T1 cancer cells treated with both Sorafenib and radiation.

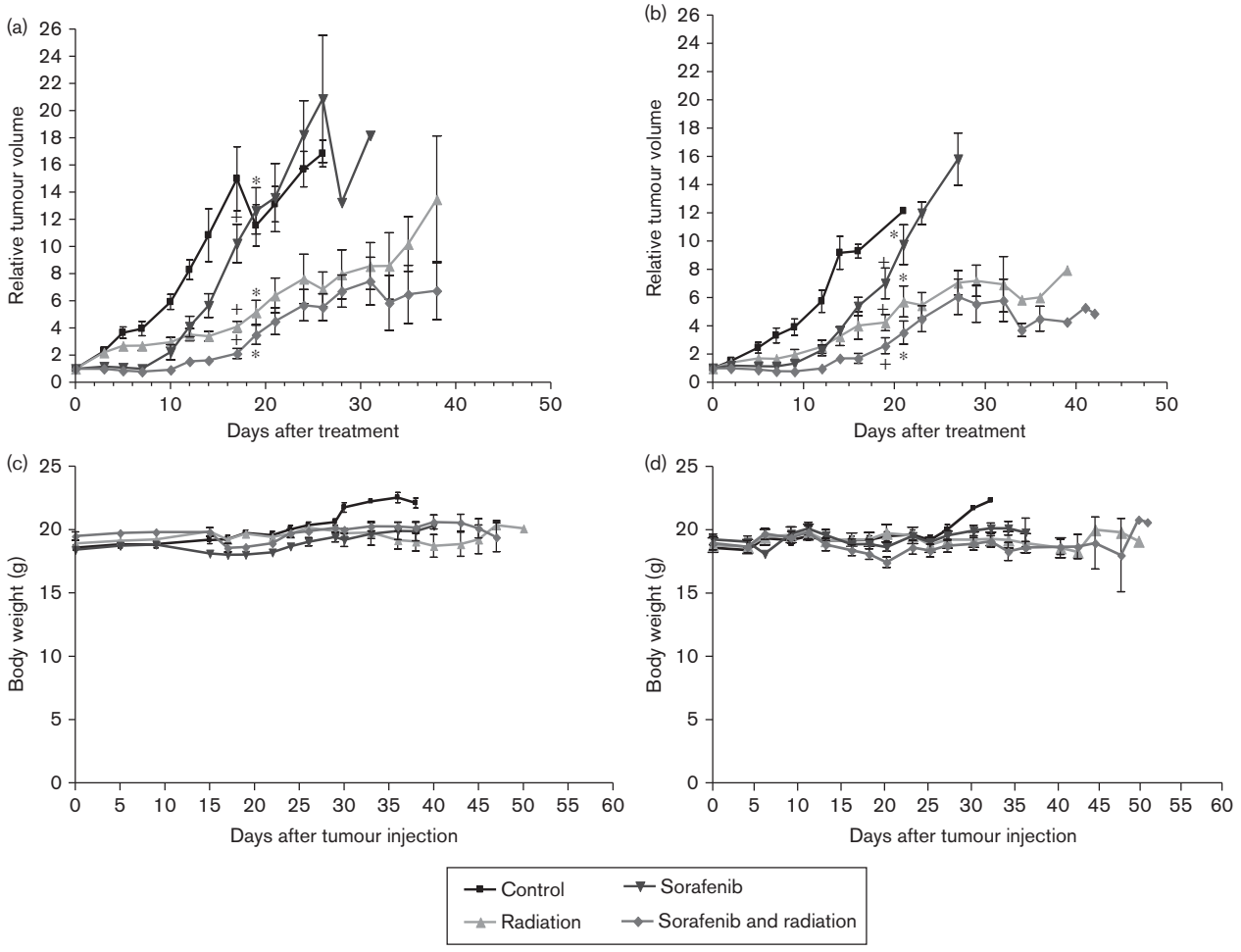
In this study, we have also shown that radiation prolongs the antitumour activity of Sorafenib *in vivo*. Tumours implanted in mice treated with Sorafenib alone started to grow rapidly immediately after the drug treatment was stopped (day 6–8) whereas the inhibitory effect of Sorafenib was longer when radiation was added (tumours started to grow gradually starting day 14). This could be of relevance as cytostatic agents such as Sorafenib usually show temporary and reversible antitumour activity [15].

Fig. 5



Western blot analysis. 4T1 cells were grown to 80% confluency in six well plates. They were serum starved for 18 h and were treated with Sorafenib or/and radiation as indicated. Half of the plates were stimulated with vascular endothelial growth factor (VEGF) and whole-cell lysates were prepared within 1 h.

Fig. 6



(a, b) Tumour growth delay assay. 4T1 cells were injected into the right hind limb of BALB/c mice. When the mean volume of tumours reached 144 mm^3, the animals were randomly divided into four groups. There were six to eight animals in each group. Schedule A (a): Sorafenib was given to the animals 3 days before radiation, on the same day as irradiation and was continued for 3 days after irradiation (+: day 17, irradiated group vs. combined treatment: $P=0.0309$; Sorafenib-treated group vs. combination: $P=0.0002$; *: day 19, irradiated group vs. combined treatment: $P=0.1710$; Sorafenib-treated group vs. combination: $P=0.0255$). Schedule B (b): 15 Gy of radiation was delivered 2 h before the start of Sorafenib treatment (+: day 19, irradiated group vs. combined treatment: $P=0.0316$; Sorafenib-treated group vs. combination: $P=0.0255$; *: day 21, irradiated group vs. combined treatment: $P=0.0815$; Sorafenib-treated group vs. combination: $P=0.0180$). When the tumour size reached a maximum of 2400 mm^3, the mice were euthanized. Tumour volume was calculated using: $(L \times W^2)/2$ and was normalized by dividing the tumour volume of each animal in the treatment groups by the mean tumour volume of the same group. Error bars, SEM. (c, d) Variations of body weight of mice treated with Sorafenib (60 mg/kg) and radiation alone and the combined treatment. Error bars, SEM.

Moreover, in our in-vivo model, Sorafenib increased the radiation response significantly as an early response (Fig. 6a and b). It will be interesting to evaluate different schedules and sequences of this combination to determine whether a longer response can be achieved.

Our in-vitro data demonstrated a higher radiation response when Sorafenib was added before or concurrent with radiation versus after radiation. This was in disagreement with our in-vivo results, which showed no significant difference between the Sorafenib treatment after and concurrent with radiation. The difference between our in-vitro and in-vivo outcome could be due to the interaction of tumour cells with each other and with their microenvironment. In-vitro assays are performed in a much shorter time period than in-vivo experiments. The difference could also be related to hypoxia and hypoxia-induced radiation resistance. Although antiangiogenic agents have been shown to stabilize neovasculature and improve blood perfusion, Sorafenib might not have done so, resulting in the formation of hypoxic regions inside the tumours and therefore reduced radiation response.

Recently, Suen *et al.* [32] and Plastaras *et al.* [26] have shown that the combination of Sorafenib and radiation enhances the radiation response in colorectal cancer cells *in vivo* and this response is schedule dependent. In their studies, irradiation before Sorafenib treatment appears to be the most efficient schedule [26,32]. The different outcome between their study and ours is perhaps due to the use of different tumour models and also the different radiation schedules. The mice in our study were irradiated with a single radiation dose either before or concurrent with Sorafenib administration (schedules A and B) whereas in the two mentioned studies, fractionated radiation was used over a longer period of time.

It has been shown that Sorafenib, being a cytostatic agent [33], can induce radiation response especially in fractionated schedules as it blocks regrowth (through its antiangiogenic properties) between fractions [3]. Presently, in our laboratory, more in-vitro/in-vivo studies are ongoing to test Sorafenib with fractionated radiotherapy in metastatic breast cancer models while more microenvironment studies will guide us through the complex mechanism of this combination.

There are several trials combining Sorafenib with radiation and other cytotoxic modalities [34] that are ongoing or have been completed in the clinical setting. Some results showed that the combination did not improve the efficacy of treatment as 40% of the patients did not receive Sorafenib at all due to early disease progression. Perhaps better results can be achieved with a better design or modified combinations. Other trials including a phase I/II study of cisplatin and radiation in combination with Sorafenib in cervical cancer, a phase I/II trial of radiation therapy and Sorafenib for unrespectable liver metastases and Sorafenib combined with radiation in HCC are ongoing. Depending on the outcome of these clinical trials, the protocol for patients might change and patients with cancer might benefit from the new combination therapies. Nevertheless, a better understanding of the mechanism of action of antiangiogenic agents and, more specifically, multitargeting agents is crucial to better design a clinical trial and to rationally choose the target patient population.

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Conflicts of interest

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