Synergistic Activity of Paclitaxel, Sorafenib, and Radiation Therapy in advanced Renal Cell Carcinoma and Breast Cancer[1,2]

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Abstract

Advanced cancer has been shown to be associated with a higher percentage of epigenetic changes than with genetic mutations. Preclinical models have shown that the combination of paclitaxel, sorafenib, and radiation therapy (RT) plays a crucial role in renal cell carcinoma (RCC) and breast cancer. This study aimed to investigate the involvement of mitochondrial cytochrome c–dependent apoptosis in the mechanism of action of a combination of paclitaxel, sorafenib, and RT in RCC and breast cancer. RCC and breast cancer cell lines were exposed to paclitaxel and sorafenib alone or combined in the presence of radiation, and cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The synergistic anticancer effects of the combination therapy on cell cycle and intracellular signaling pathways were estimated using flow cytometry and immunoblot analysis. RCC and breast cancer cell line xenograft models were used to examine the antitumor activity *in vivo*. Our results suggest that paclitaxel, sorafenib, and RT synergistically decreased the viability of RCC and breast cancer cells and significantly induced their apoptosis, as shown by caspase-3 cleavage. Paclitaxel, sorafenib, and radiation cotreatment reduced antiapoptotic factor levels in these cells and, thereby, significantly reduced the tumor volume of RCC and breast cancer cell xenografts. The current study suggests that paclitaxel, sorafenib, and radiation cotreatment was more effective than cotreatment with paclitaxel or sorafenib and radiation. These findings may offer a new therapeutic approach to RCC and breast cancer.

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Introduction

Over the past several decades, research and development of cancer chemotherapeutics have significantly improved, notably after the discovery of a few small molecule inhibitors; however, the control of human malignancies is still a critical concern [1–4]. Breast cancer is the most common type of malignancy in women [5]. Approximately 20%-30% of patients with early-stage breast cancers will experience a recurrence with distant metastatic disease [6]. Renal cell carcinoma (RCC) is the most common kidney cancer, accounting for 2%-3% of

all adult malignancies [7–9]. In addition, in approximately 30% of patients, RCC has the propensity to spread to other organs, usually the brain (11%), adrenal glands (15%), liver (41%), bone (42%), lymph nodes (55%), and lungs (70%) [7–9]. Established research studies indicate that metastasis to the breast from extramammary cancer is still less common with primary breast tumors (1%-2% of breast cancers) [10–12], and RCC metastasis to the breast is rare, occurring only in 3% of all cases [13,14]. Traditional cancer therapy, radiation therapy (RT), and chemotherapy are not efficacious in the treatment of advanced breast cancer and RCC [15]. The American Thyroid Association recommends surgery with locoregional RT with or without systemic therapy in cases of resectable disease, and RT with or without systemic chemotherapy in cases of unresectable disease [16]. The combination of chemotherapy and RT might treat micrometastatic disease and influence long-term survival [17,18]. Nevertheless, it also potentially induces morbidity and mortality. The anticancer effects of paclitaxel and sorafenib are already well known. The current study provides evidence that cotherapy with paclitaxel, sorafenib, and RT has significant anticancer activity in preclinical models, suggesting it could be a potential novel clinical approach for patients with advanced thyroid cancer.

Materials and Methods

Cell Culture

Caki-1 and MDA-MB-231 cells were obtained from American Type Culture Collection and grown in Roswell Park Memorial Institute-1640 medium with 10% fetal bovine serum. The cells were authenticated using short tandem repeat profiling, karyotyping, and isoenzyme analysis.

Cell Viability Assay

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and trypan blue staining. Cells were seeded in 96-well plates at 5×10^3 cells/well and incubated overnight to achieve 70% confluency, and they were incubated with the various treatments for the indicated times prior to cell viability determination using the MTT reagent according to the manufacturer's protocol. The absorbance was measured at 550 nm. The data were expressed as a percentage of the signal observed in vehicle-treated cells and presented as the mean ± SEM of triplicate experiments.

Irradiation

For *in vitro* experiments, Caki-1 and MDA-MB-231 cells were irradiated using a Faxitron X-ray system (Faxitron Bioptics, Tucson, AZ) at 5 Gy in combination with paclitaxel and sorafenib or either agent alone. For *in vivo* experiments, the mice were treated using the small animal radiation research platform (high-resolution, small animal radiation research platform with x-ray tomographic guidance capabilities/PMID; 18640502). The tumors were irradiated using a circular beam with a 1-cm diameter with three consecutive daily exposures to 3 Gy.

Flow Cytometry Analysis of Cell Cycle

Cells were treated with RT plus paclitaxel or sorafenib, or a combination of both agents in Roswell Park Memorial Institute-1640 medium containing 10% fetal bovine serum for 40 hours, harvested by trypsinization, and then fixed with 70% ethanol. The cells were stained for total DNA using phosphate-buffered saline (PBS) containing 40 μg/ml propidium iodide and 100 μg/ml RNase I for 30 minutes at 37°C. The cell cycle distribution was then analyzed using the FACSCalibur flow cytometer (BD Biosciences, San Jose,

CA). The proportions of cells in the sub-G_0/G_1, G_0/G_1, S, and G_2/M phases were analyzed using the FlowJo v8 software for MacOSX (Tree Star, Ashland, OR). This experiment was repeated thrice, and the results were averaged.

Immunoblot Analysis

Equal amounts of protein (20 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The antibodies against cyclin D1 and p21 were obtained from Abcam (Cambridge, UK). B-cell lymphoma-2 (Bcl-2), Apaf-1, caspase-3, and β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for CCAAT/enhancer-binding protein homologous protein (CHOP) and Bcl-2-associated X protein (BAX) were purchased from Cell Signaling Technology (Danvers, MA).

Immunofluorescence Analysis and Confocal Imaging

Cytochrome c release from the mitochondria was analyzed using immunofluorescence staining. Cells were grown in glass-bottom dishes (MatTek, Ashland, MA), fixed with 4% formaldehyde solution (R&D Systems, Abingdon, UK) for 10 minutes, and then permeabilized with 0.5% Triton X-100 (in PBS) for 10 minutes. The slides were air-dried, washed with PBS, and incubated with anti–cytochrome c (1:25; Abcam, Cambridge, UK) in 3% bovine serum albumin in PBS. After washing with PBS, the slides were incubated with Alexa 488 (1:200; Molecular Probes, Eugene, OR), and the nuclei were stained with Hoechst 33342 (Life Technologies, Grand Island, NY) for visualization. The images were observed under a confocal microscope (LSM Meta 700, Zeiss, Oberkochen, Germany) and were analyzed using the Zeiss LSM image browser, version 4.2.0121.

### Human Breast Cancer and RCC Xenografts

MDA-MB-231 (breast cancer) and Caki-1 (RCC) cells were cultured *in vitro* and then injected subcutaneously into the upper left flank region of female BALB/c nude mice (2.0×10^7 cells/mouse). After 7 days, tumor-bearing mice were grouped randomly ($n = 10$/group) and treated with 25 mg/kg paclitaxel (intraperitoneally), 60 mg/kg sorafenib (orally), or a combination of 15 mg/kg paclitaxel and 25 mg/kg sorafenib. The paclitaxel or sorafenib was administered once every 2 days for a total of 10-12 injections. The tumor size was measured every other day using calipers, and the tumor volume was estimated using the following formula: $L \times S2/2$ (where L and S are the longest and shortest diameters, respectively). Animals were maintained under specific pathogen-free conditions, and all experiments were approved by the Animal Experiment Committee of Yonsei University.

Immunohistochemistry

All relevant tissues samples were fixed in 10% neutral-buffered formalin and embedded in paraffin wax following standard protocols, tissue sections (5 μm) were cut and dewaxed, and then antigen retrieval was performed in citrate buffer (pH 6) using an electric pressure cooker set at 120°C for 5 minutes. The sections were incubated for 5 minutes in 3% hydrogen peroxide to quench the endogenous tissue peroxidase, and all tissue sections were counterstained with hematoxylin, dehydrated, and mounted on slides for observation.

Image Analysis

The MetaMorph 4.6 software (Universal Imaging Co., Downington, PA) was used for computerized quantification of the immunostained target proteins.
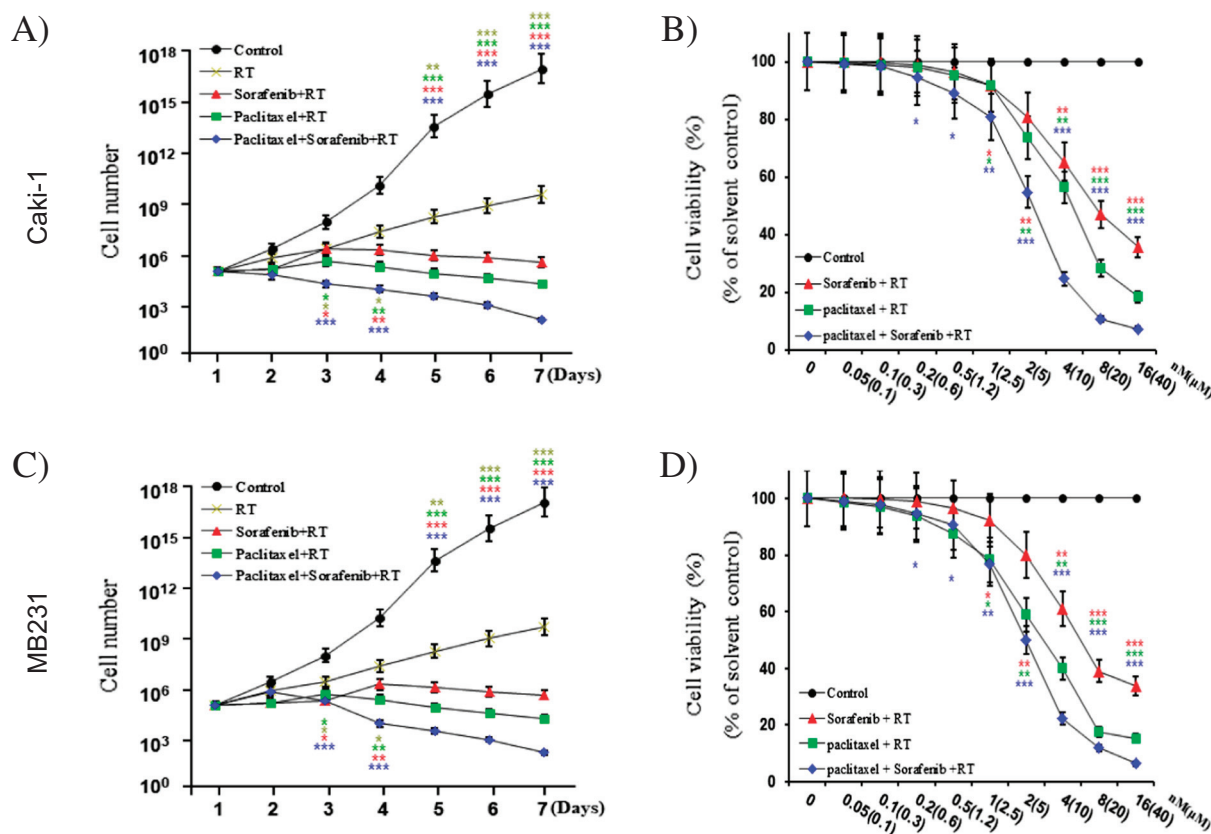
Figure 1. Cotreatment with paclitaxel, sorafenib, and RT suppressed cancer cell proliferation. Effects of various combinations of paclitaxel, sorafenib, and RT on renal cell carcinoma (RCC, Caki-1; A and B) and breast cancer (MB-231; C and D) cell using cell proliferation and viability assays. Points indicate mean (%) of value observed in the solvent-treated control. All experiments were repeated at least three times. The data represent the mean ± SD. Experiments were repeated at least three times with similar results. $*P < .05$ vs. control, $** P < .01$ vs. control, $*** P < .005$ vs. control.

Statistical Analysis

The statistical analyses were performed using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). The results were analyzed using a one-way analysis of variance followed by Bonferroni *post hoc* test. Values are expressed as means ± SEM, and *P* values <.05 were considered statistically significant.

Results

Synergistic Anticancer Effects of Cotreatment with Paclitaxel, Sorafenib, and RT in RCC and Breast Cancer

To estimate the synergistic anticancer effects of paclitaxel or sorafenib and RT on RCC and breast cancer cells, we examined the proliferation of Caki-1 and MB-231 cells in the presence and absence of the compounds with or without RT (Figure 1). The combination of paclitaxel and sorafenib suppressed cell proliferation more effectively than either agent did alone or with RT (Figure 1, *A* and *C*) in a dose-dependent manner (Figure 1, *B* and *D*). Paclitaxel, sorafenib, and RT cotreatment had a lower half-maximal inhibitory concentration (IC_{50}) than that of paclitaxel or sorafenib with RT treatment in RCC and breast cancer cells (Table 1).

These results indicate that this cotreatment may offer a new approach for targeting RCC and breast cancer using low doses of anticancer drugs.

Paclitaxel, Sorafenib and RT in Combination Induced Cell Cycle Arrest in RCC and Breast Cancer Cells

The most important process in cancer cell growth is the cell cycle progression. We next determined the synergistic anticancer effects of paclitaxel or sorafenib with and without RT as well as RT alone on cell cycle progression using propidium iodide staining and flow cytometry. Cotreatment with paclitaxel and sorafenib with RT significantly increased the sub-G_0/G_1 phase arrest more than RT alone

Table 1. IC_{50} Determination Using a Cell Proliferation Assay

Cell Line	Hisopathology	Animal	Cell Proliferation IC_{50}^a				
			Paclitaxel (nM)	Sorafenib (μM)	Paclitaxel + RT	Sorafenib + RT	Paclitaxel + Sorafenib + RT
Caki-1	Kidney cancer	Human	7.3 (±0.3)	10.5 (±0.2)	5.6 (±0.3)	7.8 (±0.4)	3.1 (±0.5) + 5.2 (±0.1)[a]
MB-231	Breast cancer	Human	4.1 (±0.1)	7.5 (±0.4)	3.6 (±0.4)	5.9 (±0.3)	2.2 (±0.2) + 3.3 (±0.3)[a]

Paclitaxel, Sorafenib, and RT combination treatment has a lower IC_{50} than each agent used alone or Paclitaxel and Sorafenib with RT combination. Each data point represents the mean of three independent MTT assays for IC_{50} performed in triplicate.

Table 2. Combination Treatment of Paclitaxel, Sorafenib and RT Induced Sub-G0/G1 Phase (Apoptotic Cells) in RCC and Breast Cancer Cells

A. Caki-1

Status	Sub-G_0G_1	G_0G_1	S	G_2/M
Control	1.7 ± 0.02	34.8 ± 0.01	37.2 ± 0.03	26.3 ± 0.03
RT	4.5 ± 0.01	46.5 ± 0.02	32.3 ± 0.03	16.7 ± 0.01
Sorafenib + RT	18.9 ± 0.03	39.5 ± 0.02	34.4 ± 0.03	7.2 ± 0.02
Paclitaxel + RT	27.8 ± 0.01	38.5 ± 0.04	29.2 ± 0.03	4.5 ± 0.01
Paclitaxel + sorafenib + RT	54.1 ± 0.02	27.9 ± 0.03	16.6 ± 0.02	1.4 ± 0.01

B. MB-231

Status	Sub-G_0G_1	G_0G_1	S	G_2/M
Control	0.9 ± 0.01	34.8 ± 0.03	35.7 ± 0.02	28.6 ± 0.02
RT	3.5 ± 0.03	43.8 ± 0.04	35.2 ± 0.05	17.5 ± 0.02
Sorafenib + RT	10.8 ± 0.02	47.3 ± 0.01	29.5 ± 0.02	12.4 ± 0.05
Paclitaxel + RT	22.5 ± 0.04	39.4 ± 0.01	31.5 ± 0.05	6.6 ± 0.05
Paclitaxel + sorafenib + RT	48.7 ± 0.01	32.5 ± 0.06	14.7 ± 0.03	4.1 ± 0.03

Cell cycle analysis for quantitation of DNA content with propidium iodide (RCC: Table 2A and breast cancer cell: Table 2B). Cells were exposed to the indicated inhibitors, harvested, and stained with propidium iodide before analysis using flow cytometry and FlowJo v8 software. Experiments were repeated at least three times, with similar results.

or either paclitaxel or sorafenib with RT did ($P < .05$). This observation suggests that this cotreatment induced cell cycle arrest and apoptosis of RCC and breast cancer cells (Table 2, *A* and *B*). Furthermore, this result also showed that paclitaxel, sorafenib, and RT induced the most potent apoptosis of the tested treatments. Moreover, the synergistic anticancer effect of paclitaxel, sorafenib, and RT was remarkably observed at lower doses than required for either drug with RT.

These findings suggest that this cotreatment would enable the use of lower doses of these anticancer drugs than those currently used, which would reduce their associated side effects and toxicity.

Synergistic Anticancer Effect of Paclitaxel, Sorafenib, and RT Was Induced by Endoplasmic Reticulum Stress–Dependent Cell Cycle Arrest in RCC and Breast Cancer Cells

To further assess the synergistic anticancer effect of paclitaxel, sorafenib, and RT, immunoblot analysis was carried out to examine levels of well-known cell cycle– and apoptosis-related markers. Following treatment with the test combination, proapoptotic signaling pathway molecules were evaluated to determine the effects on induction of cell-cycle progression (cyclin D1) and arrest (p21), endoplasmic reticulum stress (CHOP), and apoptosis (BAX, Apaf-1, and cleaved-caspase 3), as well as antiapoptotic protein levels (Bcl-2), using immunoblot analysis in RCC (Figure 2A) and breast cancer cell lines (Figure 2B). Cotreatment with paclitaxel, sorafenib, and RT remarkably increased p21, CHOP, BAX, Apaf-1, and cleaved-caspase 3 expression levels, whereas the antiapoptotic protein Bcl-2 was significantly reduced.

Consequently, these data proved that paclitaxel, sorafenib, and RT effectively induced cell cycle arrest and apoptosis of RCC and breast cancer cell lines, and the synergistic effect was likely mediated *via* caspase cleavage and inhibition of the Bcl-2 pathway.
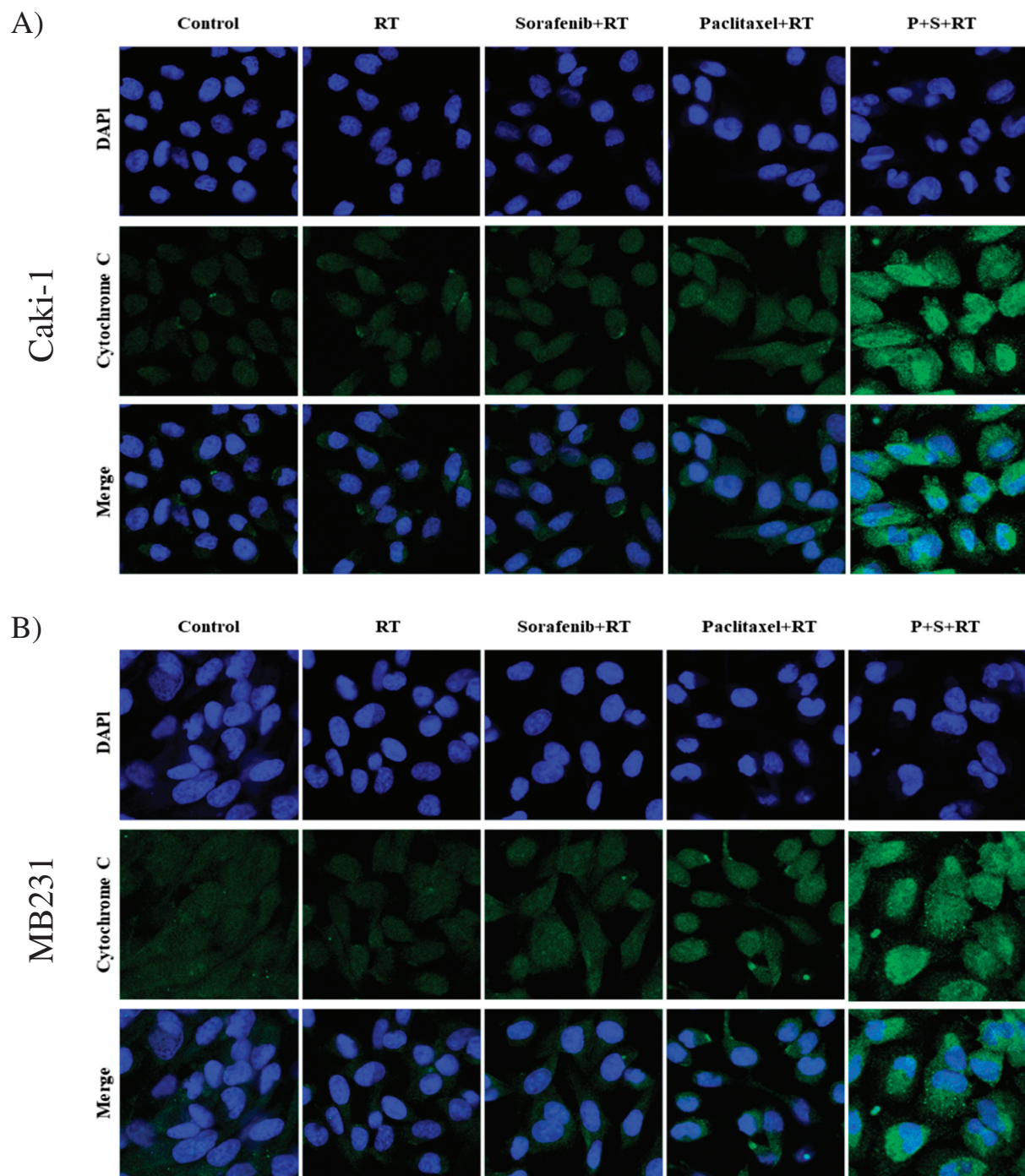
Cotreatment with Paclitaxel, Sorafenib, and RT Induced Cytochrome C Released into RCC and Breast Cancer Cell Cytosol

Cytochrome c release into the cytosol from the mitochondria is a crucial event in the apoptotic process. Moreover, DNA damage induces apoptosis by releasing cytochrome c from the mitochondria.
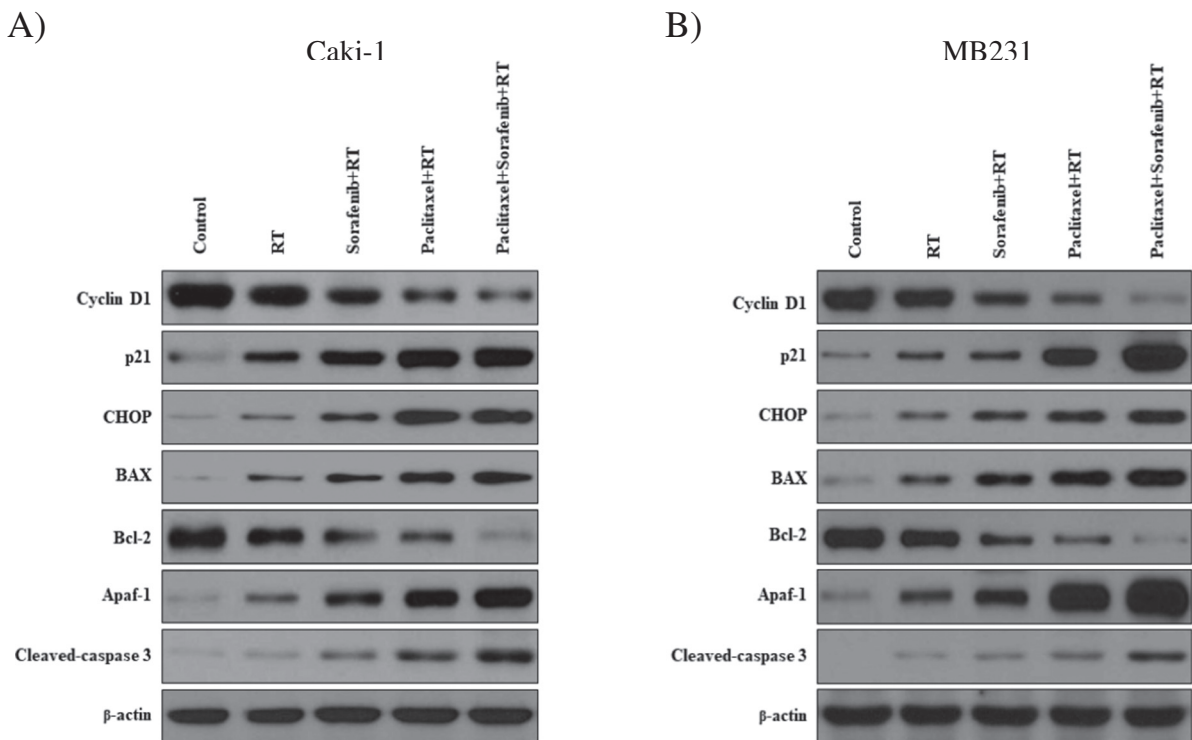
Figure 2. RT, paclitaxel, and sorafenib combination was the most induced cancer cell cycle arrest and apoptosis. Cell cycle analysis of the indicated cell lines following exposure to various combinations of paclitaxel, sorafenib, and RT. Paclitaxel, sorafenib, and RT combination potently induced cell cycle arrest and apoptosis of RCC (Caki-1; A) and breast cancer (MB-231; B) cells. Cells were exposed to the indicated inhibitors.
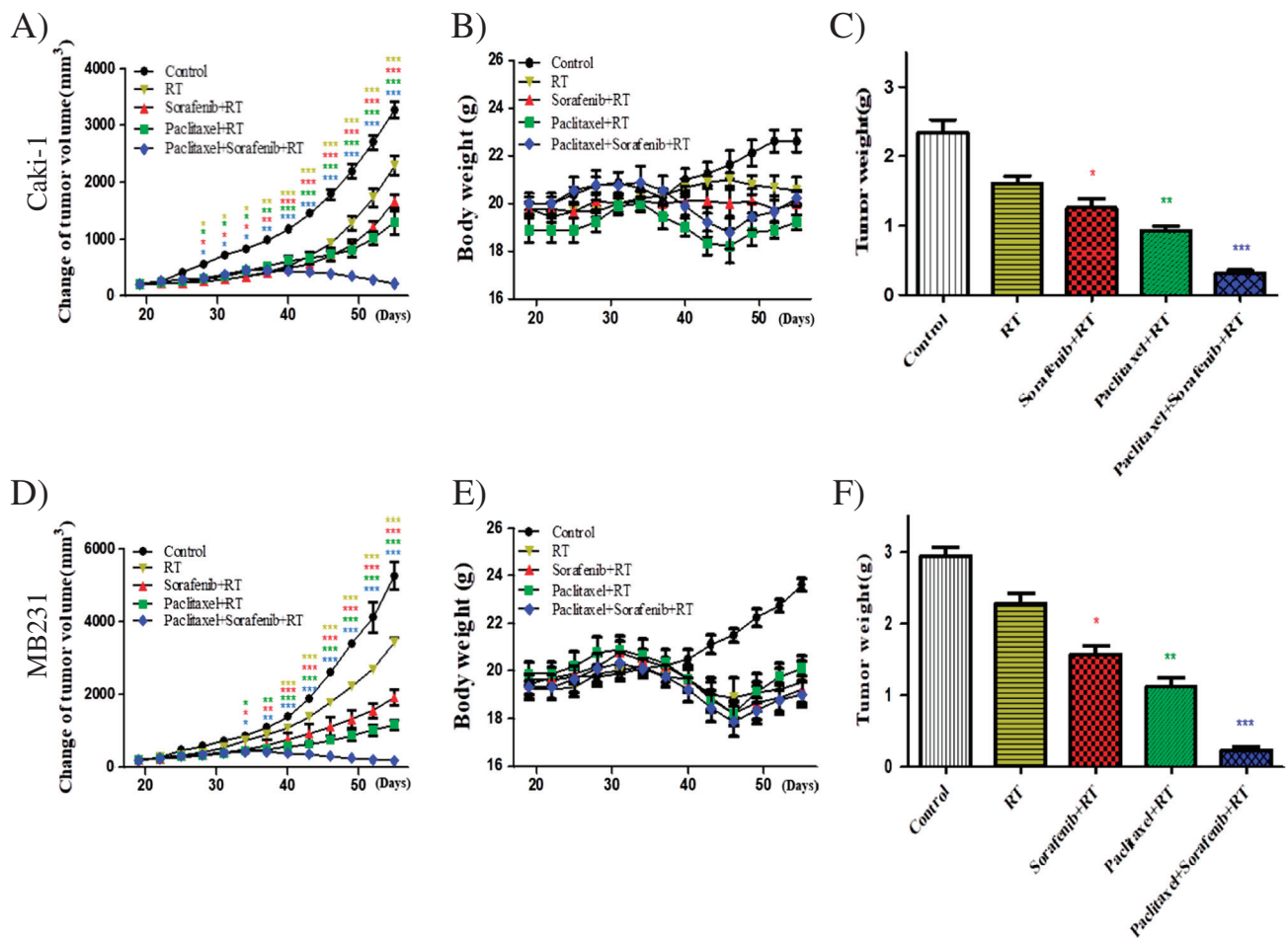
Figure 3. RT, paclitaxel, and sorafenib combination significantly induced apoptosis mediated by induction of cytochrome c release. Immunofluorescence assay: cotreatment with paclitaxel, sorafenib, and RT showed the most significant induction of cytochrome c release into cytosol, indicating apoptosis of RCC (A) and breast cancer (B) cells.

To evaluate the apoptotic mechanisms of cotreatment with paclitaxel, sorafenib, and RT, we next carried out immunofluorescence to assess the expression of cytochrome c. Immunofluorescent cytochemical staining showed that the level of cytochrome c released into the cytosol of the RCC and breast cancer cell lines was significantly increased by cotreatment with paclitaxel, sorafenib, and RT than any of the other treatments. This result suggests that cotreatment with paclitaxel, sorafenib, and RT induced apoptosis through a cytochrome c–dependent pathway in RCC (Figure 3A) and breast cancer (Figure 3B) cells.

Therefore, the apoptosis induced by the synergistic anticancer effect of the cotreatment was likely mediated through cytochrome c–dependent pathways in RCC and breast cancer cells.

Cotreatment with Paclitaxel, Sorafenib, and RT Suppressed Tumor Growth of Mouse Xenografts In Vivo

Cotreatment with paclitaxel, sorafenib, and RT significantly suppressed Caki-1 (RCC, Figure 4A) and MB-231 (breast cancer, Figure 4D) cell xenograft tumors. No evidence of systemic toxicity or treatment-related death was found in the RCC (Figure 4B) and breast

Figure 4. RT, paclitaxel, and sorafenib combination induced highest tumor shrinkage of cancer cell xenografts *in vivo*. Combination of paclitaxel, sorafenib, and RT induced more potent inhibition of tumor progression than paclitaxel or sorafenib with RT or RT only did in RCC (Caki-1; A) and breast cancer (MDA-MB-231; D) cell xenografts ($n = 10$ mice/group). Athymic nude mice with established tumors were treated with the indicated drugs or RT. Data represent the mean tumor volumes. The compounds had no significant effect on body weight in RCC (Caki-1; B) and breast cancer (MDA-MB-231, E) cell xenografted mice. Cotreatment with paclitaxel, sorafenib, and RT showed the highest reduction of tumor weights of the dissected weight in RCC (Caki-1, C) and breast cancer (MDA-MB-231, F) cell xenografts. $*P < .05$, $**P < .01$, and $***P < .005$ vs. control.

cancer (Figure 4E) cells. Furthermore, there was no significant effect on the body weight of the mice cotreated with paclitaxel, sorafenib, and RT. Furthermore, the tritherapy group showed remarkably smaller tumor volumes than those of the groups treated with paclitaxel or sorafenib and RT (Figure 4, C and F).

This result suggests that the paclitaxel, sorafenib, and RT combination induced the most efficient tumor shrinkage of mouse xenografts among the tested combinations.

Cotreatment with Paclitaxel, Sorafenib, and RT Suppressed Tumor Growth by Downregulating Antiapoptotic Factor Expression in RCC and Breast Cancer Xenografts

The determination of antiapoptotic activity is a crucial factor in evaluating the biological behavior of tumorigenesis. In particular, Bcl-2 is the most characterized protein involved in the control of apoptotic cell death. Therefore, we detected this marker in RCC (Caki-1) and breast cancer (MB-231) cell xenograft tumors using immunohistochemistry and found that the paclitaxel, sorafenib, and the RT–cotreated group showed the highest decrease in Bcl-2 expression (Figure 5, A and B) among the tested combinations, further confirming the potent

anticancer activity of the tritherapy in the RCC and breast cancer cell xenograft models.

Discussion

RCC has been identified to contribute to approximately 3% of adult malignancies and 90%-95% of neoplasms developed in the kidney [19]. Breast cancer is the most frequently diagnosed life-threatening cancer in women and the leading cause of cancer-related deaths among women [5]. These diseases are characterized by a scarcity of early warning signals, diverse clinical manifestations, and resistance to radiation therapy and chemotherapy.

RCC and breast cancer are malignancies with a very high mortality rate. Complete surgical removal in combination with adjuvant therapy (such as RT and chemotherapy) is the standard treatment [20,21]. However, this strategy is inadequate, and despite such treatment interventions, the mortality rate remains extremely high because of drug resistance [22–24]. Studies have evaluated the effect of combining chemotherapeutic agents and tyrosine kinase inhibitors [25–27], and a recent research proved that sorafenib, a tyrosine kinase inhibitor, potentiated the effect of paclitaxel against ovarian cancer [28]. The
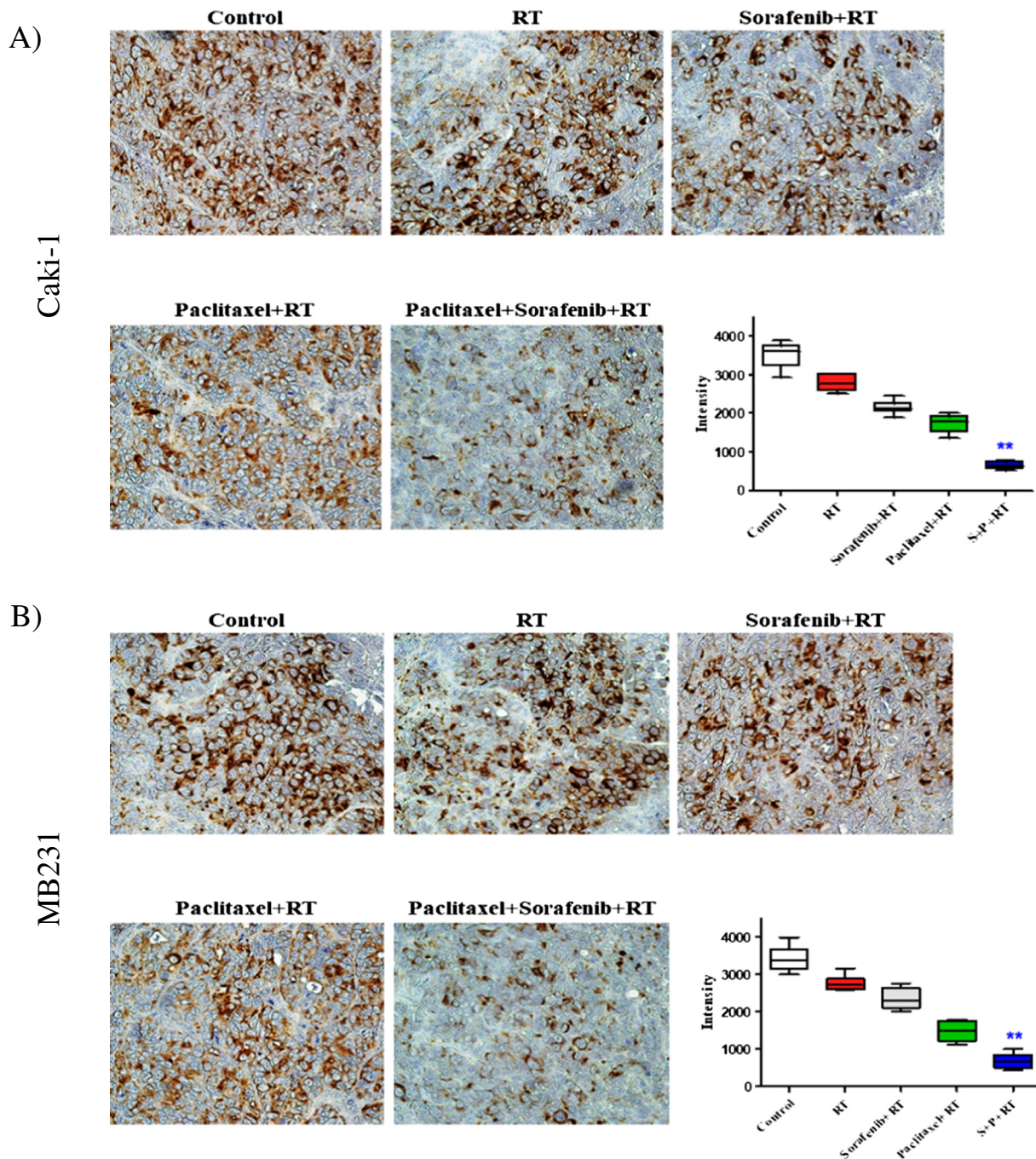
Figure 5. RT, paclitaxel, and sorafenib combination affected antiapoptosis-related proteins in tumor tissues derived from RCC and breast cancer cells. Immunohistochemical analysis of Bcl-2 proteins in tumor tissues following the indicated treatments. Each assay was performed in triplicate, and representative images are displayed. $P < .05$, $P < .01$, and $P < .005$ vs. control. MetaMorph 4.6 image-analysis software was used to quantify the immunostained target protein.

cotreatment synergistically inhibited colony formation and tumor growth, while it increased cell cycle arrest and apoptosis [29]. The synergistic anticancer effect of sorafenib and paclitaxel was investigated in experimental models of breast cancer with bone metastases, which revealed antiangiogenic, anticancer, and antiresorptive effects [30]. Moreover, sorafenib sensitized hepatocellular carcinoma cells to lethal doses of paclitaxel by reducing hepatoma upregulated protein, which correlates with paclitaxel resistance [31]. Paclitaxel is well known to have radiosensitizing effect in numerous cancers, and at low doses, it showed a better effect in combination with RT than that of chemotherapy or RT alone [32]. Therefore, using paclitaxel as a cytotoxic drug and a radiosensitizer might be a valuable strategy in reducing radiotoxicity by decreasing the radiation dose.

This study demonstrated the synergistic cytotoxicity of cotreatment with paclitaxel and sorafenib plus RT in RCC and breast cancer cell lines, both *in vitro* and *in vivo*. This cotreatment induced a more significant increase in RCC and breast cancer cell apoptosis than RT did with either sorafenib or paclitaxel. This result proposes a novel

clinical approach that targets RCC and breast cancer with low-dose anticancer drugs in combination with RT, which would reduce the toxic side effects of anticancer treatments.

Acknowledgements

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