

Enhanced cell growth inhibition by thiacremonone in paclitaxel-treated lung cancer cells

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Abstract Activation of nuclear factor kappa-B (NF- κ B) is implicated in drug resistant of lung cancer cells. Our previous data showed that thiacremonone inhibited activation of NF- κ B. In the present study, we investigated whether thiacremonone enhanced susceptibility of lung cancer cells to a common anti-cancer drug paclitaxel by further inhibition of NF- κ B. Thus, we used the threefold lower doses of IC₅₀ values (50 μ g/ml thiacremonone and 2.5 nM paclitaxel). We found that combination treatment with thiacremonone and paclitaxel was more susceptible (combination index; 0.40 in NCI-H460 cells and 0.46 in A549 cells) in cell growth inhibition of two types of lung cancer cell lines compared to a single agent treatment. Consistent with the combination effect on cancer cell growth inhibition, the combination treatment further induced apoptotic cell death and arrested the cancer cells in G2/M phase accompanied with a much lower expression of cdc2 and cyclin B1, and inhibited colony

formation. Much more inactivation of NF- κ B and greater expression of NF- κ B target apoptosis regulated genes such as caspase-8 and PARPs were found by the combination treatment. Molecular model and pull down assay as well as MALDI-TOF analysis demonstrated that thiacremonone directly binds to p50. These data indicated that thiacremonone leads to increased apoptotic cell death in lung cancer cell lines through greater inhibition of NF- κ B by the combination treatment with paclitaxel.

Keywords Thiacremonone · Paclitaxel · NF-kappaB · Apoptotic cell death · Lung cancer

Introduction

Nuclear factor kappaB (NF- κ B) mediates the promotion of tumor growth, angiogenesis, metastasis and chemotherapeutic resistance (Brown et al. 2008). Constitutive activation of NF- κ B has been described in a large number of tumors (Lind et al. 2001). NF- κ B is also constitutively active in lung cancer cells

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and human tumor samples (Chen et al. 2011). NF- κ B may be critical in the development of drug resistance in lung cancer cells (Kim et al. 2000). In the lungs, conditions that increase the risk of primary lung cancer development have been associated with NF- κ B activation (Christman et al. 2000; Anto et al. 2002; Fabbri et al. 1998). Some drugs against lung cancer frequently induce drug resistance (Chiou et al. 2003) through the activation of NF- κ B (Aggarwal et al. 2005). Therefore, agents that are able to inhibit NF- κ B might be considered as an adjuvant approach in combination with chemotherapeutics.

Paclitaxel (Taxol) is one of the most extensively used anti-neoplastic agents (Rowinsky et al. 1992). Unfortunately, patients treated with paclitaxel or other chemotherapeutic drugs experience significant systemic side effects (Kelly et al. 2001). Recent studies have revealed the potential of combining paclitaxel with other agents for both prevention and treatment of cancers (Zheng et al. 2006; Perez et al. 2007). Genistein enhances the antitumor effects of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) by the inactivation of NF- κ B (Gadgeel et al. 2009). Combination therapy with sesquiterpene lactone parthenolide shows remarkable cytotoxicity against human lung adenocarcinoma cells A549 through complete inhibition of NF- κ B activation (Fang et al. 2010; Hayashi et al. 2010). The combination therapy with Bortezomib and Cetuximab has been moderately effective through concomitant blockade of NF- κ B and EGFR signaling in extensively pretreated patients with non-small cell lung (Dudek et al. 2009). Our previous study showed that ginsenoside Rg3 inhibits NF- κ B, and enhances the susceptibility of prostate cancer cells to docetaxel (Kim et al. 2010). In addition, obovatol augments cell growth inhibition by docetaxel through the inactivation of NF- κ B (Lee et al. 2009). Therefore, the inhibition of NF- κ B can be a therapeutic target for the combination treatment of lung cancer.

Garlic has been known to inhibit the growth of several human cancer cells (Hong et al. 2000). In addition, several research groups have demonstrated the importance of sulfur compounds in the preventative effects against lung cancer development (Milner 2006). In a previous study, we showed that thiacremonone induced growth inhibition of human colon cancer cells through the inactivation of NF- κ B (Ban et al. 2009). Thus, in the present study, we investigated whether thiacremonone enhances the growth inhibitory effect of paclitaxel in lung cancer cells through the inactivation of NF- κ B via direct binding to p50.

Materials and methods

Materials

Characterization of a novel sulfur compound isolated from garlic (named thiacremonone) was described elsewhere

(Ban et al. 2007). The structure is shown in Fig. 6. Thiacremonone was resolved in 0.01 % dimethyl sulfoxide (DMSO), and administered in a dose of 50 μ g/ml. Paclitaxel was obtained from Sigma Chem. Co. (St. Louis, MO, USA). Paclitaxel was dissolved in 0.01 % DMSO for treatment in vitro.

Cell culture

NCI-H460 and A549 human lung cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). RPMI1640, penicillin, streptomycin, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). NCI-H460 and A549 human lung cancer cells were grown in RPMI 1640 with 10 % fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in 5 % CO₂ humidified air.

Cell viability assay

To determine the cell number, cultured cells were trypsinized with TrypLE Express (Invitrogen), and then the cells were pelleted and resuspended in phosphate-buffered saline (PBS), and 0.2 % trypan blue was added to the cancer cell suspension.

Soft agar formation assay

Cells (8×10^3 per well) were suspended in BME (1 ml with 10 % FBS and 0.33 % agar) and plated over a layer of solidified BME/10 % FBS/0.5 % agar (3.5 ml) with thiacremonone and/or paclitaxel. The cultures were maintained at 37 °C in a 5 % CO₂ incubator for 10 days, and the colonies were counted under a microscope.

Western blot analysis

Western blot analysis was done as described previously (Ban et al. 2007).

Electromobility shift assay

Electromobility shift assay was done as described previously (Ban et al. 2007). The relative density of the protein bands was scanned by densitometry using MyImage, and quantified by Labworks 4.0 software (UVP Inc. Upland, CA, USA).

Detection of apoptosis

Lung cancer cells (2.5×10^5 cells/cm²) were cultured on a chamber slide (Lab-Tak II chamber slider system, NalgeNunc Int., Naperville, IL, USA), fixed in 4 % paraformaldehyde,

membrane-permeabilized by exposure for 30 min to 0.1 % Triton X-100 in phosphate-buffered saline at room temperature. TdT-mediated dUTP nick and labeling (TUNEL) assays were performed by using the in situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions.

Cell cycle analysis by flow cytometry

Subconfluent cells were treated with thiacremonone and/or paclitaxel in a culture medium for 48 h. Flow cytometric analysis was done as previously described (Ban et al. 2007). The cell cycle distribution was determined using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA).

Pull-down assays

Thiacremonone-bead conjugation was prepared as described previously (Shim et al. 2008). Briefly, p50 proteins (Millipore, Billerica, MA, USA) or cell lysate from NCI-H460 lung cancer cells were incubated with thiacremonone-Sepharose 4B beads in a reaction buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01 % Nonidet P-40, 2 µg/ml bovine serum albumin, 0.02 mM phenylmethylsulfonyl fluoride, 1 × proteinase inhibitor). The beads were washed five times with buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01 % Nonidet P-40, 0.02 mM phenylmethylsulfonyl fluoride), and proteins bound to the beads were analyzed by immunoblotting with the p50 antibodies.

Molecular modeling

Docking studies between thiacremonone and p50 subunit of NF-κB (Trott and Olson 2010) and AutodockTools. p50 subunit of NF-κB was obtained in the X-ray crystal structure of NF-κB (p65 and p50) complexed with its cognizant κB DNA element (PDB ID: 1KVX) (Chen et al. 1998). Thiacremonone in nature lacks optical activity and exists as a racemic mixture of two enantiomeric stereoisomers as it is formed by the cyclization of its natural precursor 4-hydroxy-5-mercapto-hex-4-en-2,3-dione (Fig. 5a). Therefore, we built thiacremonone in both enantiomeric forms and each was separately docked with p50 subunit of NF-κB. All rotatable bonds on the thiacremonone were allowed to rotate during the molecular simulation. The grid box was centered on the p50 subunit and the size of the grid box was adjusted to include the whole p50 subunit. Docking experiments were performed at various exhaustiveness values of the default, 25, 50, and 100. For each docking experiment, the nine best binding

modes were identified among which six of them recognized the same binding pocket with a slightly different orientation of the thiacremonone ring. The same site was unanimously recognized as the best binding site throughout all exhaustiveness values and for both stereoisomers.

HPLC purification and MALDI-TOF analysis of p50

The purified p50 proteins incubated in the absence or presence of thiacremonone was injected into a reverse-phase HPLC column (Beckman Ultrasphere, 5 µm particle size, 4.6 mm × 25 cm), equilibrated with solvent A (0.1 % trifluoroacetic acid), and eluted with a gradient of 0–100 % eluant B (100 % acetonitrile in solvent A). Fractions containing p50 were pooled and concentrated by evaporation. 10 µl of the fractions to be analyzed were applied onto target and dried out along with 10 µl of thiacremonone (1 mg/ml) matrix in distilled water. Mass spectrometry analysis by MALDI-TOF was performed using a Voyager-DE STR instrument (Applied Biosystems, Foster City, CA, USA), operating in a linear mode. Calibration was performed externally using bovine serum albumin and control p50 as standards.

Calculation of combination index

The median-effect analysis of Chou and Talalay (1984) was used to determine synergism, additivity or antagonism effects for combination of thiacremonone and paclitaxel. Median-effect plots were obtained by plotting $\log(fa/fu)$ against $\log(D)$, where D represents the concentration of each single compound alone, while fa and fu stand for the affected (values between 0 and 1) and unaffected ($1 - fa$) fraction, respectively, at each concentration D . From the median effect curves, the x -intercept ($\log IC_{50}$) and slope m were calculated for each agent and for each combination. These parameters were then used to calculate doses of the drugs either alone or in combination required to produce varying levels of cell growth inhibition according to the equation $Df = DIC_{50} (fa/(1 - fa))^{1/m}$. For each of the drug combinations tested, the combination indices (CI) were calculated according to the equation $CI = (D)Thia / (Dx) Thia + (D)Taxol / (Dx) Taxol$, where $(Dx) Thia$ was the dose of thiacremonone required to produce x percent effect alone, and $(D) Thia$ was the dose of thiacremonone required to produce the same x percent effect in combination with $(D)Taxol$. Similarly, $(Dx)Taxol$ was the dose of Paclitaxel required to produce x percent effect alone, and $(D)Taxol$ was the dose required to produce the same effect in combination with $(D)Thia$. A CI value < 1 or > 1 indicates synergy or antagonism, respectively. An additive effect is proposed when a CI value is close to 1 (i.e., between 0.9 and 1.1).

Data analysis

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

Results

Effect of the combination of thiacremonone and paclitaxel on cell growth in NCI-H460 and A549 human lung cancer cells

Our study showed that thiacremonone (0–200 $\mu\text{g}/\text{ml}$) dose-dependently inhibited cancer cell growth and NF- κB

activity, with IC_{50} values of 127 $\mu\text{g}/\text{ml}$ in NCI-H460 and 161 $\mu\text{g}/\text{ml}$ in A549 lung cancer cells (data not shown). Thus, we used the threefold lower doses of IC_{50} values (50 $\mu\text{g}/\text{ml}$ thiacremonone and 2.5 nM paclitaxel) in testing the combination effect of these agents. Treatment of NCI-H460 and A549 lung cancer cells with 50 $\mu\text{g}/\text{ml}$ thiacremonone and 2.5 nM paclitaxel alone for 48 h showed 5–30 % inhibition of cell growth (Fig. 1). However, the addition of both agents together resulted in a strong synergistic inhibitory effect on cell growth (60–80 % inhibition) in both cells (Fig. 1). We analyzed thiacremonone and paclitaxel combination effects using the median effect method of Chou and Talalay (28). The combination index (CI) for the thiacremonone–paclitaxel combination was 0.40 in NCI-H460 and 0.46 in A549 cells, respectively. We also found that the combination treatment of thiacremonone (50 $\mu\text{g}/\text{ml}$) with docetaxel (2.5 nM) or doxorubicin (2.5 μM) was more effective in growth inhibition of NCI-H460 cells (combination index; 0.54 and 0.63, respectively) than those by thiacremonone or paclitaxel alone (Supplementary Fig. S1). The

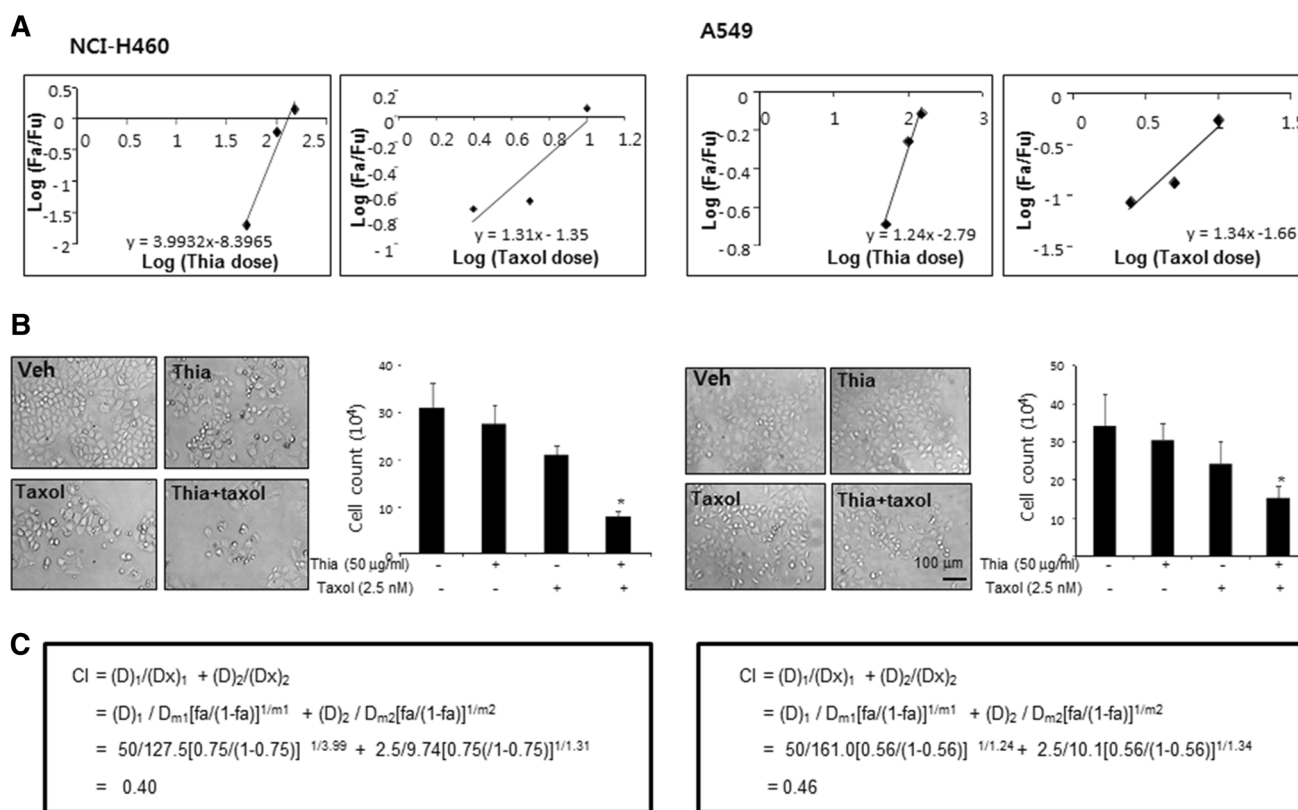


Fig. 1 Median-effect analysis of thiacremonone and paclitaxel in combination on NCI-H460 and A549 human lung cancer cell viability. **a** The median-effect plots of thiacremonone and paclitaxel are represented here. **b** Cell viability was determined after 48 h culture by cell counting using 0.2 % trypan blue as described in “Materials and methods” section, and the results were expressed as percentage of dead cells. Values are each the mean \pm SD of three experiments, each performed in triplicate. Bar indicates 100 μm . * $P < 0.05$ indicates statistically significant differences from the single treated group. **c** Quantification of synergism for thiacremonone and paclitaxel was calculated described in “Materials and methods” section. The type of interaction that occurred at each particular drug combination was determined by the calculation of combination indices (CI)

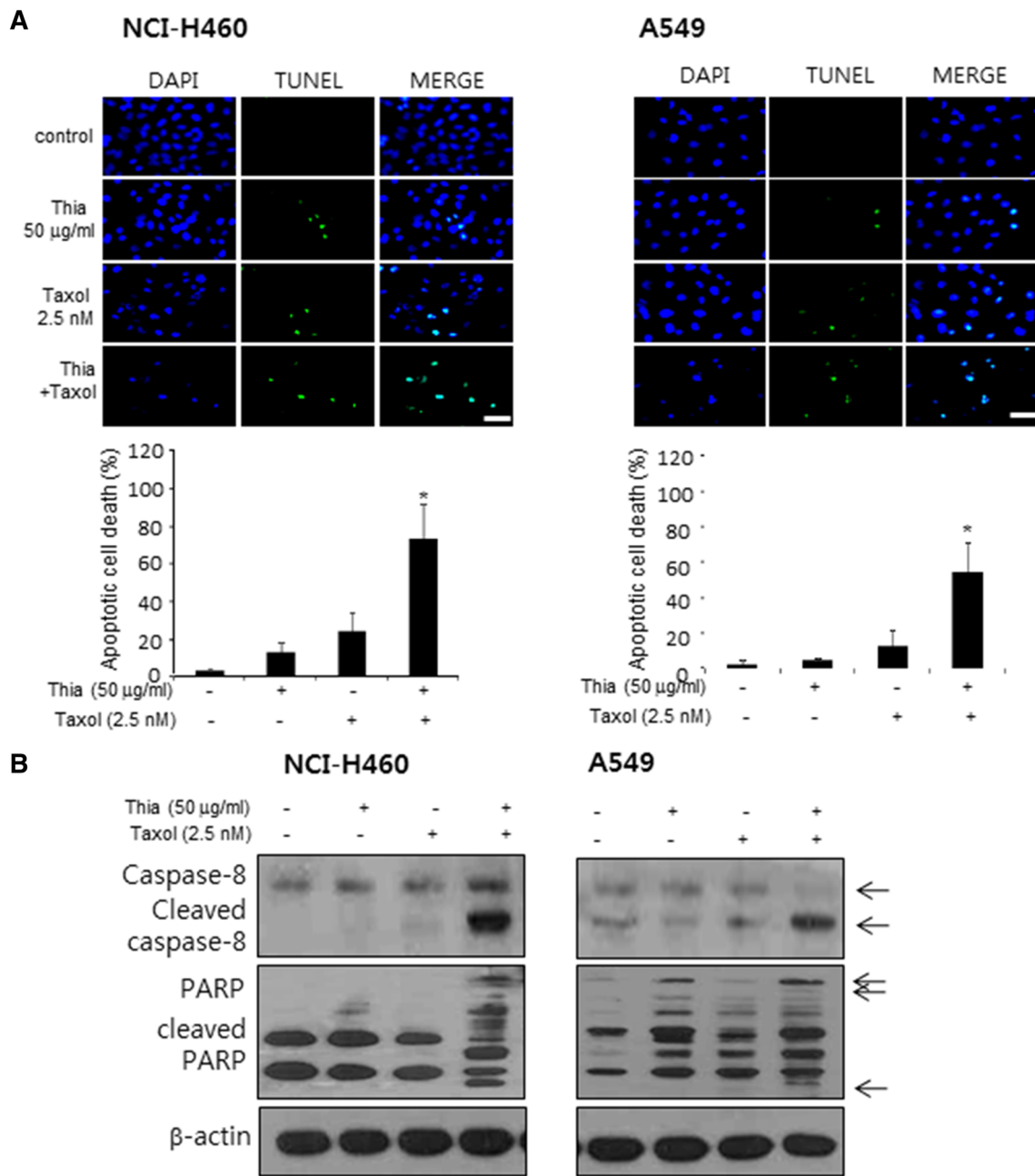


Fig. 2 Apoptotic cell death of NCI-H460 and A549 lung cancer cells by the combination treatment of thiacremonone and paclitaxel. NCI-H460 and A549 lung cancer cells were co-treated with 50 µg/ml thiacremonone and 2.5 nM paclitaxel for 48 h. **a** Apoptotic cells were examined by fluorescence microscopy after TUNEL staining (fluorescent microscopy) (*middle panels*). Total number of cells in a given area was determined by using DAPI nuclear staining (*left panels*). The apoptotic index was determined as the DAPI-stained TUNEL-positive cell number counted. *Values* are mean ± SD of three experiments, with triplicate of each experiment. *P < 0.05 indicates statistically significant differences from untreated group. *Bar* indicates 50 µm. **b** The cells were co-treated with 50 µg/ml thiacremonone and 5 nM paclitaxel for 48 h. Equal amounts of total proteins (50 µg/lane) were subjected to 12 % SDS-PAGE. Expression of caspase-8, PARP and β-actin was detected by Western blotting using specific antibodies. β-actin protein here was used as an internal control. *Each band* is representative for three independent experiments

combination treatment of thiacremonone (50 µg/ml) with cisplatin (5 nM) or doxorubicin (10 µM) was also more effective in the growth inhibition of A549 cells

(combination index; 0.60 and 0.17, respectively) than those by thiacremonone or paclitaxel alone (Supplementary Fig. S1).

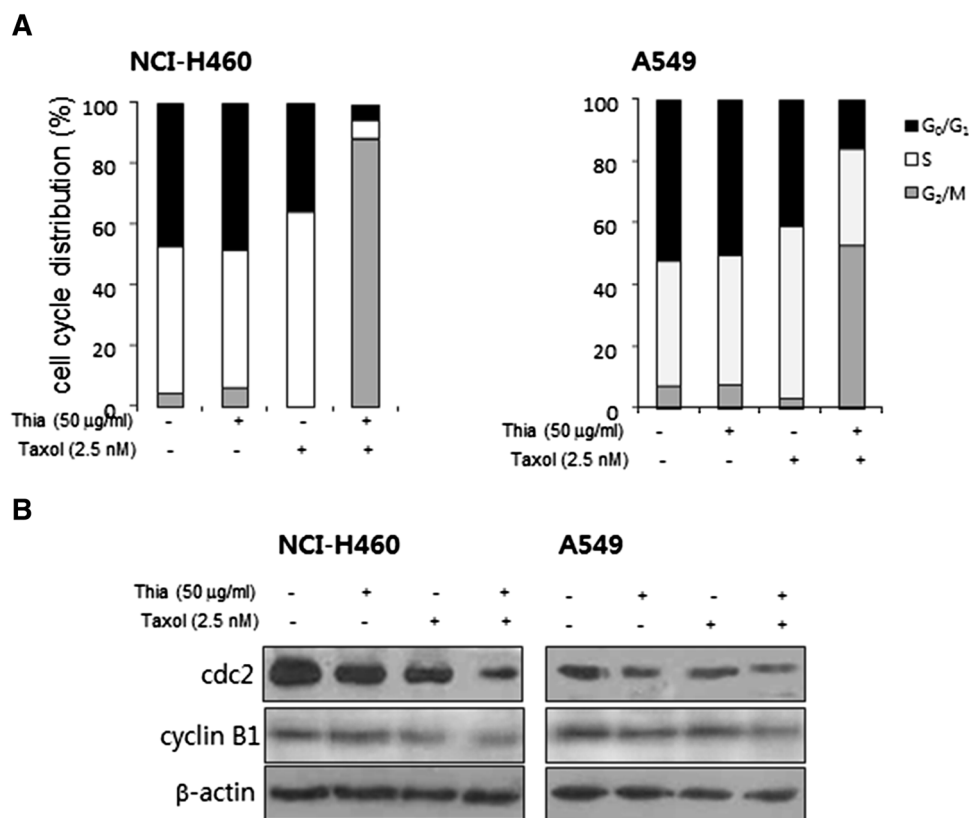


Fig. 3 Effect of the combination treatment of thiacecremonone and paclitaxel on cell cycle and the expression of cell cycle regulatory proteins in NCI-H460 and A549 lung cancer cells. Proportion of cell cycle phase was analyzed by flow cytometry. NCI-H460 and A549 lung cancer cells were treated with thiacecremonone, paclitaxel or combination for 48 h. **a** NCI-H460 and A549 lung cancer cells were co-treated with 50 µg/ml thiacecremonone and 2.5 nM paclitaxel for 48 h, and then analyzed cell cycle by flow cytometry. **b** The cells were co-treated with 50 µg/ml thiacecremonone and 5 nM paclitaxel for 48 h. Equal amounts of total proteins (50 µg/lane) were subjected to 12 % SDS-PAGE. Expression of cdc2 and cyclin B1 was detected by Western blotting using specific antibodies. β-actin protein here was used as an internal control. Each band is representative for three independent experiments

Effect of the combination of thiacecremonone and paclitaxel on apoptotic cell death in NCI-H460 and A549 human lung cancer cells

In agreement with the cell growth inhibitory effects, the combination of thiacecremonone and paclitaxel treatment significantly increased apoptotic cell numbers (DAPI-stained TUNEL positive cells) of both lung cancer cells compared to a single agent treatment (Fig. 2a). A significant increase in the expression levels of pro-apoptotic proteins, cleaved caspase-8 and cleaved PARP after the combination treatment was found in comparison to the single treatment (Fig. 2b). These results suggest that the efficiency in the inhibition of cancer cell growth by the combined treatment of thiacecremonone and paclitaxel may be a result of the combination treatment on the induction of apoptosis of cancer cells.

Effect of the combination of thiacecremonone and paclitaxel on cell cycle arrest and the levels of G₂/M regulatory proteins in NCI-H460 and A549 human lung cancer cells

As shown in Fig. 3, compared with a single treatment of thiacecremonone or paclitaxel, the combination treatment resulted in a significantly higher number of cells in the G₂/M phase (Fig. 3a). However, the number of cells at the G₀/G₁ and S phase decreased. In order to investigate the role of cyclin/Cdk complexes in the synergic anti-proliferative activity of combined therapy with thiacecremonone and paclitaxel, the expression of G₂/M regulators; cyclin B1 and cdc2 was analyzed in lung cancer cells. The treatment with thiacecremonone and/or paclitaxel resulted in a significant reduction in cyclin B1 and cdc2 protein expression in both cell lines (Fig. 3b).

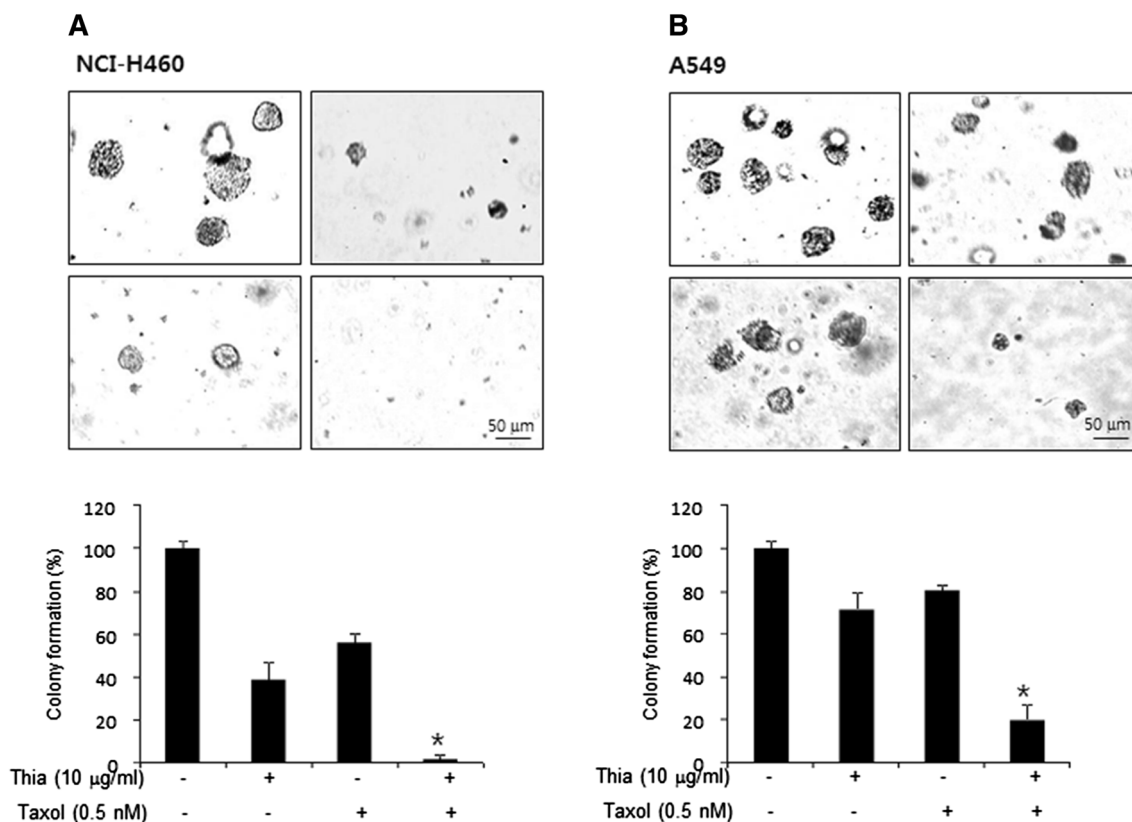


Fig. 4 Effect of a combination of thiacremonone with paclitaxel on colony formation of NCI-H460 and A549 lung cancer cells. The colonies from two cell lines, NCI-H460 and A549, treated with thiacremonone and paclitaxel as either single agents or a combination were examined under microscope as described in “Materials and methods” section. Values are each the mean \pm SD of three experiments, each performed in triplicate. * $P < 0.05$ indicates statistically significant differences from the single treated group

Effect of the combination of thiacremonone and paclitaxel on colony formation

We next determined the colony formation of NCI-H460 and A549 cells in soft agar by the combination treatment of the cells with thiacremonone and paclitaxel. The combination treatment resulted in a strong synergistic inhibitory effect on colony formation (80–90 % inhibition) in both cells (Fig. 4).

Effect of the combination of thiacremonone and paclitaxel on NF- κ B activation in NCI-H460 and A549 human lung cancer cells

Constitutional activation of the DNA binding activity of NF- κ B was observed in lung cancer cells, and treatment with paclitaxel (2.5 nM) or thiacremonone alone (50 µg/ml) slightly reduced the activated DNA binding activity of NF- κ B in NCI-H460 and A549 lung cancer cells. However, the combination of thiacremonone and paclitaxel resulted in a synergistic and significant inhibition of the translocation of p50 and p65 into the nucleus (Fig. 5a) in both cell

lines. In addition, the combination of 50 µg/ml thiacremonone and 2.5 nM paclitaxel resulted in a synergistic inhibitory effect on the DNA binding activity of NF- κ B (Fig. 5b).

Binding between thiacremonone and p50

We anticipated in the molecular docking experiment that only one of the two stereoisomers of thiacremonone would bind to p50 subunit of NF- κ B or at least one would have a much higher binding affinity than the other (Fig. 6a). However, both stereoisomers were found, to our surprise, to bind at the identical binding site with very similar conformation and orientation of the five membered thiacremonone ring frame (Fig. 6b). Both exhibited a binding affinity of -5.8 kcal/mol. The binding pocket for thiacremonone is formed by two beta-sheet peptide segments (Arg356, and Val412, Leu437–Leu440) and a loop (Gly361–Gly365) in close proximity to the DNA binding site of p50 (Fig. 6c). Essentially identical peptides interact with thiacremonone in both stereoisomers. (R) and (S) thiacremonones are shown to form two and three hydrogen bonds with the surrounding

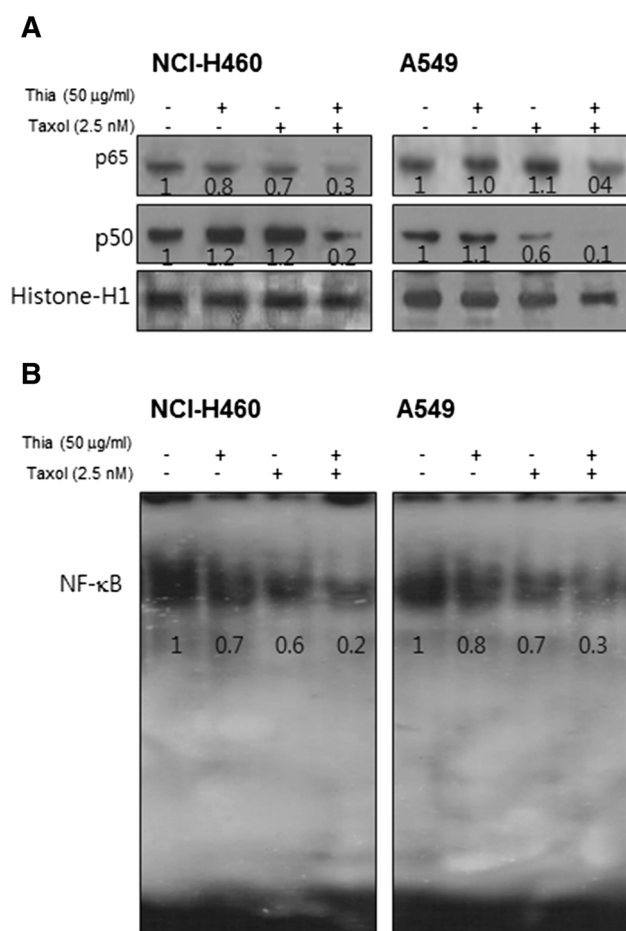


Fig. 5 Effect of the combination treatment of thiacremonone and paclitaxel on NF-κB activation in NCI-H460 and A549 lung cancer cells. **a** and **b** NCI-H460 and A549 lung cancer cells were co-treated with 50 µg/ml thiacremonone and 2.5 nM paclitaxel for 30 h. The activation of NF-κB was investigated using Western blot and EMSA as described in “Materials and methods” section. **a** Equal amounts of nuclear proteins (50 µg/lane) were subjected to 12 % SDS-PAGE. Expression of p50, p65 and Histone H1 was detected by western blotting using specific antibodies. Histone H1 protein here was used as an internal control. Each band is representative for three independent experiments. **b** Nuclear extract was incubated in binding reactions of ³²P-end-labeled oligonucleotide containing the κB sequence. Quantification of band intensities from three independent experimental results performed by densitometry (Imaging System) and the value under each band indicated as fold difference from the untreated control group

amino acids, respectively. Among these, one hydrogen bond interaction (between C1=O and NH of Val412) is conserved in both models. The MALDI-TOF spectrum of vehicle- and thiacremonone-incubated p50 showed a peak of $m/z = 49.327$ and 49.586 , respectively (Fig. 6d). The interaction was also assessed in a pull-down assay using thiacremonone-Sepharose 4B beads, and p50 (Millipore, Billerica, MA, USA) was then detected by immunoblotting with anti-p50. Cell lysates containing p50 from human NCI-H460 lung cancer cells (Fig. 6e).

Discussion

Although early-stage lung cancer is highly treatable, no effective treatment is available for late-stage lung cancer that follows surgery, radiation, and chemotherapy (Goffin et al. 2010). Parthenolide dramatically lowered the effective dose of paclitaxel needed to induce cytotoxicity (Gao et al. 2010). Vorinostat enhances the efficacy of carboplatin and paclitaxel in patients with advanced NSCLC (Ramalingam et al. 2010). In addition, carboplatin/paclitaxel chemotherapy with bevacizumab is an accepted standard treatment for advanced nonsquamous NSCLC (Reynolds et al. 2009). The addition of bevacizumab to paclitaxel plus carboplatin in the treatment of selected patients with NSCLC has a significant survival benefit (Sandler et al. 2006). The novel systemic therapy regimen is feasible in patients with advanced NSCLC (Cohen et al. 2012). The central finding of the present study is that thiacremonone strongly enhanced the therapeutic efficiency of paclitaxel by growth inhibition and induction of apoptosis through the inactivation of NF-κB via binding to p50 subunit in lung cancer cells. These combination effects of thiacremonone with other chemotherapeutics on cancer cell growth inhibition and NF-κB activity was also found. These data suggest that inactivation of NF-κB by thiacremonone may contribute to increase in cancer cell susceptibility against conventional chemotherapeutics.

It is known that many chemotherapeutic agents induce activity of NF-κB, causing drug resistance in cancer cells (Kim et al. 2000). Thus, we speculated that inhibition of NF-κB by thiacremonone is a profound contributor to the increase of susceptibility of lung cancer cells against chemotherapeutics. It is clearly demonstrated in the present study that the combination of thiacremonone and paclitaxel or other chemotherapeutics block the constitutive activation of NF-κB even though lower doses of thiacremonone and paclitaxel (threefold lower dose of its IC₅₀ values on cell growth inhibition and NF-κB activity) which only slightly decreased NF-κB activity or no effect on it. Supporting our hypothesis, several combinations of therapeutic treatments have shown to enhance cancer cell susceptibility through coordinating the inhibition of NF-κB (Ali et al. 2008; Lee et al. 2008). More proper inactivation of NF-κB by curcumin and chemotherapeutic enhance cancer cell killing effects (Aggarwal et al. 2005). Sulindac enhances arsenic trioxide-mediated apoptosis in 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, while the levels NF-κB were not altered significantly by As₂O₃ or sulindac treatment alone (Lee et al. 2008). A significant reduction in cell viability, induction of apoptosis, NF-κB activity, and expression of antiapoptotic genes was reported in

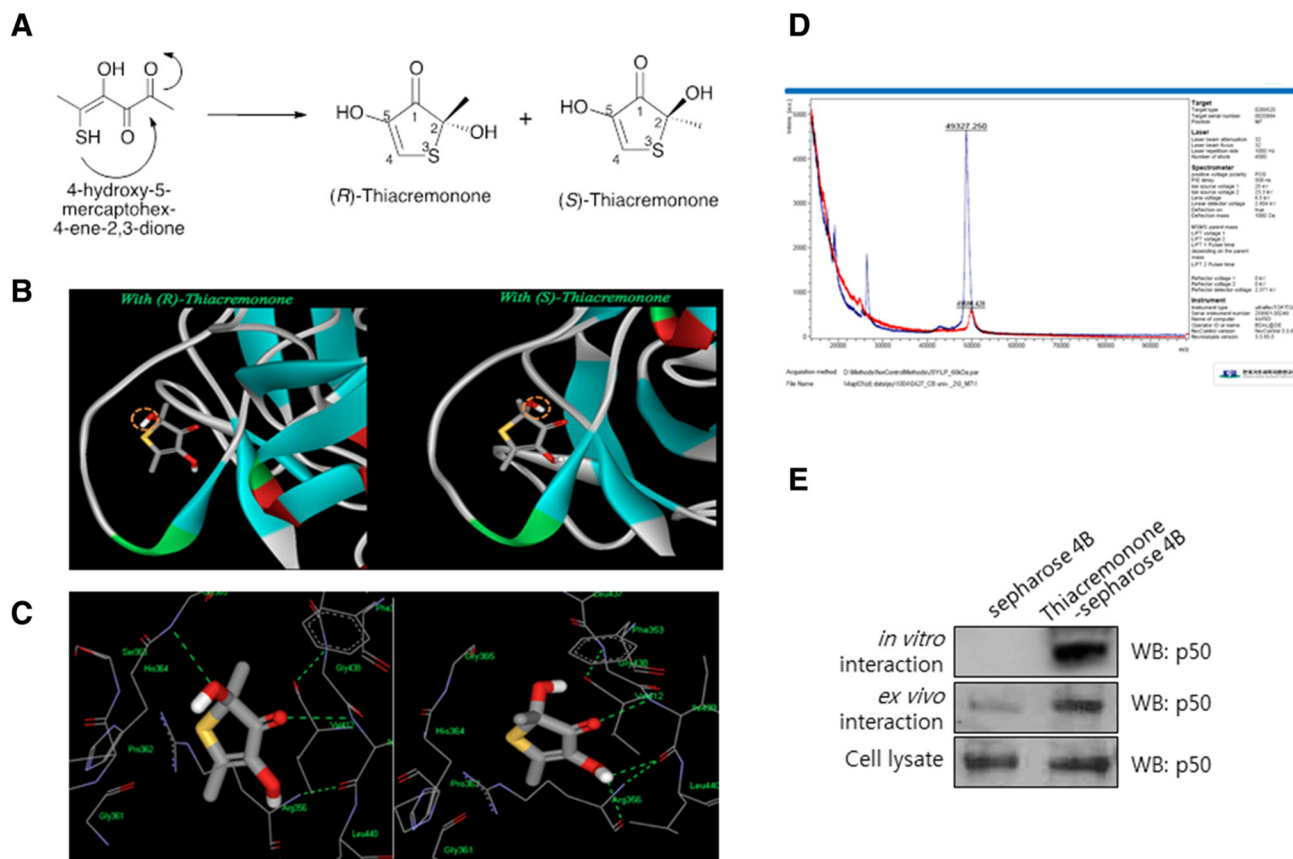


Fig. 6 Modeling study of the binding of thiacremonone binding to p50 protein. **a** Formation of two thiacremonone stereoisomers in nature. **b** Binding models between two thiacremonone stereoisomers and p50 subunit of NF- κ B. **c** Detailed interaction of two thiacremonone stereoisomers (represented by stick model) with surrounding amino acids of p50. **d** the recombinant wild type p50 proteins purified were treated with vehicle (control) or thiacremonone (50 μ g/ml) for 30 min at 37 $^{\circ}$ C and subsequently purified by reverse-phase HPLC. The fractions of the p50 peak were collected and analyzed by MALDI-TOF mass spectrometry. **e** Thiacremonone binds to p50. p50 was incubated with thiacremonone-agarose 4B beads in reaction buffer overnight at 4 $^{\circ}$ C. After washing, the proteins bound with thiacremonone-agarose 4B beads were resolved by SDS-PAGE and analyzed by Western blot using a p50 antibody (*top*) and quantified (*bottom*) using the Image software program (National Institutes of Health, Bethesda, USA)

pancreatic cancer PC-3 cells when treated with a combination of erlotinib and B-DIM compared to either agent alone (Ali et al. 2008). The combination of all-trans retinoic acid and paclitaxel synergistically induced apoptosis and inhibited NF- κ B activity in human glioblastoma U87MG xenografts in nude mice (Karmakar et al. 2008). Taken together, the blocking ability of thiacremonone on NF- κ B activity could be significant for combination treatment with chemotherapeutics including paclitaxel in lung cancer cell growth inhibition.

Down stream target gene expression by NF- κ B is implicated in the sensitization of cancer cells to chemotherapeutic agents. Apoptosis is an important mechanism to eliminate unwanted cells, and deregulation of this process is implicated in pathogenesis in the development of cancer (Thompson 1995). A group of intracellular proteases called caspases are responsible for the deliberate disassembly of the cell into apoptotic bodies during apoptosis (Thornberry

and Lazebnik 1998). It was found that consistent with the increase of apoptosis, the expression of apoptotic proteins active caspase 8 and PARP was dose dependently increased *in vitro*. Caspase-8 activation is known to play an important role in regulation of chemotherapy-induced apoptosis (Ferreira et al. 2000). Several proteins such as cFLIP expressed by the action of NF- κ B act to prevent the activation of caspase-8 (Scaffidi et al. 1999). In addition, caspase-8 not only triggers and amplifies the apoptotic process at cytoplasmic sites but can also act as an executioner at nuclear levels. Caspase-8 can cleave PARP to the 85-kDa fragment (Medema et al. 1998; Muzio et al. 1996). Several compounds contribute to caspase-8 dependent apoptotic pathway in cancer cells (Kondoh et al. 2004; Ying et al. 2012). Fisetin induces apoptosis in human cervical cancer HeLa cells through ERK1/2-mediated activation of caspase-8-dependent pathway (Ying et al. 2012). Kaurene diterpene induces apoptosis in human leukemia cells through a

caspase-8-dependent pathway (Kondoh et al. 2004). More recently, the mechanism of activation of caspase-8 in chemoresistant cells has been investigated in detail. Curcumin enhances Apo2L/TRAIL-induced apoptosis by activation and cleavage of procaspase-8 and -9 in chemoresistant ovarian cancer cells (Wahl et al. 2007). In addition, cisplatin enhances rhTRAIL-induced apoptosis in cisplatin-resistant ovarian cancer cells, and induction of caspase 8 protein expression is the key factor of rhTRAIL sensitization (Duiker et al. 2011). Thus, the activation of caspase-8 by the combination of thiacecronone with paclitaxel could be significant in the increase of susceptibility of cancer cells.

Transcriptional regulation of cyclin as a cell cycle check point to control cell growth and differentiation was reported to be conducted via NF- κ B (Guttridge et al. 1999). Cyclin B1 is the regulatory subunit of cyclin-dependent kinase 1 (Cdk1) and key molecules for G₂-M transition during the cell cycle. In general, taxol is a microtubule stabilizing agent that arrests cells in the G₂ and M phases of the cell cycle in variety of cancer cells (Li et al. 1999). Paclitaxel induces accumulation of G₂-M by reduction of the expression of cyclin B1 and cdc2 in human osteosarcoma cell line (Li et al. 1999). Several taxol-based combination chemotherapy for malignant have synergistic effects on suppression of cancer cell growth through G₂/M phase arrest. Targeting cyclin B1 sensitizes human breast cancer cells to taxol, suggesting that specific cyclin B1 targeting is an attractive strategy for the combination with conventionally used agents in gynecological cancer therapy (Androic et al. 2008). GL331 (a new potent topoisomerase II (Topo II) poison)-induced perturbation of cell cycle progression dramatically over-rode the patterns of mitotic arrest induced by paclitaxel, and the mechanism could be the inhibition of cyclin B1/CDC2 kinase and MAD2 check protein activities (Huang et al. 2001). In addition, Yuan and his colleagues reported that cyclin B1 is an essential molecule for tumor cell survival and aggressive proliferation, suggesting that the downregulation of cyclin B1, might become an interesting strategy for antitumor intervention, especially in combination therapy. Similar to these reports, our results showed that about 60–80 % of the cells treated with paclitaxel and thiacecronone arrested in the G₂/M phase with down expression of cyclin B1 and cdc2. These data suggest that G₂/M phase arrest could be significant in the thiacecronone and paclitaxel combination therapy.

In the present study, docking modeling, pull-down assay and MALDI-TOF of analysis showed that thiacecronone might directly bind to p50, and then block the activity of NF- κ B. p50 is a potential therapeutic target for clinical consideration in cancer patients in which these pathways are involved. Many research groups have reported that

target protein such as p50 has been known to form to hydrogen bonds with a hydroxyl group of small molecule causing inhibition of its activity (Lee et al. 2002; Shim et al. 2008). By the pull-down assay using thiacecronone-agarose bead, we also found that a sulfur group of thiacecronone bind to a hydroxyl group of tyrosine residue of p50. Very similar to our findings, Chen et al., demonstrated that benzyl selenocyanate (BSC) and 1,4-phenylenebis (methylene) selenocyanate (p-XSC) may exert their chemopreventive activity by inhibiting NF- κ B through covalent modification of p50 subunit of NF- κ B (Chen et al. 2007). Cernuda-Morollón and colleagues proved that that 15d-PGJ2 covalently modifies p50 using MALDI-TOF spectrum (Cernuda-Morollon et al. 2001). Therefore, thiacecronone interferes with p50, thus increase susceptibility of cancer cells against chemotherapeutics. Therefore, thiacecronone could be potentially useful in combination with other chemotherapeutic agents for the treatment of different types of cancers.

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