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## Sequential treatment with ursolic acid chlorophenyl triazole followed by 5-fluorouracil shows synergistic activity in small cell lung cancer cells

Rui-Xia Zhu<sup>1</sup>, Lei Zhao<sup>2</sup>, Yu-Kun Zhang<sup>3</sup>, Peng Luo<sup>4</sup>, Si-Hai Liu<sup>1</sup> and Jin-Guang Wang<sup>2</sup>

<sup>1</sup>Department of Oncology, Zaozhuang Mining Group Central Hospital, Zaozhuang, 277 800, China;

<sup>2</sup>Department of Thoracic Surgery, The First Affiliated Hospital of Dalian Medical University, Dalian,

116 011, China; <sup>3</sup>Department of Thoracic Surgery, Taian Central Hospital, Taian, 271 000, China;

<sup>4</sup>Shanghai Topgen Biopharm Co., Ltd, Shanghai 200 000, China.

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### Abstract

Combination therapy has prolonged the survival of patients with small cell lung cancer (SCLC), an aggressive neoplasm characterized by a high rate of metastasis. In the present study the effect of sequential treatment of ursolic acid chlorophenyl triazole (UACT) followed by 5-fluorouracil (5-FU) on human small cell lung cancer cells was investigated. The results revealed a synergistic effect of the sequential treatment with UACT and 5-FU combination on cytotoxic activities, NF- $\kappa$ B protein activation, repression of TNF-induced NF- $\kappa$ B-dependent reporter gene expression, and TNF-induced COX-2, MMP-9 and Cyclin D1 activation in H209 cells. The synergism in apoptotic cell death was observed in H209, H69, 87-5, and Lu135 cells. The synergistic effect of UACT and 5-FU was observed at a concentration of 50 nM of UACT and 20  $\mu$ M of 5-FU. These results indicate that UACT and 5-FU combination can be a promising chemotherapeutic regimen in the treatment of SCLC.

### Introduction

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 (Rostiet al., 2006). In addition topoisomerase I inhibitors (irinotecan) Nodaet al., 2002; Hannae-tal., 2006) and topotecan (Ardizzonietal., 1997) and amrubicin (AMR) (Oheet 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 (Nodaet 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. 5-fluorouracil (5-FU) and

oral fluoropyrimidine have been shown to be effective in gastrointestinal tract and other cancers (Takiuchi and Ajani, 1997).

There are reports that demonstrate oral fluoropyrimidines as promising candidate in combination chemotherapy (Ichinoseet 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 (TS) protein (Johnston et al., 1994).



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, antiallergic, antibacterial, antiviral, antitumor and cytotoxic activity being the most intriguing (Li et al., 2005; Kim et al., 2002; Harmandet 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 (Mutoet al., 1990). Ursolic acid arrests the cell cycle progression in the G1 phase and induces apoptosis (Hsuet al., 2004). However the poor bioavailability under *in vivo* hinders its clinical applications (Saraswatet 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 (Baiet 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.

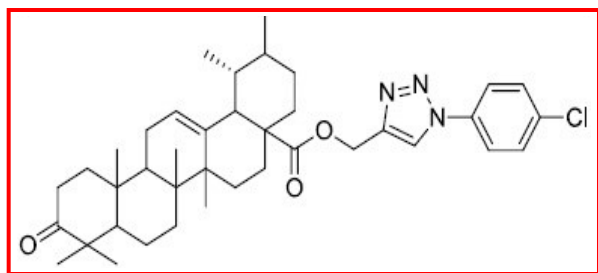


Figure 1: Structure of ursolic acid chlorophenyltriazole (UACT)

## 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  $-20^{\circ}\text{C}$ . 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  $-20^{\circ}\text{C}$ .

### 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  $\mu\text{g}/\text{mL}$ ), and 4 mmol/L glutamine in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

### 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: equiactive doses (50:50 cytotoxic ratio) of the two agents ( $\text{IC}_{50}$ ), higher relative doses of UACT/5-FU (25:75 cytotoxic ratio;  $\text{IC}_{25}$  of UACT/ $\text{IC}_{75}$  of 5-FU), and higher relative doses of 5-FU/ UACT ( $\text{IC}_{75}$  of 5-FU/ $\text{IC}_{25}$  of UACT). For sequential combinations, serial dilutions of equiactive 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  $\text{CI} < 0.8$ ,  $\text{CI} = 0.8-1$ , and  $\text{CI} > 1$  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  $1 \times 10^4$  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  $1 \times 10^5$  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  $\mu\text{L}$  medium containing various concentrations of 5-FU or UACT. After incubation for the indicated duration, 10  $\mu\text{L}$  solution of MTT was added to each well and the plates were again incubated for 4 hours at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  incubator. 100  $\mu\text{L}$  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  $\mu\text{g}/\text{mL}$  leupeptin, 1 mM phenylmethyl sulfonyl fluoride, 10 mM NaF, 40 mM  $\beta$ -glycerophosphate and 2 mM

$\text{Na}_3\text{VO}_4$ ) 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  $\mu\text{g}$  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 chemi-luminescence detection (Amersham Pharmacia Biotech) using goat anti-rabbit IgGs coupled to horseradish peroxidase as a secondary antibody (Amersham Pharmacia Biotech). An anti- $\beta$ -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  $-20^\circ\text{C}$  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

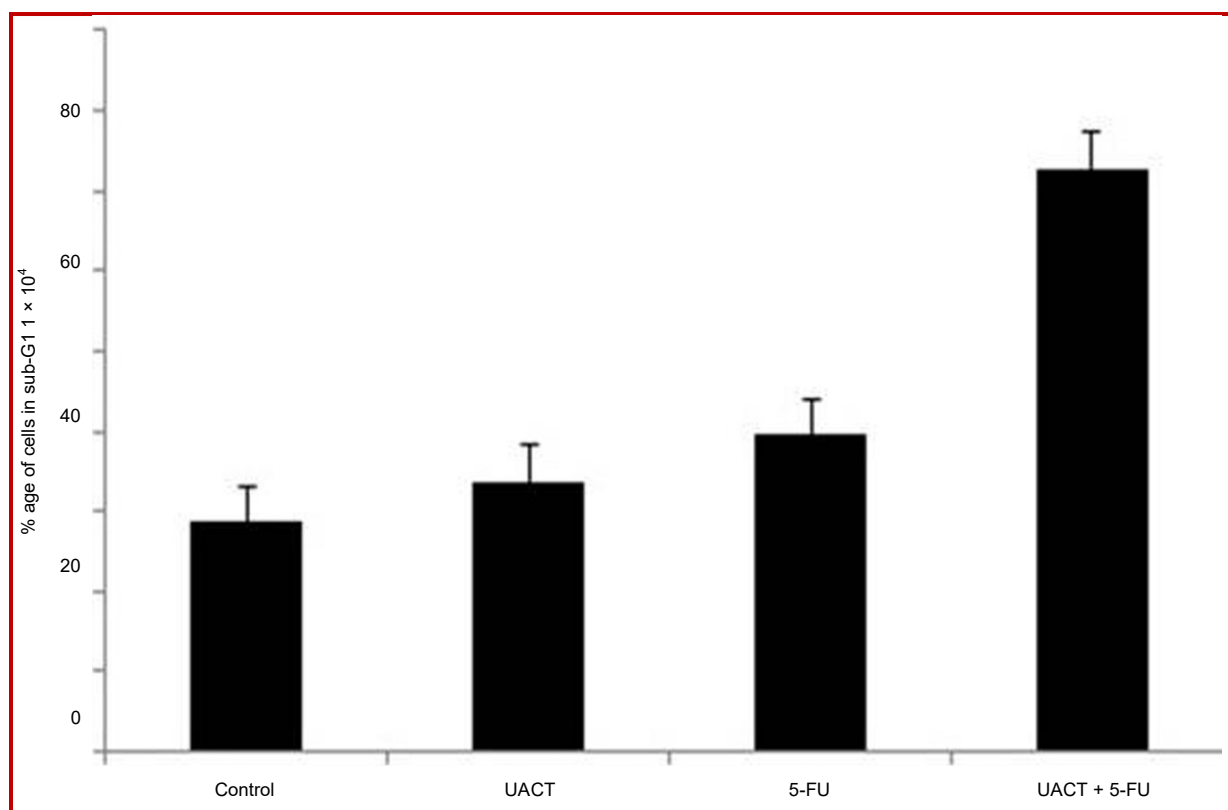


Figure 2: Flow cytometric analysis of the cytotoxic activities of UACT and 5-FU sequential combination in H209 cells.

Control, untreated H209 cells for 72 hours; UACT, H209 cells treated with 50  $\mu\text{M}$  of UACT for 24 hours followed by normal medium for an additional 48 hours; 5-FU, H209 cells treated with 20  $\mu\text{M}$  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

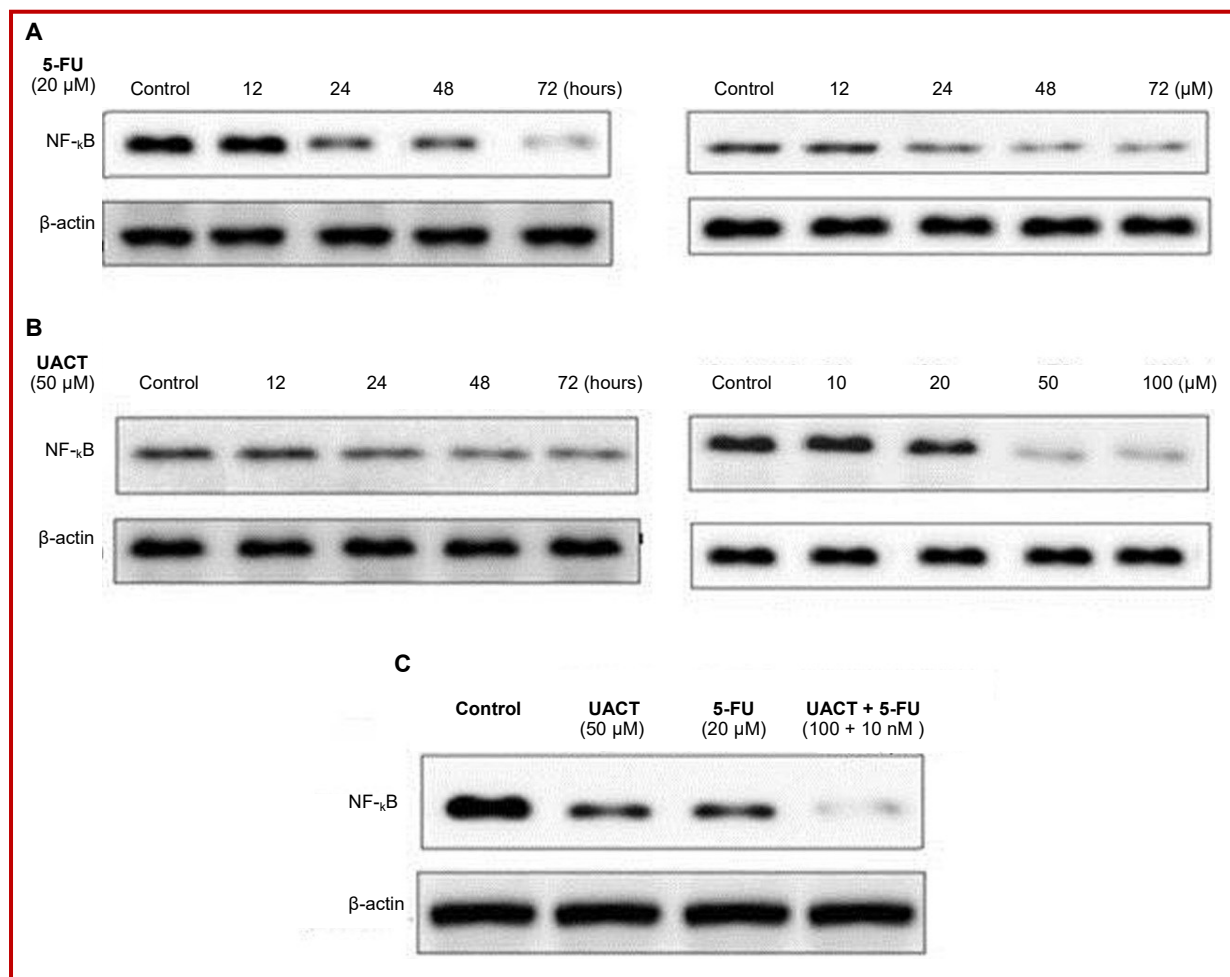


Figure 3: Effect of 5-FU and UACT on NF-κB protein expression in H209 cells

(A) H209 cells were treated with 20 μM of 5-FU for indicated time (left) and a range of 5-FU concentrations for 72 hours (right). (B) H209 cells were treated with 50 μM of UACT for 72 hours (left) and a range of indicated UACT concentrations for 72 hours. (C) H209 cells were untreated or treated with 50 μM of UACT alone for 24 hours, 20 μM 5-FU alone for 24 hours, or 100 nM of UACT for 24 hours followed by 10 μM 5-FU for 24 hours. Total cell lysates of each treatment were subjected to Western blot analysis with an anti-NF-κB antibody as described in Materials and Methods. β-actin was used as a loading control

effect due to UACT and 5-FU. These results suggest that UACT 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 I<sub>κ</sub>Bα. TNF, PMA, H<sub>2</sub>O<sub>2</sub>, 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 for NF-κB inhibition (Figure 3).

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

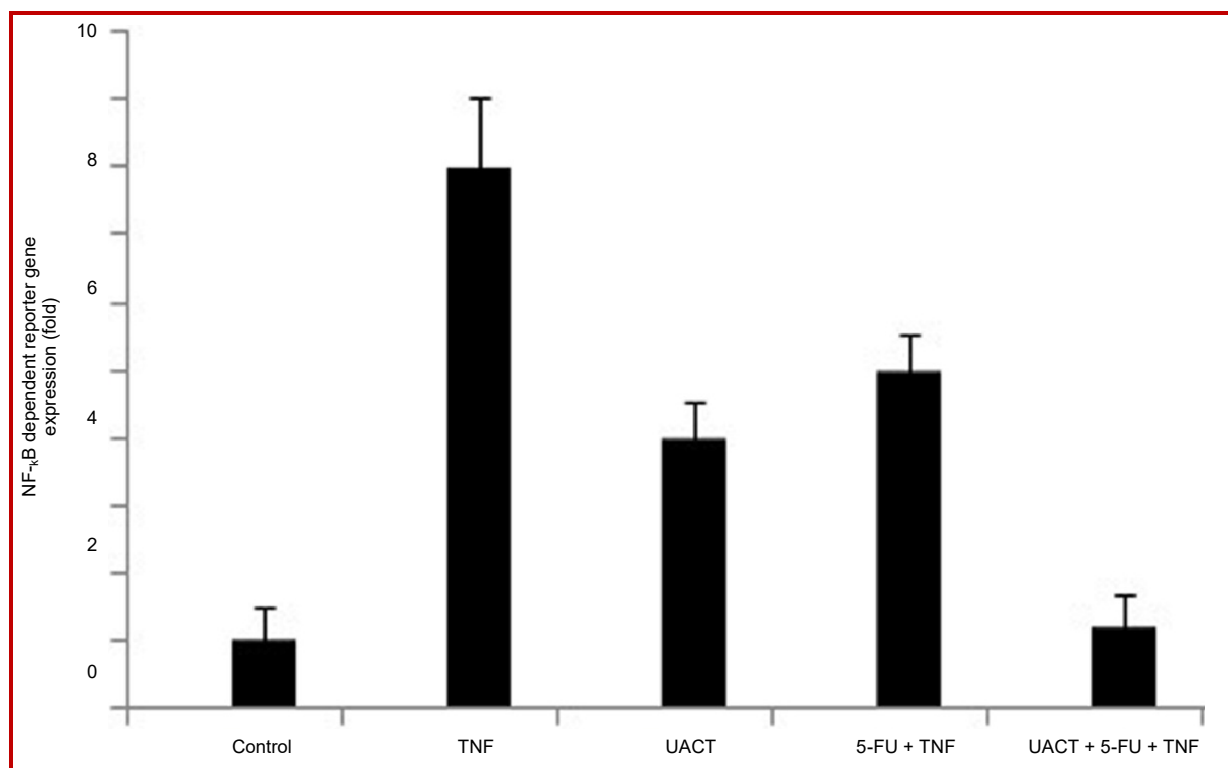


Figure 4: UACT and 5-FU inhibited TNF-induced NF-κB-dependent reporter gene (SEAP) expression

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

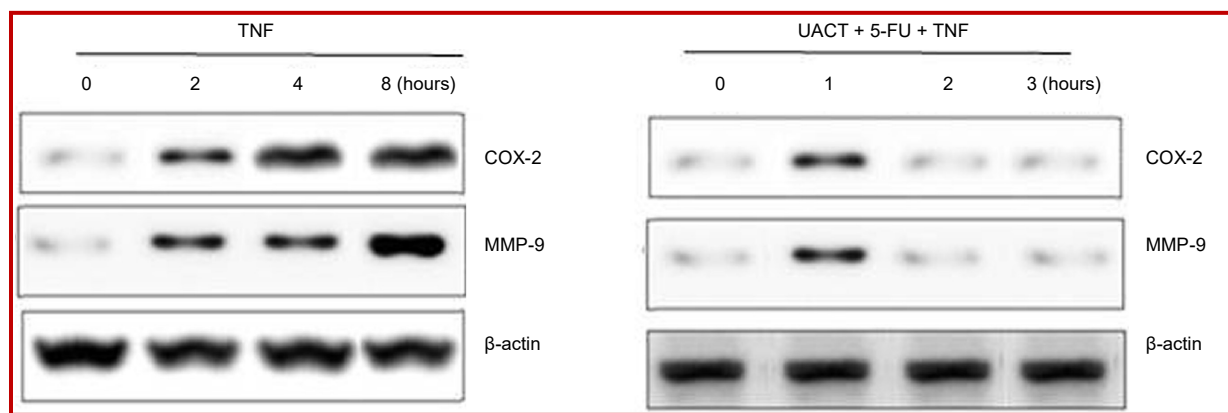


Figure 5: Sequential treatment with combination of UACT followed by 5-FU inhibited induction of COX-2 and MMP-9 by TNF

H209 cells ( $2 \times 10^6$  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. *B*, UACT inhibited TNF-induced cyclin D1. H209 cells ( $2 \times 10^6$  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

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 indica-



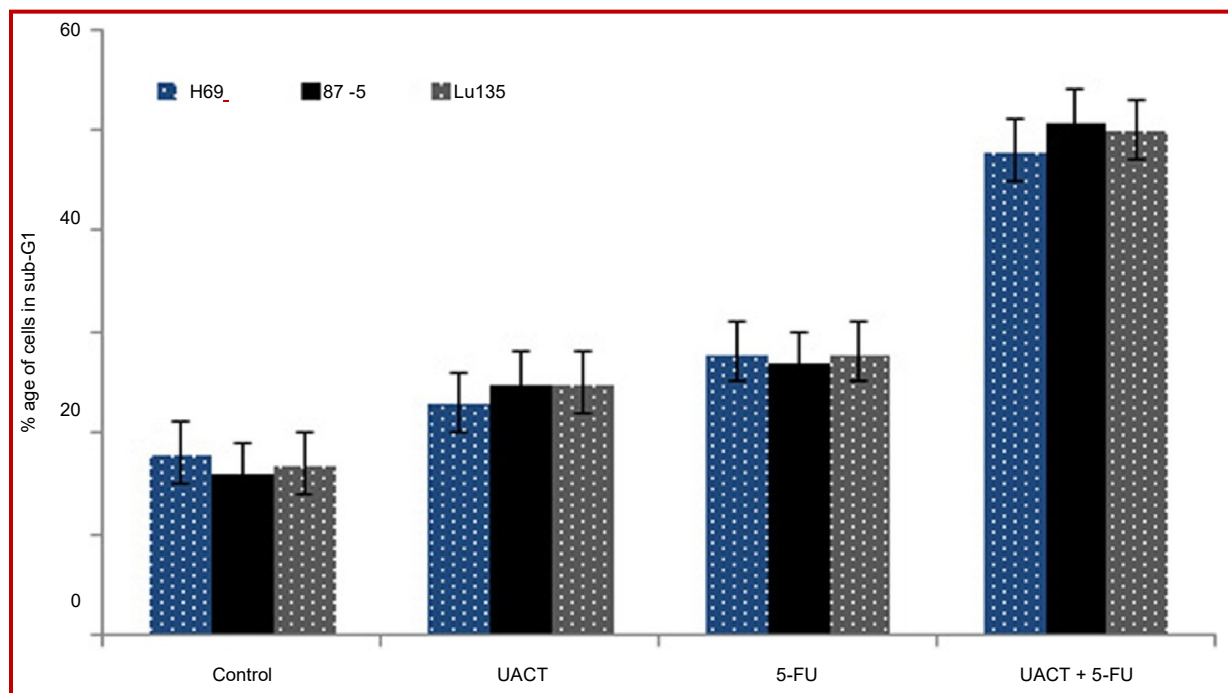


Figure 6: Effect of the sequential treatment with UACT and 5-FU combination on H69, 87-5 and Lu135 SCLC cell lines

H69, 87-5 and Lu135 cells were treated with sequential combination of UACT for 24 hours followed by 5-FU for 24 hours

ted 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  $IC_{50}$  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.

## 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 (Matsushima et al., 1991). In this study we observed that sequential treatment with a combination of UACT 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 UACT 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- $\kappa$ B by cigarette smoke condensate through phosphorylation and degradation of I $\kappa$ B $\alpha$ . NF- $\kappa$ B has also been reported to be activated by TNF, PMA, H<sub>2</sub>O<sub>2</sub>, and okadaic acid (Anto et al., 2002). NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B inhibition. Treatment with the sequential combination of UACT and 5-FU decreased the time of inhibition of NF- $\kappa$ B 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- $\kappa$ B-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 synergistic manner. The similar results were observed for cyclin D1 expression.

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### Conflict of Interest

Authors declare no conflict of interest

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**Author Info**

Si-Hai Liu (Principal contact)  
e-mail: liusihai277@gmail.com