Among lung neoplasms, small cell lung cancer (SCLC) has a high rate of metastasis and accounts for 12 to 16% (Jackman and Johnson, 2005). Combination therapy using cisplatin and etoposide have prolonged the survival of patients (Rosti et al., 2006). In addition, topoisomerase I inhibitors (irinotecan) Noda et al., 2002; Hanna et al., 2006) and topotecan (Ardizzoni et al., 1997) and amrubicin (AMR) (Ohe et al., 2005) are in regular clinical practice. An 87% response rate and 12.8-month median survival time (MST) has been achieved in extensive-disease SCLC using combination of irinotecan and cisplatin (Noda et al., 2002). Although first-line CDDP-based chemotherapies successfully achieved high response rates but there is disease progression after few years. Thus, potent alternative agents and new integrated therapeutics are needed.

There are reports that demonstrate oral fluoropyrimidines as promising candidate in combination chemotherapy (Ichinose et al., 2004) or monotherapy (Kato et al., 2004; Nakagawa et al., 2005; Kawahara et al., 2011) against advanced non-small cell lung cancer (NSCLC). Complete remission of SCLC using a combination treatment of topotecan and 5-FU in a Phase I clinical trial (Sbar et al., 2002) and a 77% initial response rate with combination regimen of 5-FU and cisplatin (Morere et al., 1994) was achieved. The synergism of 5-FU (in vitro) or UACT (in an animal model) and vinorelbine against NSCLC (Matsumoto et al., 2004) was attributed to vinorelbine-induced suppression of thymidylate synthase protein (Johnston et al., 1995).
Ursolic acid, a pentacyclitriterpene present abundantly in the peels of Maluspumila Mill. (Ma et al., 2005) exhibits a wide range of pharmacological properties, including anti-inflammatory, anti-allergic, antibacterial, antiviral, antitumor and cytotoxic activity being the most intriguing (Li et al., 2005; Kim et al., 2002; Harmand et al., 2005; Choi et al., 2000; Kanjoormana et al., 2010). It was ranked as one of the most promising tumor preventive medication by Japanese researchers (Muto et al., 1990). Ursolic acid arrests the cell cycle progression in the G1 phase and induces apoptosis (Hsu et al., 2004). However the poor bioavailability under in vitro hinders its clinical applications (Saraswat et al., 2000). It has been reported that keeping polar substituents at the C-28 position of ursolic acid enhanced its antitumor potential against BGC-823 cell lines (Bai et al., 2011). In the present study the effect of sequential treatment of UACT (Figure 1) followed by 5-FU on small cell lung cancer cells was investigated.

**Figure 1:** Structure of ursolic acid chlorophenyltriazole (UACT)

### Materials and Methods

**Chemicals and reagents:** 5-FU (Zhejiang Weishi Biotechnology Co., Ltd. China), UACT (Sigma-Aldrich, St. Louis, MO, USA), were dissolved in dimethyl sulfoxide and stored at -20°C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chemical Industries) was dissolved in phosphate-buffered saline (PBS) and stored at -20°C.

**Cell lines and cultures:** Human SCLC cell lines H69,H209, Lu135 and Lu139, and 87-5 were purchased from American Type Culture Collections. All of these SCLC cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin (50 units/mL), streptomycin (500 μg/mL), and 4 mmol/L glutamine in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

**Drug combination studies:** Drug combination studies were based on concentration effect curves generated as plot of the fraction of unaffected (surviving) cells versus drug concentration after 72 or 96 hours (in the case of sequential experiments) of treatment. To explore the relative contribution of each agent to the synergism, serial dilutions of doses of the two agents (5-FU and UACT) in combination were tested with different molar ratios: equi-active doses (50:50 cytotoxic ratio) of the two agents (IC₅₀), higher relative doses of UACT/5-FU (25:75 cytotoxic ratio; IC₅₀ of UACT:IC₅₀ of 5-FU), and higher relative doses of 5-FU/ UACT (IC₅₀ of 5-FU/IC₅₀ of UACT). For sequential combinations, serial dilutions of equi-active doses were tested in three different sequences of treatment: simultaneous (both drugs were given together 24 hours after seeding and cell growth assessment was done after 96 hours) or when each one was applied 24 hours before the other (UACT or 5-FU was added 24 hours after seeding followed by the indicated combined drug after 24 hours, and cell growth assessment was done after 72 hours, so that cells were exposed for 96 hours to the first applied drug and for 72 hours to the following combined agent. The combination index (CI) was calculated using Chou-Talalay equation using CalcuSyn (Biosoft) software. The CI<0.8, CI=0.8-1, and CI>1 indicate synergistic, additive, and antagonistic effects, respectively. The reduction in dose of each drug in synergistic combination to produce a given effect is represented by dose reduction index (DRI).

**MTT assay:** To 96-well flat bottom multiplates (BD Falcon, Franklin, NJ) containing 1 x 10⁵ cells per well various concentrations of the 5-FU and UACT were added together in simultaneous combinations of drugs. The plates were then incubated for 72 hours. For sequential combinations, the plates containing 1 x 10⁵ cells per well in 6-well flat bottom multiplates (Sumitomo Bakelite Co., Tokyo, Japan) were treated with different concentrations of 5-FU or UACT. The cells after harvesting were washed with PBS and then incubated in 100 μL medium containing various concentrations of 5-FU or UACT. After incubation for the indicated duration, 10 μL solution of MTT was added to each well and the plates were again incubated for 4 hours at 37°C in 5% CO₂ incubator. 100 μL of 0.04 N HCl in 2-propanol was added to dissolve formazan crystals and absorption was measured by a microplate reader (MPR-A4i; Tosoh Corporation, Tokyo, Japan) at 570 nm (reference filter 650 nm). All the measurements were carried out in triplicate.

**Cell lysis and Western blot analysis:** The cells were washed twice in PBS. Then, a modified radioimmune precipitation lysis buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethyl sulfonyl fluoride, 10 mM NaF, 40 mM β-glycerophosphate and 2 mM Na₃VO₄) 2 mL was added to the cells. The lysate was centrifuged to remove the insoluble material. The Bio-Rad Protein assay (Bio-Rad, CA) was used to determine the concentration of protein. The lysates containing 30 μg of total cellular protein were loaded and resolved by electrophoresis on a 10% polyacrylamide gel. The proteins were analyzed by Western blotting and visualized by enhanced chemiluminescence detection (Amersham Pharmacia Biotech) using goat anti-rabbit IgGs coupled to hors eradish peroxidase as a secondary antibody (Amersham Phar-
macia Biotech). An anti-ß-actin antibody (Sigma, Tokyo, Japan) was used as a loading control.

**Analysis of cell cycle kinetics:** For analysis of cell cycle kinetics cells were harvested, fixed in 70% ethanol, and stored at -20°C until analysis. After nuclear DNA staining with propidium iodide, flow cytometry was done in duplicate by a FAC Scan flow cytometer (Becton Dickinson). For each sample, 20,000 events were stored and cell cycle analysis was done by the ModFit LT software (Verity Software House). FL2 area versus FL2 width gating was done to exclude doublets from the G2-M region. The percentage of apoptotic cells was calculated in the subdiploid region of the DNA content, registered as FL2 signals in linear scale. To avoid cell debris contamination due to necrotic cell death, cells were selected by side scatter versus DNA signals (FL2) gating.

**Statistical analysis:** A paired t test using Sigma Stat software (Systat Software) was used for statistical analyses. Differences were considered to be statistically significant at p<0.05. All measurements were done in quadruplicates or triplicates as indicated and each experiment was repeated at least three times.

**Results**

Treatment of H209 cells with UAFT and 5-FU at their equipotent doses increased the cell population in sub-G1 to 38.4 and 41.5% respectively whereas in control only 20.2% cell population was in Sub-G1 phase (**Figure 2**). However, treatment of H209 cells with sequential combination of UAFT and 5-FU increased the Sub-G1 cell population to 51.3%, which exceeded the additive effect due to UAFT and 5-FU. These results suggest that UAFT and 5-FU exhibit synergistic effect on induction of apoptosis in H209 cells.

Exposure of H209 cells to UACT, 5-FU or their sequential combination showed a synergistic effect on activation of NF-κB protein levels. There are reports that cigarette smoke condensate can activate NF-κB through phosphorylation and degradation of IκBα. TNF, PMA, H2O2 and okadaic acid are other potent activators of NF-κB (**Anto et al., 2002**). The results from Western blot analysis demonstrated that exposure of H209 cells to either UACT or 5-FU inhibited activation of NF-κB protein. However, when H209 cells were treated with sequential combination of UACT and 5-FU, there was a decrease in both concentration as well as time duration.

**Figure 2:** Flow cytometric analysis of the cytotoxic activities of UACT and 5-FU sequential combination in H209 cells.

Control, untreated H209 cells for 72 hours; UACT, H209 cells treated with 50 µM of UACT for 24 hours followed by normal medium for an additional 48 hours; 5-FU, H209 cells treated with 20 µM of 5-FU for 48 hours and UACT + 5-FU, H209 cells treated with sequential combination of UACT and 5-FU (100 nM and 10 nM respectively). Cells were fixed, stained and analyzed by flow cytometry as described in Materials and Methods. The Sub-G1 cell population is expressed as a percentage of total cell counts.
Treatment of H209 cells with UACT at a concentration of 50 µM for 72 hours caused a significant decrease in NF-κB protein. However, treatment with the sequential combination of UACT and 5-FU decreased the time of inhibition to 24 hours and the decreased level continued for the 72 hours. There was a significant inhibition in sequential combination only at 100 nM concentration of UACT.

The synergistic effect of UACT and 5-FU on TNF-induced NF-κB-dependent reporter gene expression, was determined by transiently transfecting UACT, 5-FU, UACT + 5-FU-pretreated or untreated cells, with the NF-κB-regulated SEAP reporter construct and then stimulating the cells with TNF. There was 5-6 times enhancement in SEAP activity over the vector control after stimulation with TNF (Figure 4). However, pretreatment of H209 cells with UACT and 5-FU inhibited TNF-induced NF-κB-dependent SEAP expression by 69 and 54% respectively at an UACT concentration of 100 µM. On the other hand, UACT + 5-FU-pretreated cells showed 95% inhibition of TNF-induced NF-κB-dependent SEAP expression was observed at just 50 µM concentration of UACT. These results demonstrate synergistic effect of 5-FU on UACT.

Activation of NF-κB by TNF via the involvement of IKK induces phosphorylation and degradation of IκBα. There are reports that COX-2 and MMP-9, the genes regulated by NF-κB are induced on treatment with TNF. The investigation of the effect of sequential combination of UACT and 5-FU revealed a synergistic inhibition on COX-2 and MMP-9 induction by TNF in H209 cells. Treatment of UACT and 5-FU pre-treated cells with TNF followed by Western blot analysis clearly indicated the inhibition of TNF-induced expression of COX-2 and MMP-9 in a synergistic manner (Figure 5). The similar results were observed for cyclin D1 expression (not shown).

The sequential treatment with UACT and 5-FU combination showed synergistic cytotoxic activity in H69
cells. To evaluate the generality of this synergism in other SCLC cell lines, we tested the sequential combination effects in H69, 87-5, and Lu135 cells. The cells were treated with UACT at its IC50 concentrations for 24 hours, harvested and washed with PBS and then were exposed to various concentrations of 5-FU for 24 hours. Results from cytometry analysis showed the accumulation of cells in Sub-G1 clearly indicating synergistic effect. The synergistic effects were detected in all the SCLC lines tested in these experiments (Figure 6). Including the results from H209 cells, the synergistic effects of sequential treatment with UACT followed by 5-FU were observed in all the 4 SCLC cell lines tested in our study.

Figure 4: UACT and 5-FU inhibited TNF-induced NF-κB-dependent reporter gene (SEAP) expression

H209 cells treated with UACT and 5-FU at 100 nM and 10 nM were transiently transfected with a NF-κB containing plasmid linked to the SEAP gene. After 24 hours in culture with 1 nM TNF, cell supernatants were collected and assayed for SEAP activity. Results are expressed as fold activity over the activity of the vector control.

Figure 5: Sequential treatment with combination of UACT followed by 5-FU inhibited induction of COX-2 and MMP-9 by TNF

H209 cells (2 x 10^6 cells/mL) were left untreated or incubated with 50 µM UACT for 72 hours and then treated with 0.1 nM TNF for different time periods. Whole-cell extracts were prepared and analyzed by Western blotting using antibodies against COX-2 and MMP-9. UACT inhibited TNF-induced cyclin D1. H209 cells (2 x 10^6 cells/mL) were left untreated or incubated with 50 µM UACT for 72 hours and then treated with 0.1 nM TNF for different time periods. Whole-cell extracts were prepared, and 80 µg of the whole-cell lysate were analyzed by Western blotting using antibodies against cyclin D1. Combination of UACT and 5-FU inhibited the induction of COX-2 and MMP-9 by TNF through synergistic effect.
Discussion

Complete remission of SCLC using a combination treatment of topotecan and 5-FU in a Phase I clinical trial (Sbar et al., 2002) and a 77% initial response rate with combination regimen of 5-FU and cisplatin (Morere et al., 1994) inspired us for this study. Accumulation of cells in G1 phase may be due to decline in levels of cyclin D1 because D-type cyclins are involved in progression of cells from the G1 phase of the cell cycle to S phase (Matsushime et al., 1991). In this study we observed that sequential treatment with a combination of UAFT and 5-FU increased accumulation of cells in sub-G1 phase in H209 cells. The enhancement was greater than the simple sum of increments caused due to UACT and 5-FU separately. The effect of sequential combination of UAFT and 5-FU was also analysed in H209, 87-5, and Lu135 cells. The results from the cytometry analysis showed the accumulation of cell population in Sub-G1 similar to that observed for H209 cells. The synergistic effects were detected in all the SCLC lines tested in these experiments.

There are reports for activation of NF-κB by cigarette smoke condensate through phosphorylation and degradation of IκBα. NF-κB has also been reported to be activated by TNF, PMA, H2O2, and okadaic acid (Anto et al., 2002). NF-κB regulates the expression of a number of genes with significant role in induction of tumor (Pahl, 1990; Garg and Aggarwal, 2002). These include anti-apoptosis genes, COX-2, MMP-9, genes encoding adhesion molecules, chemokines, inflammatory cytokines, and iNOS; and cell cycle-regulatory genes (e.g., cyclin D1). Therefore, the molecules having tendency to inhibit NF-κB activation can suppress carcinogenesis and have therapeutic potential (Garg and Aggarwal, 2002; Banerjee et al., 2002). The therapeutic role of phytochemicals in prevention and treatment of cancer has been indicated (Wattenberg, 1990; Sporn and Suh, 2000; Sporn and Suh, 2002). The results from Western blot analysis demonstrated that exposure of H209 cells to sequential combination of UACT and 5-FU, exhibited synergistic effect on NF-κB inhibition. Treatment with sequential combination of UACT and 5-FU decreased the time of inhibition of NF-κB to 24 hours and the decreased level continued for the 72 hours. UACT + 5-FU-pretreated H209 cells showed 95% inhibition of TNF-induced NF-κB-dependent SEAP expression at just 50 nM concentration of UACT. The investigation of the effect of sequential combination of UACT and 5-FU on COX-2 and MMP-9 induction by TNF in H209 cells also revealed a synergistic inhibition. UACT and 5-FU pretreated cells on treatment with TNF followed by Western blot analysis clearly indicated the inhibition of TNF-induced expression of COX-2 and MMP-9 in a

Figure 6: Effect of the sequential treatment with UACT and 5-FU combination on H69, 87-5 and Lu135 SCLC cell lines

H69, 87-5 and Lu135 cells were treated with sequential combination of UACT for 24 hours followed by 5-FU for 24 hours.
synergistic manner. The similar results were observed for cyclin D1 expression.

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