Thiacremonone Augments Chemotherapeutic Agent–Induced Growth Inhibition in Human Colon Cancer Cells through Inactivation of Nuclear Factor-κB

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Abstract

Chemotherapeutic strategies commonly use multiple agents to overcome drug resistance and to lower drug toxicity. Activation of nuclear factor-κB (NF-κB) is implicated in drug resistance in cancer cells. Previously, we reported that thiacremonone, a novel sulfur compound isolated from garlic, inhibited NF-κB and cancer cell growth with IC_{50} values about 100 μg/mL in colon cancer cells. In the present study, we tested whether thiacremonone could increase susceptibility of cancer cells to chemotherapeutics through inactivation of NF-κB. Colon cancer cells were cotreated with thiacremonone (50 μg/mL, half dose of IC_{50}) and lower doses of each chemotherapeutic agent (half dose of IC_{50}) for 24 hours. NF-κB activity was completely abrogated in cells treated with a combination of thiacremonone and docetaxel, whereas thiacremonone on its own did not alter NF-κB activity. This combined drug effect was also found with other anticancer drugs in colon cancer and in other cancer cells. In good correlation with inhibition of cell growth and NF-κB activity, the combination treatment also regulated NF-κB target genes. Oral treatment of mice with thiacremonone (1 mg/kg) by administering it in drinking water for 4 weeks significantly augmented docetaxel (1 mg/kg, i.p., four times)–induced decrease of tumor growth accompanied with regulation of NF-κB activity and NF-κB target genes. These results warrant carefully designed clinical studies investigating the combination of thiacremonone and commonly used chemotherapeutic agents for the treatment of human cancers. (Mol Cancer Res 2009;7(6):870–9)

Introduction

Nuclear factor-κB (NF-κB) mediates the promotion of tumor growth, angiogenesis, metastasis, and chemotherapeutic resistance through the expression of genes participating in malignant conversion and tumor promotion (1, 2). Constitutive activation of NF-κB has been described in a large number of tumors. NF-κB is also constitutively active in colon cancer cells. Colon cancer cell lines and human tumor samples as well as nuclei of stromal macrophages in sporadic adenomatous polyps showed increased NF-κB activity (3, 4). It has also been reported that intrinsically or constitutively activated NF-κB may be critical in the development of drug resistance in cancer cells (5, 6). Therefore, agents that are able to inhibit NF-κB might be considered as an adjuvant approach in combination with chemotherapeutics for the treatment of various cancers.

Docetaxel, a semisynthetic taxoid produced from the needles of the European yew (*Taxus baccata*) tree, is one of the most important new and active chemotherapeutics in the market. Docetaxel has potential activity in the treatment of refractory human solid tumors, including colon cancers (7). Although docetaxel possess potential cell killing activity in a variety of tumor cells, the use of high doses of this agent has shown to induce different levels of toxic reactions; however, low or moderate doses of docetaxel does not show significant antitumor activity in patients (8, 9). Thus, the use of docetaxel as a monotherapy for cancer has been precluded (10, 11).

Alternatively, a combination therapy using more than one anticancer drug to achieve optimum results against cancer has been shown (6, 12). The effects of a combination of protein-bound polysaccharide (PSK) and docetaxel treatment against a pancreatic cancer cell line, NOR-P1, indicated that PSK rendered tumor cells more susceptible to cytotoxicity induced by a low dose of docetaxel, causing an efficient execution of apoptosis. Furthermore, the enhanced apoptosis was attributed to the suppression of NF-κB activation by PSK, followed by inactivation of cellular inhibitor of apoptosis protein 1 (c-IAP1) and augmentation of caspase-3 activity, both of which are regulated by NF-κB (6). BAY 11-7085, a known pharmacologic inhibitor of IκB phosphorylation, inhibits both basal and transient induction of IκB phosphorylation and NF-κB activity, and increases the cytotoxic efficacy of paclitaxel in both *in vitro* and *in vivo* ovarian cancer models (12). Other combination cytotoxic effects of chemotherapeutics with nontoxic dietary supplements have been shown (13, 14). Silibinin, which is effective against prostate cancer, was able to synergize the therapeutic efficacy

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of doxorubicin in advanced human prostate carcinoma DU145 cells (13). The combination of curcumin with cisplatin resulted in a synergistic antitumor activity through a lowering of the NF-κB activity (14).

Garlic contains numerous pharmacologically active substances, including sulfur and selenocompounds, which have been shown to alter the activation of several carcinogens. These compounds have also been shown to inhibit cancer cell growth and/or to induce cell death (15, 16). Sulfur compounds isolated from garlic, including diallyl sulfide, *S*-allylmercaptocysteine, and ajoene, have been known to increase the activity of enzymes involved in the metabolism of carcinogens (17), and have antioxidant activity (18) and protective activity against lipid peroxidation and hepatic toxicity *in vitro* and *in vivo* (19, 20). Several recent studies have also shown that these sulfur compounds are able to inhibit the growth of several human cancer cells, including breast, hepatoma, and lymphocytic leukemia, as well as lung cancer cells in culture (21, 22). In addition, several research groups have shown the importance of sulfur compounds in the preventative effect against colon cancer development (23, 24). We previously isolated a novel sulfur compound from garlic and identified it as thiacremonone; subsequent studies showed that it exerted inhibitory effects on colon cancer cell growth through inactivation of NF-κB and target gene expression within a hundred micromolar (100-150 μg/mL) concentration range (25). In the present study, we examined whether the inhibitory ability of thiacremonone on NF-κB activity augmented cancer cell killing activity of conventional chemotherapeutics when these agents were coadministered with thiacremonone.

Results

Effect of a Combination of Thiacremonone and Docetaxel on NF-κB Activation in SW620 and HCT116 Human Colon Cancer Cells

We were interested to determine whether thiacremonone (Fig. 1A) inhibited colon cancer cell growth by inactivation of NF-κB because activation of NF-κB plays a critical role in cancer cell survival and drug resistance. Our previous study showed that thiacremonone (30-150 μg/mL) dose dependently inhibited cancer cell growth and NF-κB activity, with IC_{50} values of 105 μg/mL in SW620 and 130 μg/mL in HCT116 colon cancer cells (25). We also observed that docetaxel (1-50 nmol/L) inhibited cell growth and NF-κB activity dose dependently with IC_{50} values of about 10 nmol/L in colon cancer cells (Supplementary Fig. S1A). Thus, we used the half doses of IC_{50} values (50 μg/mL thiacremonone and 5 nmol/L docetaxel) in testing the combination effect of these agents.

We first tested the combination effect of thiacremonone and docetaxel on the NF-κB activity. The doses of each compound alone did not alter NF-κB but slightly inhibited cancer cell growth. However, the combination of thiacremonone and docetaxel resulted in a synergistic and significant inhibition of the transcriptional activation of NF-κB (Fig. 2A) in the two colon cancer cell lines. Constitutional activation of the DNA binding activity of NF-κB was observed in colon cancer cells, and treatment with docetaxel (5 nmol/L) and thiacremonone alone (50 μg/mL) did not change the activated DNA binding activity of NF-κB in SW620 and HCT116 colon cancer cells. However, consistent with synergistic inhibition of the transcriptional activity of NF-κB, the combination of 50 μg/mL thiacremonone and 5 nmol/L docetaxel resulted in a synergistic inhibitory effect on the DNA binding activity of NF-κB (Fig. 2B). To test the combination effect of thiacremonone with other chemotherapeutics, we treated colon cancer SW620 as well as HCT 116 cells with thiacremonone and 5-fluorouracil (100 nmol/L) or oxaliplatin (10 nmol/L) and found that these combinations also inhibited NF-κB synergistically (Supplementary Fig. S1B). The combined effect of thiacremonone with docetaxel as well as other well-prescribed chemotherapeutics on the NF-κB activity was also observed in other cancer cells, including liver HepG2, prostate PC-3, stomach

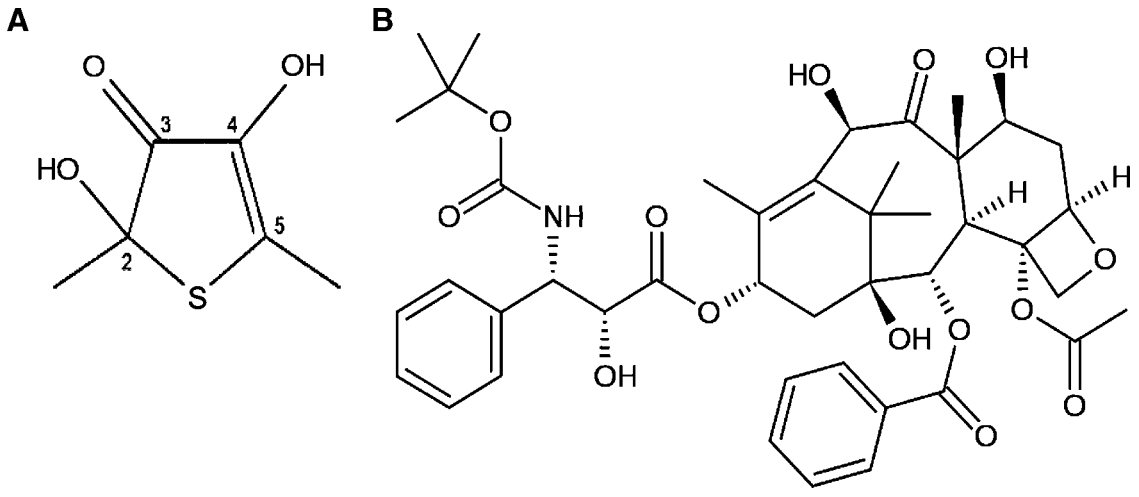

FIGURE 1. Structure of thiacremonone (**A**) and docetaxel (**B**).

NKM-45, and lung NCI-H460 cancer cells (Supplementary Fig. S1C).

Effect of a Combination of Thiacremonone and Docetaxel on Cell Growth in SW620 and HCT116 Human Colon Cancer Cells

Subsequent studies were undertaken to determine whether colon cancer cells were more sensitive to the cytotoxic effect exerted by a combined regimen of thiacremonone with docetaxel. Treatment of SW620 and HCT116 colon cancer cells with 50 μg/mL thiacremonone and 5 nmol/L docetaxel alone for 24 hours showed 5% to 30% inhibition of cell growth (Fig. 2C). Paralleled with the synergistic inhibition of NF-κB, the addition of both agents together resulted in a strong synergistic inhibitory effect on cell growth (70-80% inhibition) in SW620 and HCT116 (Fig. 2C). The combined effect of thiacremonone with other chemotherapeutics, 5-fluorouracil (100 nmol/L) and oxaliplatin (10 nmol/L), on cancer cell growth inhibition was also observed in colon cancer cells (Supplementary Fig. S2A). Moreover, these combination effects of thiacremonone with other chemotherapeutics on cancer cell growth inhibition was also found in other cancer cells, including liver (HepG2), stomach (MKN-45), lung (NCI-H460), and prostate (PC-3) cancer cells (Supplementary Fig. S2B). These results suggest that the combination of thiacremonone with lower doses of chemotherapeutics elicited significantly greater inhibition of cancer cell growth compared with either agent alone and this combination effect may not be cancer cell type and chemotherapeutic specific.

Effect of a Combination of Thiacremonone and Docetaxel on Apoptotic Cell Death in SW620 and HCT116 Human Colon Cancer Cells

Cell death can contribute to cell growth inhibition. We thus evaluated apoptotic cell death in colon cancer cells using 4′,6-diamidino-2-phenylindole (DAPI) and TdT-mediated dUTP nick and labeling (TUNEL) staining analysis by fluorescence microscopy. In agreement with the cell growth inhibition studies, the combination of thiacremonone and docetaxel treatment significantly increased apoptotic cell numbers (DAPI-stained TUNEL positive cells) of colon cancer cells compared with single-agent treatment (Fig. 3). These results suggest that the efficiency in inhibition of cancer cell growth by the combined treatment of cells with thiacremonone and docetaxel may have been a result of the induction of apoptosis of cancer cells.

Effect of a Combination of Thiacremonone and Docetaxel on the Expression of Apoptotic Regulatory Proteins in SW620 and HCT116 Human Colon Cancer Cells

NF-κB activation in cancer cells correlates with the resistance to apoptosis and the control of antiapoptotic proteins as well as apoptotic regulatory proteins. To elucidate the relationship between the induction of apoptosis and the expression of their regulatory proteins by combination treatment, expression of apoptosis-related proteins was investigated. Cells were treated with a combination of thiacremonone (50 μg/mL) and docetaxel (5 nmol/L), and whole-cell extract was subjected to Western blotting. Our data showed that the combination treatment substantially inhibited the expression levels of all tested

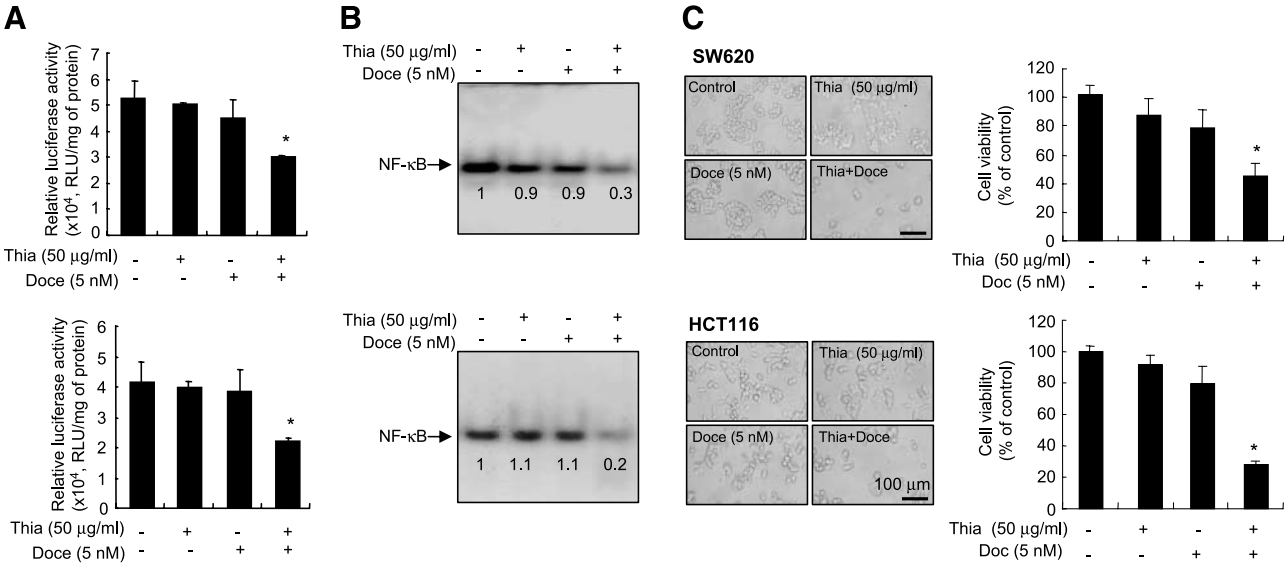

FIGURE 2. Effect of the combination treatment of thiacremonone and docetaxel on cell viability and NF-κB activation in SW620 and HCT116 colon cancer cells. **A.** SW620 and HCT116 colon cancer cells were transfected with pNF-κB-Luc plasmid (5× NF-κB) for 4 h. The transfected cells were incubated with 400 μL of fresh medium for 24 h and then cotreated with 50 μg/mL thiacremonone and 5 nmol/L docetaxel for 6 h. Columns, mean of three independent experiments done in triplicate; bars, SD. RLU, relative luciferase activity in unstimulated cells. **B.** SW620 and HCT116 colon cancer cells were cotreated with 50 μg/mL thiacremonone and 5 nmol/L docetaxel for 1 h. Nuclear extract was incubated in binding reactions of ^{32}P-end–labeled oligonucleotide containing the κB sequence. The activation of NF-κB was investigated using EMSA as described in Materials and Methods. Quantification of band intensities from three independent experimental results was done by densitometry (Imaging System) and the value under each band was indicated as fold difference from the untreated control group. **C.** SW620 and HCT116 colon cancer cells were cotreated with 50 μg/mL thiacremonone and 5 nmol/L docetaxel. Cell viability was determined after 24-h culture by cell counting using 0.2% trypan blue as described in Materials and Methods, and the results were expressed as the percentage of dead cells. Columns, mean of three experiments, each done in triplicate; bars, SD. Bar, 100 μm. *, $P < 0.05$, statistically significant differences from the control group.

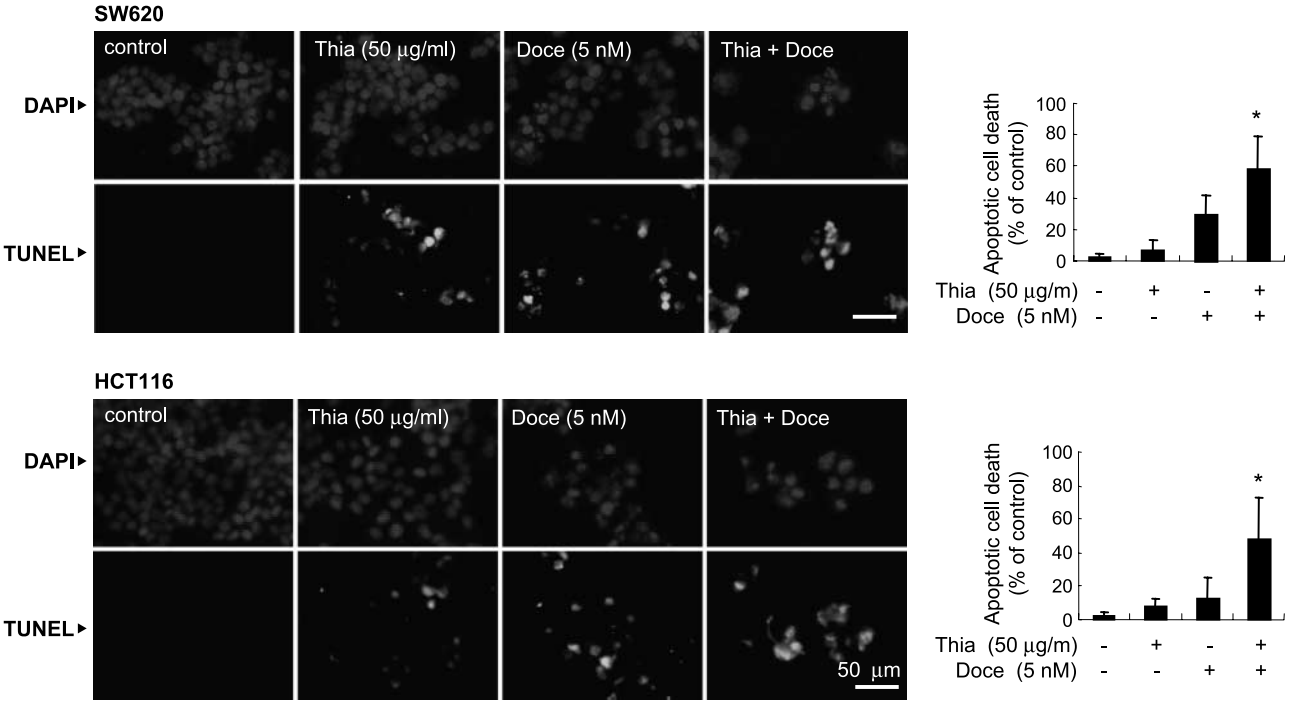

FIGURE 3. Apoptotic cell death of SW620 and HCT116 colon cancer cells by the combination treatment of thiacremonone and docetaxel. SW620 and HCT116 and colon cancer cells were cotreated with 50 μg/mL thiacremonone and 5 nmol/L docetaxel for 24 h. Apoptotic cells were examined by fluorescence microscopy after TUNEL staining (fluorescent microscopy; *bottom panels*). Total number of cells in a given area was determined by using DAPI nuclear staining (*top panels*). The apoptotic index was determined as the DAPI-stained TUNEL-positive cell number was counted. Columns, mean of three experiments, with triplicate of each experiment; bars, SD. *, $P < 0.05$, statistically significant differences from the untreated group. Bar, 50 μm.

marker proteins that favor cell survival. A significant decrease in the expression levels of Bcl-2, XIAP, and cIAP-1 was observed in the combination treatment, whereas an increase in the expression levels of proapoptotic proteins, Bax, and active form of caspase-3 (including cleaved caspase-3) and caspase-9 (including cleaved caspase-9) after combination treatment was found compared with single treatment (Fig. 4).

In vivo *Effect of a Combination of Thiacremonone and Docetaxel on Tumor Growth in a Xenograft Model*

The tumor growth in colon cancer xenograft–bearing nude mice following treatment with a combination of thiacremonone and docetaxel or both these agents alone was investigated. To determine the optimum doses for thiacremonone, SW620 colon cancer–bearing nude mice (100-300 mm^3) were treated by orally administering thiacremonone at different doses ranging from 5, 10, and 30 mg/kg, in drinking water for 4 weeks. The mice treated with 5, 10, and 30 mg/kg thiacremonone showed different inhibiting responses to tumor (32-50% inhibition of tumor volume) compared with the control group. Hence, we selected a dose of 1 mg/kg as optimum for the combination treatment with docetaxel (1 mg/kg, i.p., once a week for 28 days), which showed 32% to 50% tumor growth inhibition by 5, 10, and 30 mg/kg (data not shown). In the SW620 xenograft studies, thiacremonone (1 mg/kg) was administered orally for 10 days before an i.p. injection of docetaxel (1 mg/kg) once per week for 4 weeks, in mice with tumors ranging from 100 to 300 mm^3 (Fig. 5A). The mice were weighed twice per week. The changes in body weight between the control, thiacremonone-docetaxel combination therapy, or thiacremonone and docetaxel single-treatment mice ($n = 10$) were not remarkably different during the experiment (data not shown). An analysis of the tumor volume and weight of the mice showed that both thiacremonone and docetaxel individually resulted in a slight antitumor activity (Fig. 5B-D). The combined treatment exerted a superior suppression of the tumor growth. The combined group showed marked regression of tumor weight (0.39 ± 0.14 g) compared with the control groups (1.0 ± 0.36 g), thiacremonone groups (0.88 ± 0.40 g), and docetaxel groups (0.62 ± 0.37 g) alone (Fig. 5B). The combined group also showed marked regression of tumor volume (1.22 ± 0.55 cm^3) compared with the control groups (1.81 ± 0.08 cm^3), thiacremonone groups (1.71 ± 0.19 cm^3), and docetaxel groups (1.50 ± 0.13 cm^3) alone (Fig. 5C). In another set of experiments with the same treatment, we showed that the combination of thiacremonone and docetaxel treatment significantly prolonged the survival rate of mice compared with the single treatment (Fig. 5D). The immunohistochemical analysis of tumor sections by H&E staining, and proliferation antigens against Ki-67 (a cell proliferation marker) staining, revealed a greater inhibitory effect exerted by the combination of thiacremonone and docetaxel on the tumor cell growth compared with the single treatment (Fig. 6A). Combination therapy was also accompanied by a greater increase in cleavage caspase-3 reactive cell number in the tumor tissue (Fig. 6B) as well as apoptotic cell death (Fig. 6C).

Effect of a Combination of Thiacremonone and Docetaxel on the DNA-Binding Activity of NF-κB and the Expression of Proapoptotic and Antiapoptotic Genes In vivo

We also tested the effect of the combination of thiacremonone and docetaxel on NF-κB activity using an *in vivo* model. Similar to the inhibitory effects reported *in vitro*, the combination of thiacremonone and docetaxel significantly inhibited DNA binding activity of NF-κB in tumor tissues (Fig. 7A). Western blotting analysis also showed the decreased expression of p65 and p50 in the nucleus after a combination treatment with thiacremonone and docetaxel compared with the single treatments (Fig. 7A). We also characterized the immunohistochemical staining pattern of the NF-κB subunits p65 and p50 in tumor tissues. There was a trend toward decreased intensity of nucleus staining of p65 and p50 in the combination of thiacremonone and docetaxel-treated tumor tissue (Fig. 7B). These data agreed with the inhibitory effects of a combination of thiacremonone and docetaxel in the DNA binding activity of NF-κB *in vitro* and *in vivo*, and indicate that inhibition of NF-κB could sensitize tumor cells against docetaxel. Although there was an increase in the levels of Bax and cleaved caspase-3, a deceased expression of Bcl-2, xIAP, and cIAP in colon cancer tissue was detected after combination treatment, as determined by Western blot analysis (Fig. 7C).

Discussion

The central finding of the present study is that thiacremonone strongly enhanced the therapeutic efficiency of docetaxel by growth inhibition and induction of apoptosis through inactivation of NF-κB in colon cancer cells. These combination effects of thiacremonone with other chemotherapeutics on cancer cell growth inhibition and NF-κB activity was also found in colon and other cancer cells, including liver, stomach, lung, and prostate cancer cells. In addition, thiacremonone and docetaxel showed strong combination efficiency *in vivo* on tumor growth inhibition. These results suggest that the combination effects of thiacremonone with chemotherapeutics are not specific to cell type and chemotherapeutic agent. These data suggest that inactivation of NF-κB by thiacremonone may contribute to increase in cancer cell susceptibility against conventional chemotherapeutics.

Several studies have reported similar beneficial effects of combination therapy using conventional chemotherapeutic agents and naturally occurring compounds in the inhibition of growth of cancer cells. For example, genistein showed synergistic combination effect with 5-fluorouracil in the induction of apoptosis in chemoresistant HT-29 colon cancer cells (26). Curcumin and celecoxib also synergistically inhibited the growth of colorectal cancer cells (27). It is known that many chemotherapeutic agents induce activity of NF-κB, causing drug resistance in cancer cells (28). Thus, we speculate that inhibition of NF-κB by thiacremonone is a profound contributor to the increase of susceptibility of cancer cells against chemotherapeutics. However, it is not clear in the present study how the combination of thiacremonone and docetaxel or other chemotherapeutics block the constitutive activation of NF-κB, because we used lower doses of thiacremonone and docetaxel (half of its IC_{50} values on cell growth inhibition and NF-κB activity), which only slightly decreased NF-κB activity or have no effect on it. It is possible that a common signal pathway in the induction of NF-κB activity was blocked by the combination of both compounds, which cannot be blunted by thiacremonone or docetaxel alone. Alternatively, multiple signal pathways in the activation of NF-κB were prevented by coordination of the blocking of each signal enforcing inactivation of NF-κB. Supporting our hypothesis, several combination of therapeutic treatments have shown to enhance cancer cell susceptibility through coordinating the inhibition of NF-κB (29-31). More proper inactivation of NF-κB by curcumin and chemotherapeutic enhance cancer cell killing effects (28). Sulindac enhances arsenic trioxide–mediated apoptosis in HCT116 colon cancer cells through the synergistic decrease in levels of NF-κB after combination treatment. This occurred by the inhibition of phosphorylation and degradation of IκB-α, whereas the levels NF-κB were not altered significantly by As_2O_3 or sulindac treatment alone (29). A significant reduction in cell viability, induction of apoptosis, NF-κB activity, and expression of antiapoptotic genes was reported in pancreatic cancer BxPC-3 cells when treated with a combination of erlotinib and B-DIM compared with either agent alone (30). Moreover, very similar to our results, the combination of all-*trans* retinoic acid and paclitaxel synergistically induced apoptosis and inhibited NF-κB activity in human glioblastoma U87MG xenografts in nude mice (31). The synergistic effect of the combination of thiacremonone and docetaxel in NF-κB activity was not clear at this study. However, the antioxidant properties of thiacremonone (32) could additionally lower the redox potential within the cell, providing further hindrance in activation of NF-κB. Simultaneous inactivation of NF-κB by diverse mechanisms

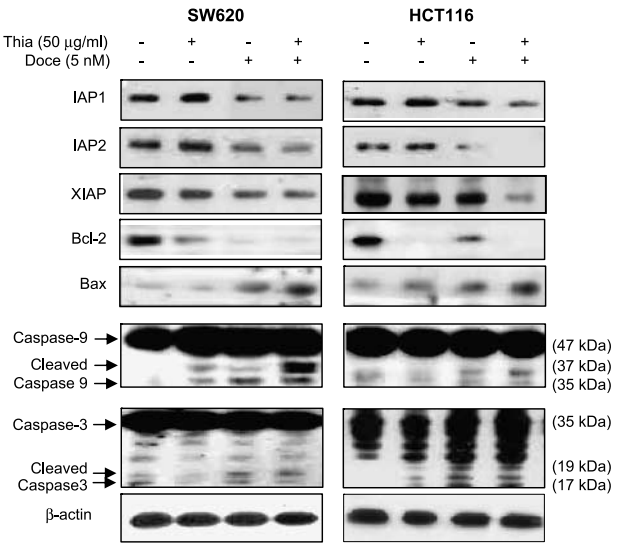

FIGURE 4. Effect of the combination treatment of thiacremonone and docetaxel on expression of apoptosis regulatory proteins in SW620 and HCT116 colon cancer cells. The cells were cotreated with 50 μg/mL thiacremonone and 5 nmol/L docetaxel for 24 h. Equal amounts of total proteins (50 μg/lane) were subjected to 12% SDS-PAGE. Expression of Bax, cleaved caspase-3, cleaved caspase-9, Bcl-2, cIAP1/2, XIAP, and β-actin was detected by Western blotting using specific antibodies. β-Actin protein was used as an internal control. Each band is representative for three independent experiments.

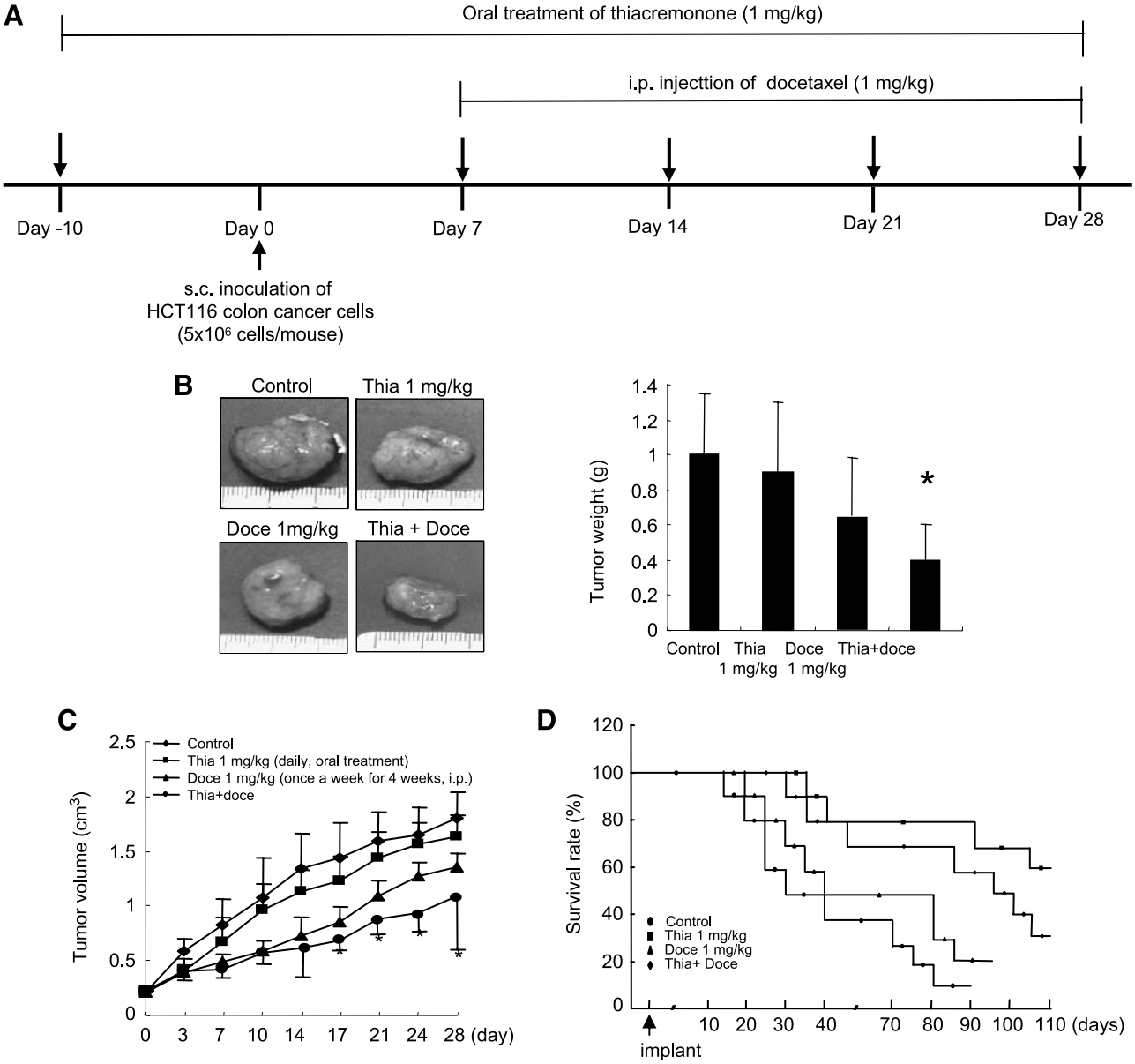

FIGURE 5. Effect of combination therapy of thiacremonone and docetaxel on the tumor growth in SW620 xenografts in an *in vivo* model. **A.** Schematic representation of the experimental protocol described in Materials and Methods. **B-D.** Tumor photographs and tumor weight, tumor volume, and survival rate. Group 1 was treated with vehicle [ethanol/Tween 80/saline (5/5/90%), i.p., once a week for 4 wk]; group 2 was orally administered with 1 mg/kg thiacremonone (1 mg/kg/d pretreatment for 10 d and continues treatment for 4 wk) alone; group 3 was treated with 1 mg/kg docetaxel (1 mg/kg/once a week for 4 wk) alone; group 4 was cotreated with 1 mg/kg thiacremonone and 1 mg/kg docetaxel. For the survival test, another set of experiments with the same treatment with same number of mice (n = 10 mice/group) was done. The survival rate and other toxic signs were observed once every 10 d for 110 d.

may be also possible by thiacremonone, which could completely eliminate NF-κB activity.

Downstream target gene expression by NF-κB is implicated in the sensitization of cancer cells to chemotherapeutic agents. It is well known that NF-κB–mediated expression of Bcl-2, IAP1/2, and survivin protects cancer cells from apoptosis, whereas Bax and caspase-3 and caspase-9 inhibit cancer cell growth and induces apoptosis (33, 34). The present data showed that the combination therapy regulated expression of NF-κB target genes *in vitro* as well as *in vivo*. It was also found that consistent with the increase of apoptosis, the expression of apoptotic proteins active caspase-3 and Bax was dose dependently increased *in vivo* and *in vitro*. Apoptosis is an important mechanism to eliminate unwanted cells and deregulation of this process is implicated in pathogenesis in the development of cancer (35). In this process, Bcl-2 inhibits apoptosis whereas Bax promotes apoptosis (36). Hence, an alteration in the levels of antiapoptotic and proapoptotic proteins is likely to influence apoptosis. NF-κB activation in cancer cells correlates with resistance to apoptosis and increased levels of antiapoptotic Bcl-2 family proteins (37). In this study, combination treatment in cells more significantly inhibited NF-κB activation and reduced Bcl-2 protein level. Caspases are cysteine

proteases that also play critical roles in the execution of apoptosis (38). These present data suggest that a down-regulation of NF-κB mediates antiapoptotic genes whereas up-regulation of apoptotic genes by the combination treatment is responsible for sensitization of cancer cells to chemotherapeutic agents.

In the present study, we showed that the combination treatment with thiacremonone and docetaxel showed strong antitumor activity *in vivo*. The antitumor activity in the present study was half of the antitumor activity of high-dose single treatment of docetaxel (10 mg/kg, i.p., once a week for 28 days), which showed significant (77% inhibition) inhibition of tumor growth. However, the present combination treatment greatly increased the survival rate of tumor-bearing mice. The single high-dose treatment with docetaxel (10 mg/kg, i.p., once a week for 28 days) showed a life span of about 45 days, whereas the combination-treated mice lived for more than 110 days. This lowering of toxicity and augmenting of cancer cell killing efficiency indicate the beneficial effects of the combination of thiacremonone with other conventional chemotherapeutics. To achieve better therapeutic efficiency, a combination of two or more chemotherapeutic agents for treatment is commonly considered. Optimization of combination chemotherapy based on the molecular mechanism of both candidate agents may improve the therapeutic index (increased therapeutic effect with low toxicity) and such therapeutic strategies are critically required for the successful treatment of patients with cancer. Our results clearly show that a combination of chemotherapeutic agents with thiacremonone, which is a dietary sulfur compound isolated from garlic, could exert great beneficial effects due to its relatively low toxicity and better antitumor activities against different types of cancers, including prostate, stomach, and lung cancers. Therefore, thiacremonone could be potentially useful in combination with other chemotherapeutic agents for the treatment of different types of cancers.

Materials and Methods

Materials

Thiacremonone was isolated from garlic as described elsewhere (32) and found to be the same compound isolated from

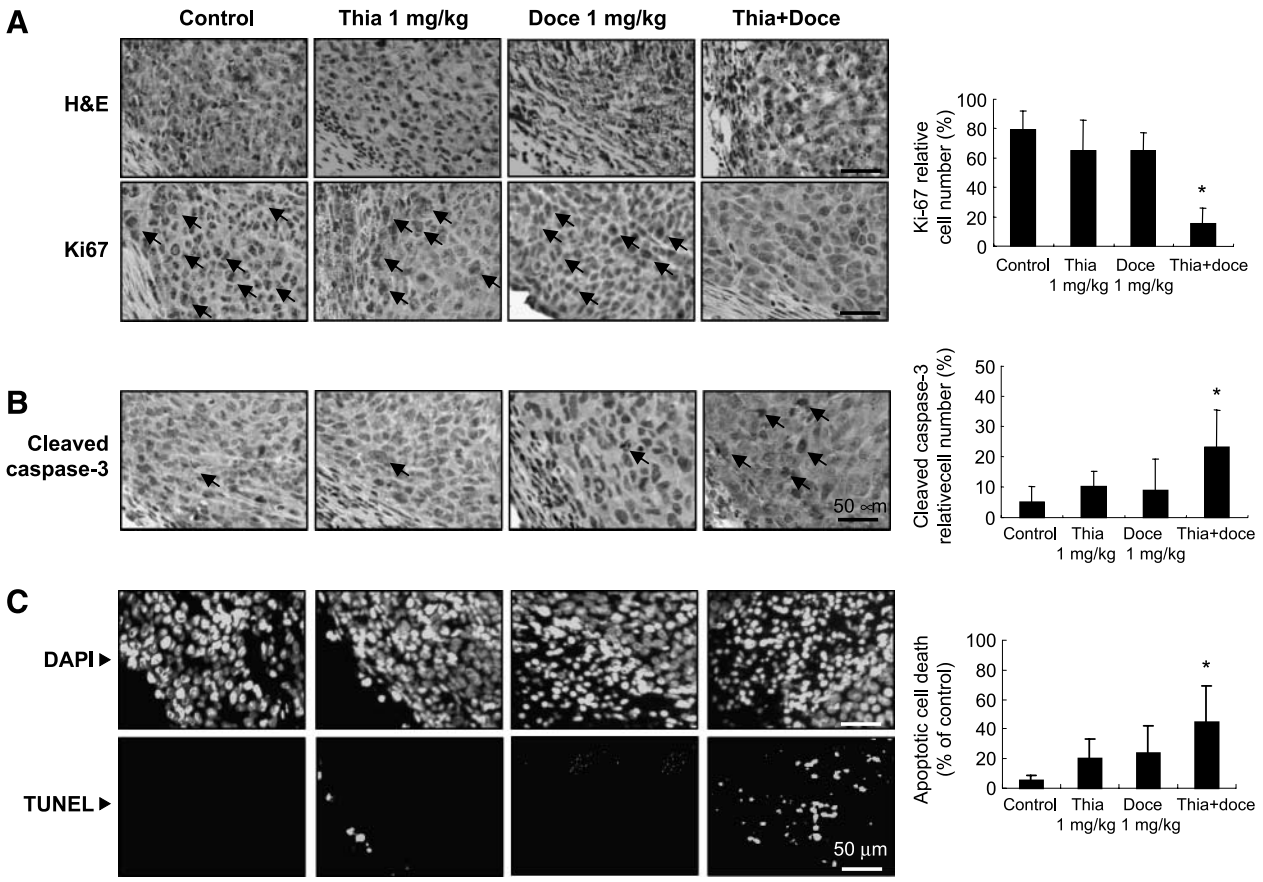

FIGURE 6. Effect of combination therapy of thiacremonone and docetaxel on tumor cell proliferation, apoptosis, and NF-κB proteins in an *in vivo* xenograft animal model. Immunohistochemistry was used to determine expression levels of H&E, Ki-67, and cleaved caspase-3 in nude mice xenograft tissues by the different treatments as described in Materials and Methods. **A.** Immunohistochemical analysis of proliferation markers H&E (*top*) and Ki-67 (*second panel*) indicates the inhibition of colon tumor cell proliferation in thiacremonone-alone or in combination with docetaxel–treated groups of animals. Quantification of Ki-67$^+$ cell as described in Materials and Methods. **B.** Immunohistochemical analysis of apoptosis marker cleaved caspase-3 indicates the induction of colon tumor cell apoptosis in thiacremonone-alone or in combination with docetaxel–treated groups of mice. Quantification of cleaved caspase-3 reactive cell was calculated as described in Materials and Methods. Apoptotic cells were examined by fluorescence microscopy after TUNEL staining. **C.** The total number of cells in a given area was determined by using DAPI nuclear staining (*fourth panels*). The apoptotic index was determined as the DAPI-stained TUNEL-positive cell numbers was counted. Columns, mean of six mice; bars, SD. *, $P < 0.05$, statistically significant differences from untreated group. Bar, 50 μm.

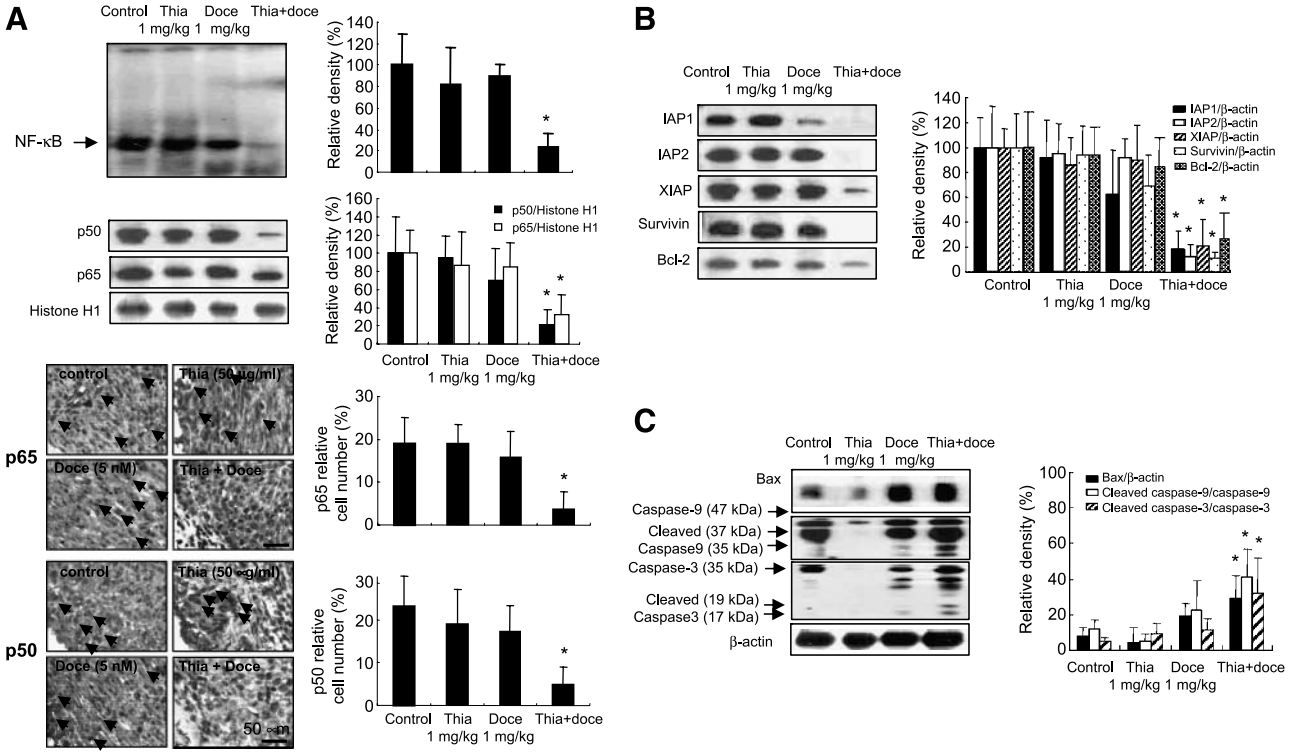

FIGURE 7. Effect of the combination treatment of thiacremonone and docetaxel on NF-κB proteins and NF-κB regulatory proteins in an *in vivo* xenograft animal model. **A.** DNA binding activity of NF-κB was determined by EMSA in the nuclear extract from xenograft tumor samples as described in Materials and Methods. Expression of NF-κB proteins (p50 and p65) was detected by Western blotting using specific antibodies in the nuclear extract from xenograft tumor samples (three samples per each group) as described in Materials and Methods. Immunohistochemical analysis of p50 and p65 indicates the inhibition of NF-κB protein expression in thiacremonone-alone or in combination with docetaxel–treated groups of animals. Quantification of p50 and p65 reactive cells was determined as described in Materials and Methods. **B** and **C.** Western blot analysis of IAP1/2, XIAP, survivin, Bcl-2, Bax, cleaved caspase-9/caspase-9, and cleaved caspase-3/caspase-3 expression in mouse tumor tissues (three samples per group) resected on day 28 untreated tumors (*lane 1*), thiacremonone 1 mg/kg–treated tumors (*lane 2*), docetaxel 1 mg/kg tumors (*lane 3*), and thiacremonone and docetaxel–cotreated tumors (*lane 4*). A representative sample from each group was stained in the picture. Columns, mean from three animal sections; bars, SD. *, $P < 0.05$, significantly different from the control group. Bar, 50 μm.

Acremonium sp. strain HA33-95 by fermentation as described by Gehrt et al. (39). The structure of thiacremonone is shown in Fig. 1A. Thiacremonone was resolved in 0.01% DMSO and administered in a dose of 50 μg/mL. Docetaxel was obtained from Samyang Genex Corporation. Docetaxel was produced by semisynthesis and purification methods (40). Briefly, crude docetaxel was obtained from semisynthesis of 13-dehydroxybaccatin III with (3*R*,4*S*)-1-*t*-Boc-3-triethylsilyloxy-4-phenylazetidin-2-one as a docetaxel side chain and then purified by recrystallization with methanol/distilled water solution. The structure of docetaxel is shown in Fig. 1B. Docetaxel was dissolved in 0.01% DMSO for treatment *in vitro*.

Cell Culture

SW620 and HCT116 human colon cancer cells, HepG2 liver cancer cells, PC3 prostate cancer cells, NCI-H460 lung cancer cells, and MKN-45 stomach cancer cells were obtained from the American Type Culture Collection. RPMI 1640, DMEM, penicillin, streptomycin, and fetal bovine serum were purchased from Invitrogen. SW620 and HCT116 human colon cancer cells, PC3 prostate cancer cells, and NCI-H460 lung cancer cells were grown in RPMI 1640 with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2 humidified air. HepG2 liver cancer cells grown in DMEM with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2 humidified air.

Cell Viability Assay

To determine the cell number, cultured cells were trypsinized with TrypLE Express (Invitrogen) and then the cells were pelleted by centrifugation for 5 min at 1,500 rpm. Cells were resuspended in 10 mL of PBS and 0.1 mL of 0.2% trypan blue was added to the cancer cell suspension. Subsequently, a drop of suspension was placed into a Neubauer chamber and the living cancer cells were counted. Cells that showed signs of staining were considered to be dead, whereas those that excluded trypan blue were considered viable. Each assay was carried out in triplicate.

Western Blot Analysis

Western blot analysis was done as described previously (25). The membranes were immunoblotted with primary specific antibodies: mouse polyclonal antibodies against p65 and p50 (1:500 dilution, Santa Cruz Biotechnology, Inc.); rabbit polyclonal for Bax (1:500 dilution, Santa Cruz Biotechnology, Inc.); and caspase-3, cleaved caspase-3, caspase-9, cleaved

caspase-9, PARP, Bcl-2, XIAP, and c-IAP-1 (1:1,000 dilution, Cell Signaling Technology, Inc.). The blot was then incubated with the corresponding conjugated anti-rabbit and anti-mouse IgG-horseradish peroxidase (1:2,000 dilution, Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were detected with the ECL Western blotting detection system. The relative density of the protein bands was scanned by densitometry using My-Image (SLB) and quantified by Labworks 4.0 software (UVP, Inc.).

Electromobility Shift Assay

Electromobility shift assay was done as described previously (25). The relative density of the protein bands was scanned by densitometry using MyImage and quantified by Labworks 4.0 software (UVP, Inc.).

Transfection and Assay of Luciferase Activity

Colon cancer cells ($2.5 \times 10^5/cm^2$) were plated in 24-well plates and transiently transfected with pNF-κB-Luc plasmid (5× NF-κB; Stratagene) using a mixture of plasmid and Lipofectamine PLUS in OPTI-MEN according to the manufacturer's specification (Invitrogen). The transfected cells were incubated with 400 μL of fresh medium for 24 h and then treated with thiacremonone 50 μg/mL and docetaxel 5 nmol/L for 6 h. Luciferase activity was measured by using the luciferase assay kit (Promega) according to the manufacturer's instructions.

Detection of Apoptosis

Colon cancer cells ($2.5 \times 10^5/cm^2$) were cultured on a chamber slide (Lab-Tak II chamber slider system, Nalge Nunc Int.), fixed in 4% paraformaldehyde, and membrane-permeabilized by exposure for 30 min to 0.1% Triton X-100 in PBS at room temperature. TUNEL assays were done by using the *in situ* Cell Death Detection Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. For DAPI staining, slides were incubated for 30 min at room temperature in the dark with mounting medium for fluorescence containing DAPI (Vector Laboratories, Inc.). The cells were then observed through a fluorescence microscope (Leica Microsystems AG). The total number of cells in a given area was determined by using DAPI nuclear staining. The apoptotic index was determined as the number of DAPI-stained TUNEL-positive stained cells divided by the total cell number counted × 100.

In vivo *Antitumor Activity Study Using a Xenograft Animal Model*

Six-week-old male BALB/c athymic nude mice were purchased from Japan SLC. Mice were housed and maintained under sterile conditions in facilities accredited by the American Association for Accreditation of Laboratory Animal Care and in accordance with the current regulations and standards prescribed by the Korea Food and Drug Administration and by Chungbuk National University. The mice were divided into four groups. Two groups of mice ($n = 30$) were orally pretreated with thiacremonone (1 mg/kg in drinking water) for 10 d. Human colon cancer cells (SW620) were injected s.c. (1×10^7 tumor cells/0.1 mL PBS/animal) with a 27-gauge needle into the right lower flanks in carrier mice. After 10 d, when the tumors had reached an average volume of 300 to 400 mm^3, the first tumor-bearing nude mice ($n = 10$) were i.p. injected with docetaxel [1 mg/kg dissolved in absolute ethanol/Tween 80/saline (5%:5%:90%, i.p.)] once a week for 4 wk with continuous treatment with thiacreonone. The second group of mice was continuously treated with thiacremonone. The third group of tumor-bearing nude mice ($n = 10$) were i.p. injected with docetaxel once a week for 4 wk. The last group of mice were treated with vehicle [ethanol/Tween 80/saline (5%:5%:90%, i.p.)] once a week, designed as the control. The weight and tumor volume of the animals were monitored twice per week. The tumor volumes were measured with vernier calipers and calculated using the following formula: $(A \times B^2) / 2$, where A is the larger and B is the smaller of the two dimensions. At the end of the experiment, the animals were sacrificed with cervical dislocation. The tumors were separated from the surrounding muscles and dermis, excised, and weighed. The tissues were investigated for the biochemical studies. For the survival test, another set of experiments with the same treatment was done. The survival rate and other toxic signs were observed once per 10 d for 110 d.

Immunohistochemistry

All specimens were fixed in formalin and paraffin-enclosed for examination. Five-micrometer-thick tissue sections were stained with H&E and immunohistochemistry was conducted. Paraffin-embedded sections were deparaffinized and rehydrated, washed in distilled water, and then subjected to heat-mediated antigen retrieval treatment. Endogenous peroxidase activity was quenched by incubation in 2% hydrogen peroxide in methanol for 15 min and then cleared in PBS for 5 min. The sections were blocked for 30 min with 3% normal horse serum diluted in PBS. The sections were then blotted and incubated with primary mouse proliferating cell nuclear antigen, Ki-67, and Bax monoclonal antibodies (1:200 dilution) at the appropriate dilution in blocking serum for 4 h at room temperature, or primary mouse anti-human p65 antibody (1:100 dilution) or primary rabbit anti-human p50 and cleaved caspase-3 polyclonal antibody (1:100 dilution) at the appropriate dilution in blocking serum overnight at 4°C. The next day, the slides were washed three times for 5 min each in PBS and incubated in biotinylated anti-mouse and rabbit antibody for 2 h. The slides were washed in PBS, followed by formation of the avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Inc.). The slides were washed and the peroxidase reaction was developed with diaminobenzidine and peroxide, then counterstained with hematoxylin, mounted in aqua-mount, and evaluated using a light microscope (×200, Olympus). A negative control was done in all cases by omitting the primary antibody. All slides were counterstained by hematoxylin. For quantification, 200 cells at three randomly selected areas were assessed and the positively proliferating cell nuclear antigen, Ki-67, p65, p50, Bax, and cleaved caspase-3–stained cells were counted and expressed as percentage of stained cells. For detection of apoptotic cell death in tumor tissues, the paraffin-embedded sections were then incubated in a mixture of labeling solution (450 μL) and enzyme solution (50 μL) for 1 h at 37°C and washed three times in 0.1 mol/L PBS for 5 min each according to the manufacturer's instructions. The sections were then incubated in DAPI for 15 min at 37°C. Finally, the sections were rinsed, mounted on slides, and coverslipped for fluorescence

microscopy (DAS microscope). Positive TUNEL stains were recorded by counting the number of positively stained DAPI cells in a defined area.

Data Analysis

Data were analyzed using GraphPad Prism 4 software (version 4.03, GraphPad Software, Inc.). Data are presented as mean ± SE. Homogeneity of variances was assessed using a Bartlett test. If variances were homogeneous, differences between groups and treatment were assessed by one-way ANOVA. If the P value in the ANOVA test was significant, the differences between a pair of means were assessed by the Dunnet's test. A value of $P < 0.05$ was considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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