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Synergistic[Q1] Anti-Tumor Activity of Isochaihulactone and Paclitaxel on Human Lung Cancer Cells

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Drug resistance frequently develops in tumors during chemotherapy. Therefore, to improve the clinical outcome, more effective and tolerable combination treatment strategies are needed. Here, we show that isochaihulactone (K8) enhanced paclitaxel-induced apoptotic death in human lung cancer cells, and the enhancing effect was related to increased NSAID-activated gene-1 (*NAG-1*) expression. CalcuSyn software was used to evaluate the synergistic interaction of K8 and paclitaxel on human lung cancer cells; the synergistic effect of K8 in combination with paclitaxel was increased more than either of these drugs alone. Furthermore, the activity of ERK1/2 was enhanced by the combination of K8 and paclitaxel, and an ERK1/2 inhibitor dramatically inhibited NAG-1 expression in human lung cancer cells. Therefore, this synergistic apoptotic effect in human lung cancer cells may be directly associated with K8-induced NAG-1 expression through ERK1/2 activation. Moreover, over-expression of NAG-1 enhanced K8/paclitaxel-induced apoptosis in human lung cancer cells. In addition, treatment of nude mice with K8 combined with paclitaxel induced phospho-ERK1/2 and NAG-1 expression in vivo. Targeting of NAG-1 signaling could enhance therapeutic efficacy in lung cancer. Our results reveal that activation of NAG-1 by K8 enhanced the therapeutic efficacy of paclitaxel in human lung cancer cells via the ERK1/2 signaling pathway.

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Cancer presents a serious clinical problem and poses significant social and economic effects on the healthcare system. Despite improved imaging and molecular diagnostic techniques, the disease still affects millions of patients worldwide (Eisenberg et al., 1998). Although taxanes and Vinca alkaloids are effective for the management of different malignancies, resistance to drugs such as paclitaxel and vincristine frequently develops in some tumors after an initial quick and promising response during chemotherapy (Jordan et al., 1998; Rafi et al., 2002; Wang et al., 2004). Therefore, to improve the clinical outcome, more effective and tolerable treatment strategies are needed. It has been reported that combinatorial agents show promise for cancer treatment because they can hit multiple targets involved in tumor development (Mukhtar and Ahmad, 1999; McCarty, 2004; Narayanan et al., 2004; Ulrich et al., 2006). Consistent with this view, there is currently increasing interest in low-dosage treatments of a combination of two or more agents with different modes of action (Tortora et al., 2003; Narayanan et al., 2004; Wong and Robertson, 2004; Banerjee et al., 2005; Lin et al., 2005; Velmurugan et al., 2005; Khor et al., 2006; Narayanan et al., 2006; Ulrich et al., 2006). Many lignan natural products have complex biological properties, making the development of synthetic or separate methods for this large class of compounds a challenge to chemists and biologists. Nan-Chai-Hu (Chai Hu of the South), the root of *Bupleurum scorzonerifolium*, is an important Chinese herb in the treatment of influenza, fever, malaria, cancer, and menstrual disorders in

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China, Japan, and many other parts of Asia. We previously reported that the crude acetone extract of *B. scorzonerifolium* causes cell-cycle arrest of A549 human lung cancer cells in the G2/M phase, formation of giant cells, and apoptosis (Chen et al., 2005; Cheng et al., 2005). The acetone extract fraction was further partitioned, and we identified a novel lignan, isochaihulactone (K8), which has anti-tumor activity against A549 cells in vitro and in vivo (Chen et al., 2006). It was previously reported that this compound was also isolated from *Bursera microphylla* (Burseraceae), and shown to have an anti-tumor effect (Cole et al., 1969). These findings indicate that K8 is a promising new anti-mitotic anticancer compound with potential for clinical applications.

We have found that several K8-induced genes are induced by nonsteroidal anti-inflammatory drugs (NSAIDs). NSAID-activated gene-1 (*NAG-1*) (also known as *MIC-1*, *GDF-15*, placental *TGF-β*, and *PLAB*) was highly induced in the presence of K8 in our previous study (Chen et al., 2007). NAG-1 is a transforming growth factor-β-like secreted protein. It was initially characterized as a p53-regulated gene (Baek et al., 2002; Bottone et al., 2002). Over-expression of NAG-1 in breast cancer cells, both in vitro and in vivo, results in growth arrest and apoptosis; similar results were observed in colon cancer cells (Baek et al., 2002; Bottone et al., 2002; Liu et al., 2003; Chintharlapalli et al., 2005; Ko et al., 2007) and following treatment of prostate cancer cells with purified NAG-1 (Liu et al., 2003). These findings suggest that NAG-1 is linked to apoptosis and that reduced expression of NAG-1 may enhance tumorigenesis. It is known that the PPARγ-dependent activation of *NAG-1* by troglitazone is due to induction of EGR-1, which in turn activates *NAG-1* (Rokos and Ledwith, 1997; Chintharlapalli et al., 2006; Yamaguchi et al., 2006; Felts et al., 2007). Like troglitazone, the PPAR-γ active 1,1-bis (3'-indolyl)-1-(*p*-substituted phenyl) methanes also induce *EGR-1*, which in turn interacts with the proximal GC-rich *EGR-1* motifs in the *NAG-1* promoter (Chintharlapalli et al., 2006). This reaction represents a pathway for induction of *EGR-1* and *NAG-1*, and these responses contribute to the induction of growth inhibition and apoptosis promoted by anti-tumor compounds in cancer cells. In addition, we found that K8 induced growth inhibition and apoptosis by activating *EGR-1* and *NAG-1* through an ERK-dependent pathway, and this did not involve activation of PI3K signaling (Chen et al., 2007).

In the current study, we demonstrate that the A549 human lung cancer cell line show hypersensitivity toward paclitaxel, and this may caused by up-regulation of the responsive factors of *NAG-1* activation via an ERK-dependent pathway. Combination chemotherapy using anticancer drugs achieves a better response rate, often exceeding the efficacy of a single treatment. Our results distinguish the mode of action of K8 as it interacts with paclitaxel in a synergistic manner and suggest a mechanism for understanding the downstream effectors of K8-induced growth inhibition in lung cancer cells.

Materials and Methods

Cell lines and cell culture

The human lung carcinoma cell line A549 (BCRC60074) was obtained from the Bioresources Collection and Research Center (BCRC, Hsin Chu, Taiwan). The human lung carcinoma cell line H1299 (CRL-5803) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The A549 and H1299 cells were maintained with RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The growth medium was changed 2–3 days per week and subcultured when 80% confluent.

Fraction purification of K8 and structure determination

B. scorzonerifolium roots were supplied by Chung-Yuan Co. (Taipei, Taiwan), and the plant was identified by Professor Lin of the National Defense Medicinal Center, where a voucher specimen was deposited (NDMCP 900801). K8 (4-benzo[1,3]dioxol-5-ylmethyl-3-(3,4,5-trimethoxyl-benzylidene)-dihydro-furan-2-one) was prepared as described previously (Chen et al., 2006) and for drug safety monitoring, our previous study (Chen *et al.*, 2006) and United States Patent application (Appl. No.: 10/690,992) indicate that K8 has no effect on normal cells or on organ function at the therapeutic dosage in vivo.

Chemicals and reagents

RPMI-1640 medium, FBS, penicillin, streptomycin, trypsin/EDTA, and a NuPAGE Bis-Tris Electrophoresis System (precast polyacrylamide minigels) were purchased from Invitrogen (Carlsbad, CA). An RNA isolation kit was purchased from QIAGEN (Valencia, CA). The PKC inhibitor GF109203X, paclitaxel (for drug safety monitoring, paclitaxel is widely used in the treatment of lung, ovarian, and breast carcinomas (Mekhail and Markman, 2002) and the first of a new class of microtubule stabilizing agents, has been hailed by National Cancer Institute (NCI) as the most significant advance in chemotherapy of the past 15–20 years (Paim et al., 2002)), vincristine, irinotecan, dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl thizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), β-actin monoclonal antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sigma (St Louis, MO). The ERK1/2 kinase inhibitor PD98059 and PI3K/AKT inhibitor LY294002 were purchased from Calbiochem (San Diego, CA). The NAG-1, caspase-3, phospho-AKT, AKT, phospho-PKC, PKC, phospho-ERK1/2 and ERK1/2 monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA). *Mycoplasma* removal reagent was obtained from Dainippon Pharmaceutical (Osaka, Japan). The Annexin V-FLOUS Staining Kit was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Polyvinylidene difluoride membranes, a bovine serum albumin protein assay kit, and western blot chemiluminescence reagents were purchased from Amersham Biosciences (Arlington Heights, IL).

Growth inhibition assay

The viability of the cells after treatment with various chemicals was evaluated using an MTT assay preformed in triplicate. Briefly, the cancer cells (5×10^3) were incubated in 96-well plates containing 200 μl of the culture medium. Cells were permitted to adhere for 12–18 h and then washed with phosphate-buffered saline (PBS). Solutions were always prepared fresh by dissolving 0.2% DMSO or drugs in culture medium and then added to the A549 cells. After 48 h of exposure, the drug-containing medium was removed, cells were washed with PBS, and the medium was replaced with fresh medium. The cells in each well were then incubated in culture medium with 500 μg/ml MTT for 2 h. After the medium was removed, 200 μl of DMSO was added to each well. Absorbance at 570 nm of the maximum was detected using a PowerWave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT). The absorbance for DMSO treated cells was considered 100%. The results were determined by three independent experiments.

Evaluation of the effect of K8 in combination with paclitaxel on the inhibition of human lung cancer cell proliferation

A commercial CalcuSyn software package (Biosoft, Cambridge, UK) was used for median-effect analysis, as described by Chou and Talalay (Chou and Talalay, 1981). The dose-effect curve was plotted using a logarithmic conversion of this equation to log (fa/fu) = mlog(D) − mlog(Dm) for each agent and then in fixed

ratio combinations. In brief, A549 or H1299 human lung cancer cells were seeded at a density of 5×10^3 cells/well in flat-bottom plates (200 μl/well). After 24 h, the culture medium was removed and the cells were washed with fresh FBS-free culture medium. By this method, six concentrations (0.625, 0.25, 2.5, 5, 10, and 20 μM) of K8 combined with six concentrations (0.625, 1.25, 2.5, 5, 10, and 20 nM) of paclitaxel were added to A549 or H1299 cells. After 48 h of exposure, the drug-containing medium was removed, the cells were washed with PBS, and the medium was replaced with fresh medium. The cells in each well were then incubated in culture medium with 500 μg/ml MTT for 4 h. After the medium was removed, 200 μl of DMSO was added to each well. Absorbance at 570 nm of the maximum was detected by a PowerWave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT). The absorbance for DMSO-treated cells was considered as 100%. According to the survival rate, we calculated the combination index (CI) based on the formula: $CI = (D)1/(Dx)1 + (D)2/(Dx)2$ ($CI = 1$ implies an additive effect, $CI > 1$ an antagonistic effect, and $CI < 1$ a synergistic effect). (Dx)1 and (Dx)2 are the drug 1 and drug 2 concentrations that inhibit cell division by 50% after 48 h. (D)1 and (D)2 are the concentrations of drug 1 combined with drug 2 that inhibit cell division by 50% after 48 h.

Detection of apoptosis

Apoptosis was analyzed according to the method described by van Engeland et al. (van Engeland et al., 1998) to detect the integrity of the cellular membrane and the externalization of phosphatidylserine. In brief, approximately 10^6 cells were grown in 35-mm diameter plates. The cells were treated with various herbal extracts and chemicals according to the experimental design and then labeled with 10 μg/ml annexin V-FLOUS and 20 μg/ml propidium iodide (PI) before harvesting. After labeling, the cells were washed with binding buffer and harvested by scraping. Cells were resuspended in binding buffer at a concentration of 2×10^5 cells/ml before analysis by flow cytometry (FACScan). The data were analyzed with WinMDI V2.8 software. The percentage of cells undergoing apoptosis was determined by three independent experiments.

Caspase activity assay

Activity of caspase-3 or caspase-9 was detected by using a fluorometric assay kit (Promega, Madison, WI) according to the manufacturer's protocol. In brief, 2×10^6 control or treated cells were lysed in 50 μl of cold lysis buffer and incubated in ice for 10 min. Cell lysate (50 μl) was added to 50 μl of reaction buffer and 5 μl of fluorogenic report substrate specific for caspase-3 in a 96-well microplate. After incubation at 37°C for 1 h, the fluorescence from the cleaved C-terminal side of the aspartate residue of DEVD-7-amino-4-trifluoromethyl coumarin was detected by a fluorescence microplate reader (Fluoroskan Ascent; Thermo Fisher Scientific, Waltham, MA), with excitation at 400 nm and emission at 505 nm.

Isolation of RNA and RT-PCR

Total cellular RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. Then, 2 μg of total RNA was reverse transcribed at 65°C for 5 min, followed by 37°C for 60 min in the presence of Omniscript Reverse Transcriptase (QIAGEN) according to manufacturer's protocol. A total of 1 μg of cDNA was amplified in the presence of 20 μmol of the following primers: NAG-1(F), 5'-ACTCCGAAGACTCCAGATTCCGA-3'; NAG-1(R), 5'-ATGCACATGGTCACTTGCACCT-3'; GAPDH(F), 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'; GAPDH(R), 5'-CAT-GTGGGCCATGAGGTCCACCAC-3' with Taq DNA polymerase (Takara Shuzo, Shiga, Japan). The thermal cycling profile was composed of an initial denaturation step at 95°C for 10 min and 30 cycles of 30 sec of denaturation at 95°C, 30 sec of annealing at 60°C, and 1 min of extension at 72°C, with a final 10-min extension step at 72°C. The intensity of the product bands was analyzed using a GS-800 calibrated imaging densitometer (Quantity One 4.0.3 software; Bio-Rad, Hercules, CA), and the levels of *GAPDH* were used as a loading control.

Western blot analysis

A549 cells were seeded in a 10-cm dish, permitted to adhere for 12–18 h, and then washed with PBS. Solutions were always prepared fresh by dissolving 0.2% DMSO or drugs in culture medium, which were then added to the cells. Cell pellets were resuspended in lysis buffer (10 nM Tris-HCl [pH 7.5], 1 mM EGTA, 0.5% 3-[3-(cholamidopropyl) dimethylammonio]-1-propanesulfonate, 10% [v/v] glycerol, 5 mM β-2-mercaptoethanol, and 0.1 mM phenyl-methylsulfonyl fluoride) and incubated on ice for 30 min. After centrifugation at $16{,}000 \times g$ for 10 min at 4°C, total cell lysates were collected. The protein concentration of the cell lysates was measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories) following the manufacturer's instructions. Aliquots (20 μg) of the cell lysates were separated by 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% skim milk overnight and probed with an appropriate dilution of primary antibodies for 2 h at room temperature. Membranes were washed three times with 0.1% Tween 20 and incubated with a 1:1,000 dilution of HRP-conjugated secondary antibody for 1 h at room temperature. All proteins were detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Waltham, MA) and quantified using a densitometer.

Anti-tumor activity in vivo

Xenograft mice were used as a model system to study cytotoxicity of K8 combined with paclitaxel in vivo; the implantation of cancer cells was carried out similar to previous reports. Female congenital athymic BALB/c nude (nu/nu) mice were purchased from the National Sciences Council (Taipei, Taiwan), and all procedures were performed in compliance with the standard operating procedures of the Laboratory Animal Center of Ilan University (Ilan, Taiwan). All experiments were carried out using 6–8-week old mice weighing 18–22 g. The animals were subcutaneously implanted with 5×10^6 A549 cells into their backs. When the tumor reached 100–250 mm^3 in volume, animals were divided randomly into control and test groups consisting of six mice per group (day 0). In our previous study, the 10 mg/kg K8 did not significantly inhibit tumor growth (Chen *et al.*, 2006). In addition, we also found that 5 mg/kg paclitaxel did not significantly inhibit tumor growth (Shoemaker et al., 2006; Skobeleva et al., 2007). Therefore, daily subcutaneous (sc) administration of 10 mg/kg K8, 5 mg/kg paclitaxel, and 10 mg/kg K8 combined with 5 mg/kg paclitaxel dissolved in a vehicle of vitamin K1 (standard) was performed from days 0 to 4 far from the inoculated tumor sites (>1.5 cm). The control group was treated with vehicle only. The mice were weighed three times a week up to 21–28 days to monitor the effects, and at the same time, the tumor volume was determined by measurement of the length (L) and width (W) of the tumor. The tumor volume at day n (TVn) was calculated as $TV(mm^3) = (LW^2)/2$. The relative tumor volume at day n (RTVn) versus day 0 was expressed according to the following formula: $RTVn = TVn/TV0$. Tumor regression (T/C [%]) in treated versus control mice was calculated using: T/C (%) = (mean RTV of treated group)/(mean RTV of control group) × 100. Xenograft tumors as well as other vital organs of treated and control mice were harvested and fixed in 10% formalin, embedded in paraffin, and cut in 4-mm sections for histologic study.

Author Proof

Immunohistochemical staining

All tumor tissues were fixed in 10% formalin at 4°C and then embedded in paraffin. Paraffin sections (5 μm) were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions. The sections were incubated with blocking solution (5% nonfat milk powder in PBS) for 60 min at room temperature, followed by a 4°C overnight incubation with anti-NAG-1, anti-phospho-ERK1/2 and anti-caspase-3 monoclonal antibodies in blocking solution separately. Subsequently, the immune complexes were visualized using the LSAB2 system (Dako North America, Carpinteria, CA), and then incubated for 15 min with 0.5 mg/ml diaminobenzidine and 0.03% (v/v) H_2O_2 in PBS. Finally, sections were counterstained with hematoxylin, mounted, observed under a light microscope at magnifications of 400×, and photographed.

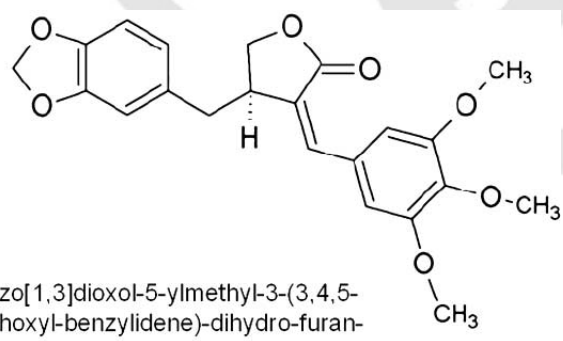
Statistical analysis

The data represent means ± SD. Statistical differences were analyzed using the Student's *t*-test. For the pairwise comparisons multiple samples, statistical differences were analyzed using the *t*-test to compare the specific pairs of groups in one-way ANOVA (LSD procedure). Values of $P < 0.05$ were considered significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

K8 synergistically increases the cytotoxicity of paclitaxel in human lung cancer cells

K8, a structural signature of lignan family compounds (Fig. 1), caused cytotoxicity in various lung cancer cell lines (Table 1), as well as in hepatocarcinoma, prostate adenocarcinoma, and oral squamous cell carcinoma cells (Chen et al., 2006). However, because it is not known whether K8 increases the sensitivity of human lung cancer cells to other anticancer chemotherapeutic drugs (paclitaxel, vincristine, and irinotecan), dose-response curves of each single chemotherapeutic drug and its combination with K8 at different doses were determined using A549 and H1299 cells and further clarify is shown in Figure 2 and Tables 2 and 3. To further investigate whether enhanced anticancer chemotherapeutic drug sensitivity occurs in human lung cancer cells, A549 and H1299 cells were exposed to K8 and shown the lethal rates of three ratios of K8 and paclitaxel in Table 2 and a CalcuSyn analysis was used to evaluate whether K8 has a synergistic effect with other anticancer drugs (Table 3). As shown in Figure 2B, A549 cells were simultaneously exposed



4-benzo[1,3]dioxol-5-ylmethyl-3-(3,4,5-trimethoxyl-benzylidene)-dihydro-furan-2-one
MW=398.41

Fig. 1. Chemical structure of K8.

TABLE 1. Growth inhibition of K8 against human lung cancer cell lines

Cell line	Origin	IC50 (μM)
A549	Lung adenocarcinoma	18.5
H460	Lung adenocarcinoma	20.0
H1299	Lung adenocarcinoma	20.5
A549-T12	Lung adenocarcinoma	10.0

IC_{50}, drug concentration that inhibits cell division by 50% after 43 h. Each value is based on at least three different experiments.

to K8 and the depolymerization agent paclitaxel at equipotent molar ratios for three generations to obtain CI plots, and were shown a dramatically synergistic effect than the other chemotherapeutic drugs. The CI values are summarized in Table 3.

Up-regulation of NAG-1 protein expression and increased apoptosis after combined K8/paclitaxel treatment

The percentage of apoptosis induced by K8 or K8 combined with paclitaxel in A549 cells is shown in Figure 3A. K8 (5 μM) induced 17.8% apoptosis compared with the control (2.2%). The apoptosis rate induced by 2.5 nM of paclitaxel alone in A549 cells was 21.5%. In addition, the synergistic increasing percentage of apoptosis induced by combined exposure to 5 μM K8 and 2.5 nM paclitaxel (2,000:1) was 38.4%.

In our previous study, K8 was shown to induce EGR-1 and NAG-1 mRNA and protein expression in A549 cells (Chen et al., 2006). To evaluate whether EGR-1 is involved in the induction of NAG-1 expression by combined K8/paclitaxel treatment, NAG-1 mRNA and protein expression was determined by RT-PCR and western blot analysis, respectively. The results showed that NAG-1 mRNA and protein expression were up-regulated following combined K8/paclitaxel treatment (Fig. 3B).

The proteolytic processing of inactive procaspases is an essential component of the death pathway in many cells. Caspases (e.g., caspase-1, -3, -8, -9, and -10) are involved in the early stages of the proteolytic cascade and induction of apoptosis. We observed increased activation of the effectors caspase-3 and caspase-9 by K8 or paclitaxel, and this increased activation was synergistically enhanced by treatment with a combination of both drugs (Fig. 3C). These results suggest that combined treatment with K8/paclitaxel induces apoptosis through a caspase-dependent mechanism.

K8 enhances paclitaxel-inhibited ERK activation followed by growth inhibition in human lung cancer cells

A role for MAPKs in the regulation of NAG-1 and NAG-1 as a novel downstream target of the PI3K/AKT/GSK-3β (PI3K/AKT) pathway has been reported in several studies (Yamaguchi et al., 2004). To determine whether the MAPK/ERK, PKC, or PI3K/AKT/GSK3 pathway plays a role in the induced growth inhibition of human lung cancer cells following combined K8/paclitaxel treatment, the A549 cells were treated with 5 μM K8 combined with various concentrations of paclitaxel (1.25–5 nM) in the presence or absence of the MEK/ERK1/2 inhibitor PD98059 (12.5, 25, and 50 μM), the PI3K/AKT inhibitor LY294002 (5, 10, and 20 μM), or the PKC inhibitor GF109203X (5, 10, and 20 μM). It was observed that an ERK inhibitor, PD98059, inhibited cell death induced by combined K8/paclitaxel treatment in a concentration-dependent manner in human A549 human lung cancer cells (Fig. 4A).

To determine which of the signaling molecular was involved in the induced growth inhibition following combined K8/

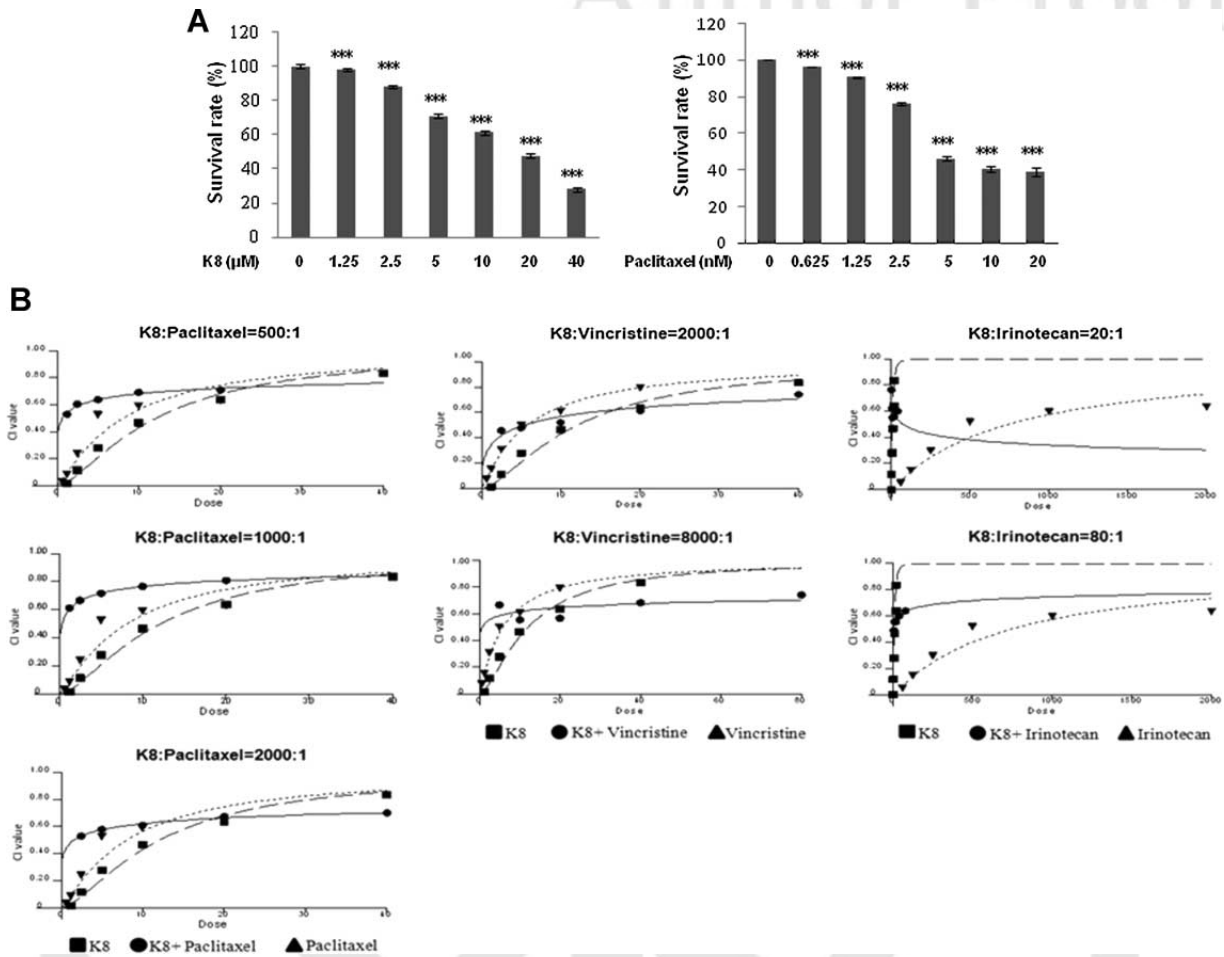


Fig. 2. K8 synergistically interacts with paclitaxel to inhibit the proliferation of human lung cancer cells in vitro. (A) A549 cells were treated with various concentrations of K8 or paclitaxel. After 48 h of incubation, the growth inhibition effect (IC_{50}) was determined by the MTT assay. The data represent the mean and SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with corresponding value for cells treated with vehicle alone. (B) CI values for the K8 and anticancer chemotherapeutic drugs (paclitaxel, vincristine, and irinotecan) combination treatments tested on A549 cells. CI values were calculated from the dose–response curves shown in part B and are given in Table 3.

paclitaxel treatment, the effect of this treatment on ERK, PKC, and AKT activation was assessed. Increased phospho-ERK expression was observed after exposure of A549 cells to combined K8/paclitaxel treatment. Furthermore, expression of PKC, phospho-PKC, AKT, phospho-AKT showed no obvious changes (Fig. 4B). To investigate a possible role for ERK in the regulation of NAG-1, A549 cells were treated with both K8 and paclitaxel in the presence or absence of the MAPK inhibitor PD98059. Western blot analysis indicated that inhibition of phospho-ERK expression (via PD98059) inhibited NAG-1 expression and increased cleavage of caspase-3 following combined treatment (Fig. 4C). These observations suggest that activation of the ERK1/2 signaling pathway is involved in the induction of NAG-1 expression and subsequent apoptosis in

TABLE 2. Lethal rates of A549 and H1299 human lung cancer cells with combined K8/paclitaxel treatment

	K8:Paclitaxel = 500:1			K8:Paclitaxel = 1,000:1			K8:Paclitaxel = 2000:1		
Cell line	K8 (μM)	Paclitaxel (nM)	Lethal rate (%)	K8 (μM)	Paclitaxel (nM)	Lethal rate (%)	K8 (μM)	Paclitaxel (nM)	Lethal rate (%)
A549	10	20	71.2	20	20	81.1	20	10	70
	5	10	69.5	10	10	76.7	10	5	67.8
	2.5	5	64	5	5	71.9	5	2.5	60.9
	1.25	2.5	60.6	2.5	2.5	66.7	2.5	1.25	58.3
	0.625	1.25	53.2	1.25	1.25	61.3	1.25	0.625	53.3
A1299	10	20	76.4	20	20	S4.7	20	10	75.3
	5	10	70.5	10	10	74.9	10	5	66.2
	2.5	5	67.9	5	5	69.1	5	2.5	61.1
	1.25	2.5	62.1	2.5	2.5	64.6	2.5	1.25	55.7
	0.625	1.25	55.6	1.25	1.25	59.8	1.25	0.625	53

TABLE 3. CI Values at ED_{50} of A549 and H1299 human lung cancer cells with combined K8/paclitaxel, K8/vincristine, or K8/irinotecan treatment

	K8/Paclitaxel			K8/Vincristine		KS/Irinotecan	
Cell line	500:1	1,000:1	2,000:1	2,000:1	8,000:1	20:1	80:1
A549	0.05431	0.08357	0.12641	0.05891	0.43677	0.48525	7.10333
H1299	0.14711	0.17624	0.20984	0.07916	0.50165	0.51787	7.81839

The combination index (CI) based on the formula: CI = (D)1/(Dx)1 + (D)2/(Dx)2 (CI = 1 implies an additive effect, CM an antagonistic effect and CI < 1 a synergistic effect).
The ED50 value is the concentration of the drug that produces 50% of the maximum response.

human lung cancer cells following combined K8/paclitaxel treatment.

NAG-1 enhances combined K8/paclitaxel toxicity in human lung cancer cells

NSAIDs induce apoptosis in colorectal cancer cells (Bottone et al., 2002). In our study, A549 cells were stably transfected with an expression vector containing the full-length *NAG-1* coding region in the sense orientation. A pooled population of cells obtained after selection with G418 was then used to determine the effect of increased NAG-1 expression. Western blot analysis indicated that these cells expressed the NAG-1 protein at a rate 2.0-fold greater than the empty vector-transfected cells (Fig. 5A). These stably transfected cells were incubated with K8 alone or combined K8/paclitaxel for 48 h, and apoptosis was determined by flow cytometry (Fig. 5B). Under all treatment conditions, a higher percentage of cells expressing NAG-1 underwent apoptosis than cells transfected with the empty vector (Fig. 5B). A colony formation assay also confirmed that over-expression of NAG-1 enhanced the cell toxicity following treatment with combined K8/paclitaxel (Fig. 5C). Our results suggest that NAG-1 is critically important for enhancing combined K8/paclitaxel toxicity in human lung cancer cells.

K8 combined with paclitaxel enhances the growth inhibition of human lung cancer xenografts in nude mice

To evaluate the in vivo anti-tumor activity of K8 alone, paclitaxel alone, and combined K8/paclitaxel, human lung cancer

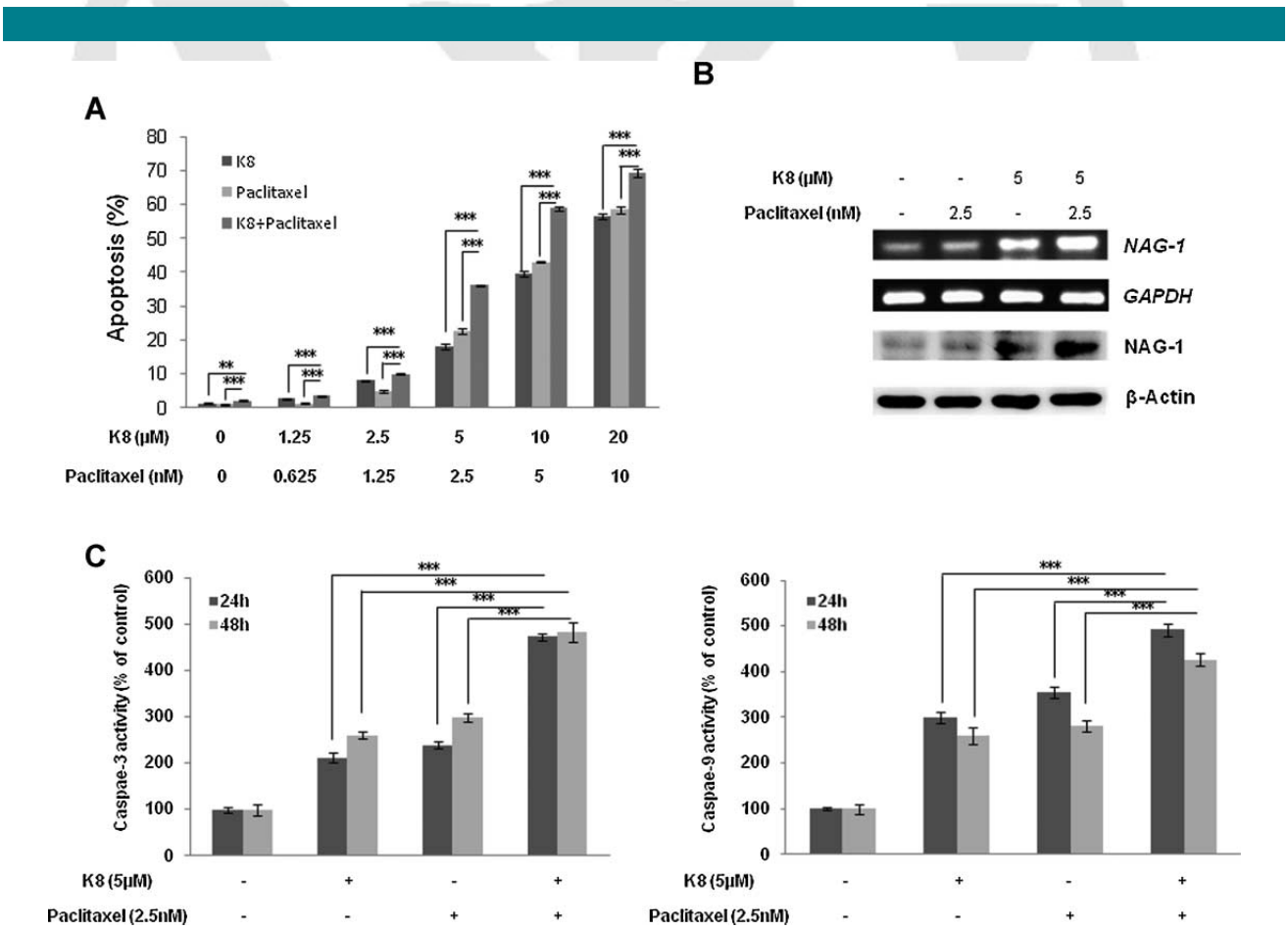


Fig. 3. Changes in NAG-1 protein expression and increased apoptosis after combined K8/paclitaxel treatment. (A) Apoptosis was measured by flow cytometry PI/annexin V staining in A549 cells and then cells were treated with 0–20 μM K8 combined with various concentrations of paclitaxel (0.625–10 nM) for 48 h as indicated. (B) A549 cells were treated with 5 μM K8 combined with 2.5 nM paclitaxel (2,000:1). In each time course, cells were collected and total mRNA and proteins were isolated for RT-PCR and western blot analysis. The expression of *GAPDH* and β-Actin were used as internal controls. (C) K8 induced A549 cell apoptosis through activated cappase-9 and caspase-3. A549 cells were treated with 5 μM K8 combined with 2.5 nM paclitaxel (2,000:1) for 24 and 48 h. The *y* axis shows relative caspase-3 (left) and caspse-9 (right) activity. The data represent the mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with corresponding values for cells treated with K8 or paclitaxel alone.

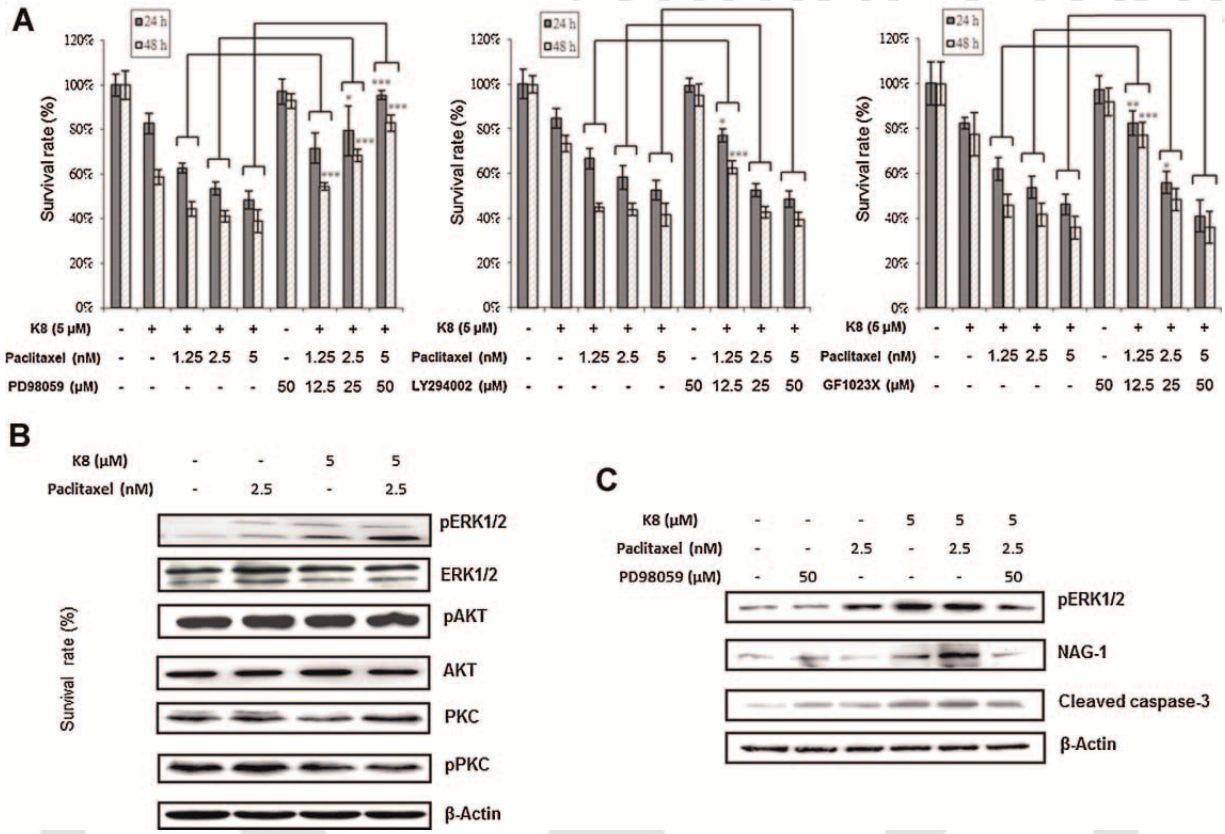


Fig. 4. Inhibition of NAG-1 expression and growth inhibition by ERK1/2 inhibitor. (A) MTT assay of A549 cells with culture in serum-containing medium pretreated with the MEK1/2 inhibitor PD98059 (12.5, 25, and 50 μM) or PKC inhibitor GF109203X (5, 10, and 20 μM) or PI3K/AKT inhibitor LY294002 (5, 10, and 20 μM) for 1 h and then treated with combined 5 μM K8/(1.25, 2.5, and 5 μM) paclitaxel for 24 h. The first sample in each graph shows the A549 cells treated with serum-containing media and no test compound as a negative control. The data represent the means ± SD of three different experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with corresponding value for cells treated with K8 combined paclitaxel treatment. (B) Western blot analysis of PKC, phospho-PKC, AKT, phospho-AKT, ERK1/2, and phospho-ERK1/2 in A549 cells after treatment with 5 μM K8 and/or combined with 2.5 nM paclitaxel (2,000:1) for 24 h. (C) A549 cells with culture in serum-containing medium pretreated with or without the MEK1/2 inhibitor PD98059 (50 μM) and then treated with 5 μM K8 and/or combined with 2.5 nM paclitaxel (2,000:1) for the indicated times. The phospho-ERK1/2, NAG-1 and cleaved caspase-3 were detected by western blotting.

xenografts were established by subcutaneous injection of A549 cells into the dorsal tissue and cells on the backs of nude mice. After the tumors reached approximately 100–250 mm^3, the mice were given a daily subcutaneous injection of K8, paclitaxel, or combined K8/paclitaxel for 5 successive days. Significant suppression of tumor growth was observed in the groups treated with combined K8/paclitaxel compared with the other groups (K8 alone or paclitaxel alone) (Fig. 6A). In addition, immunohistochemistry analysis showed increased phospho-ERK1/2 (Fig. 6B), NAG-1 (Fig. 6C), and cleaved caspase-3 (Fig. 6D) protein expression in tumor cells treated with both K8 and paclitaxel relative to the control groups in vivo.

Discussion

It has been shown that both paclitaxel and K8 enhance cell growth inhibition and apoptotic cell death. Previous data indicate that concurrent paclitaxel and radiation treatments demonstrate locoregional activity in pancreatic cancer (von Haefen et al., 2003). However, there have been few investigations into the effects of K8 on the growth of lung cancer cells. In the present study, we found that K8 also induced cell death in human lung cancer cells, and that the combination of paclitaxel and K8 had a synergistic effect on cell death (Fig. 2 and Table 3). A putative signaling pathway that could account for the actions of the combination of paclitaxel and K8 is shown in Figure 7.

Paclitaxel and K8 are implicated in the regulation of various signaling pathways involved in proliferation, differentiation, and apoptosis (Ambrosini et al., 1998; Olie et al., 2000; Bacus et al., 2001; Liu and Sun, 2003; Chen and Wong, 2008; Rahman et al., 2009). Existing data indicate that the MAPK pathway is related to paclitaxel- or K8-induced cell death in three different human cancer cell lines: HeLa cervical carcinoma, MCF7 breast cancer, and A431 squamous carcinoma cells (Bacus et al., 2001). The mitogen-induced ERK MAPKs are linked to cell proliferation and survival, whereas the stress-activated MAPKs, p38, and JNK are involved in apoptosis (Wang et al., 2004; Shih et al., 2007). Here, we found that paclitaxel or K8 alone induced ERK activation, whereas the combination of these two compounds synergistically induced activation of ERK (Fig. 4). Our results suggest that the synergistic effect on cell death was mediated by ERK signaling in human lung cancer cells. The balance between apoptotic and anti-apoptotic signaling pathways plays a role in the pathogenesis of a variety of cancers.

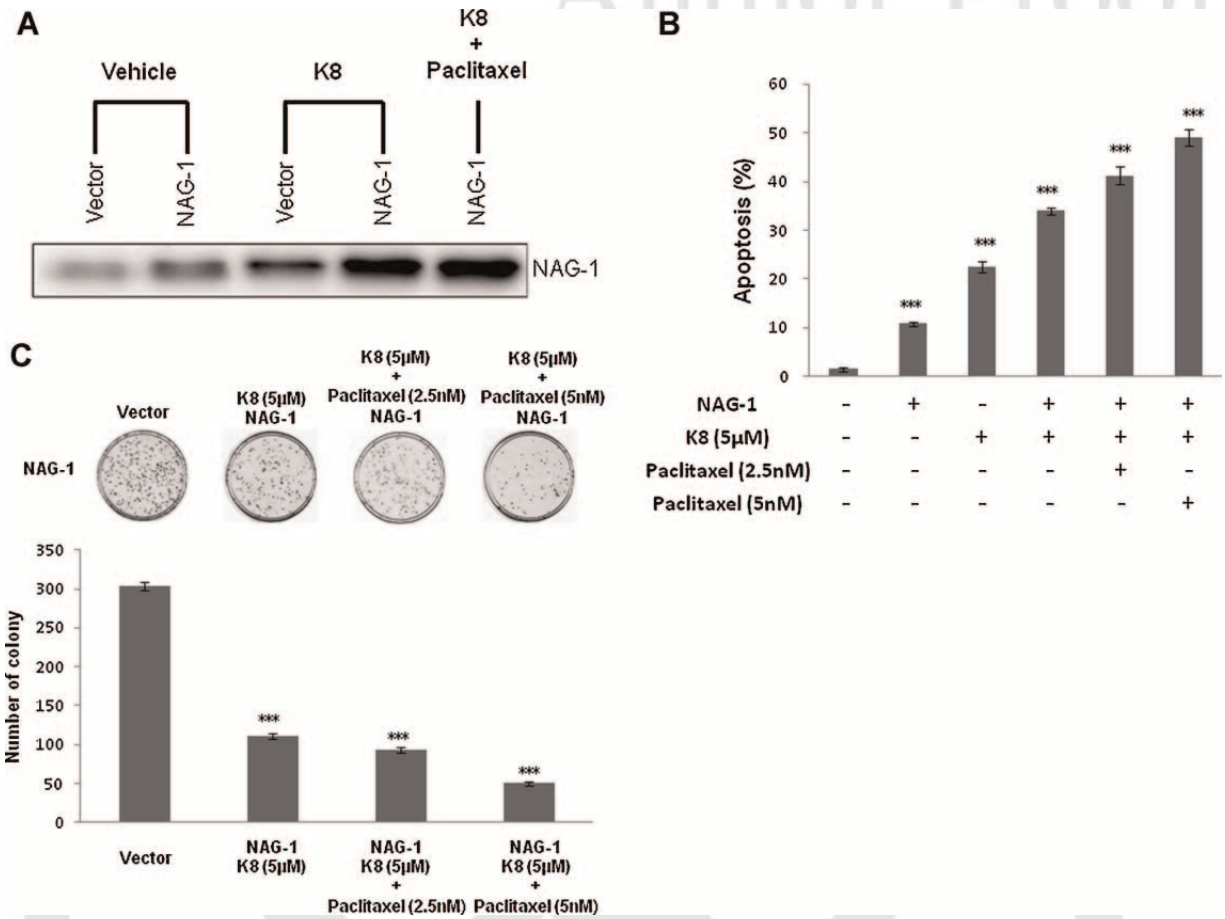


Fig. 5. Effect of NAG-1 expression on K8 combined with paclitaxel-induced apoptosis. (A) A549 cells were transfected with either pCDNA3.1 (Vector) or pCDNA3.1-NAG-1 (NAG-1), and stably transfected clones were selected by exposure to G418 (500 μg/ml) for 3 weeks. The stably transfected clones were treated with vehicle, 5 μM K8, or 5 μM K8 combined with 2.5 nM paclitaxel (2,000:1) for 24 h. (B) Apoptosis was measured by flow cytometry PI/annexin V staining in stably transfected A549 cells treated with vehicle, K8, or K8 combined with different dosages of paclitaxel (2,000:1 and 1,000:1) for 24 h. The data represent the means ± SD of three different experiments. (C) Effects of NAG-1 expression and treatment with K8 combined with paclitaxel on colony formation. The stably transfected clones were treated with vehicle, K8, K8 combined with different dosages of paclitaxel (2,000:1 and 1,000:1) for 24 h and then grown in culture medium for 2 weeks. The data represent the means ± SD of three different experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with control.

NAG-1 is a transforming growth factor-β-like secreted protein and was initially characterized as a p53-regulated gene (Baek et al., 2002; Bottone et al., 2002). In our current study, we demonstrated that over-expression of NAG-1 in A549 cells resulted in growth arrest and apoptosis. The similar results were also observed in colon cancer cells (Liu et al., 2003; Chintharlapalli et al., 2005; Chen et al., 2007; Ko et al., 2007) and for treatment of prostate cancer cells with purified NAG-1, which induces apoptosis (Liu et al., 2003). These findings suggest that NAG-1 is linked to apoptosis and that reduced expression of NAG-1 may enhance tumorigenesis. It has been demonstrated that NAG-1 is highly induced after combination treatment with sodium salicylate and PI3K/MEK1/2 inhibitor (Kim et al., 2008)• In our study, although paclitaxel alone had a mild effect on NAG-1 expression, the combination of paclitaxel and K8 remarkably up-regulated the level of NAG-1 (Fig. 5). We also demonstrated that activation of the ERK pathway led to activation of NAG-1 (Fig. 4). Taken together, we hypothesize that activation of ERK signaling might enhance the effect of paclitaxel, K8, or their combination by up-regulation of NAG-1. We further provided evidence that an ERK inhibitor, PD98059, inhibited cell death induced by paclitaxel, K8, or their combination in human A549 cells (Fig. 4A).

In conclusion, we present evidence that the combination of paclitaxel and K8 synergistically induced ERK activation, up-regulation of NAG-1, and cell death in human lung cancer cells. Transient inhibition of ERK signaling further inhibited NAG-1 expression and drug-induced cell death in vitro and in vivo. The MAPK/ERK1/2 signaling pathway has been implicated in the regulation NAG-1 because the combination of paclitaxel and K8 induced activation of phospho-ERK in A549 cells. These results suggest likely gene targets for paclitaxel treatment in combination with K8, which may be useful for future clinical applications.

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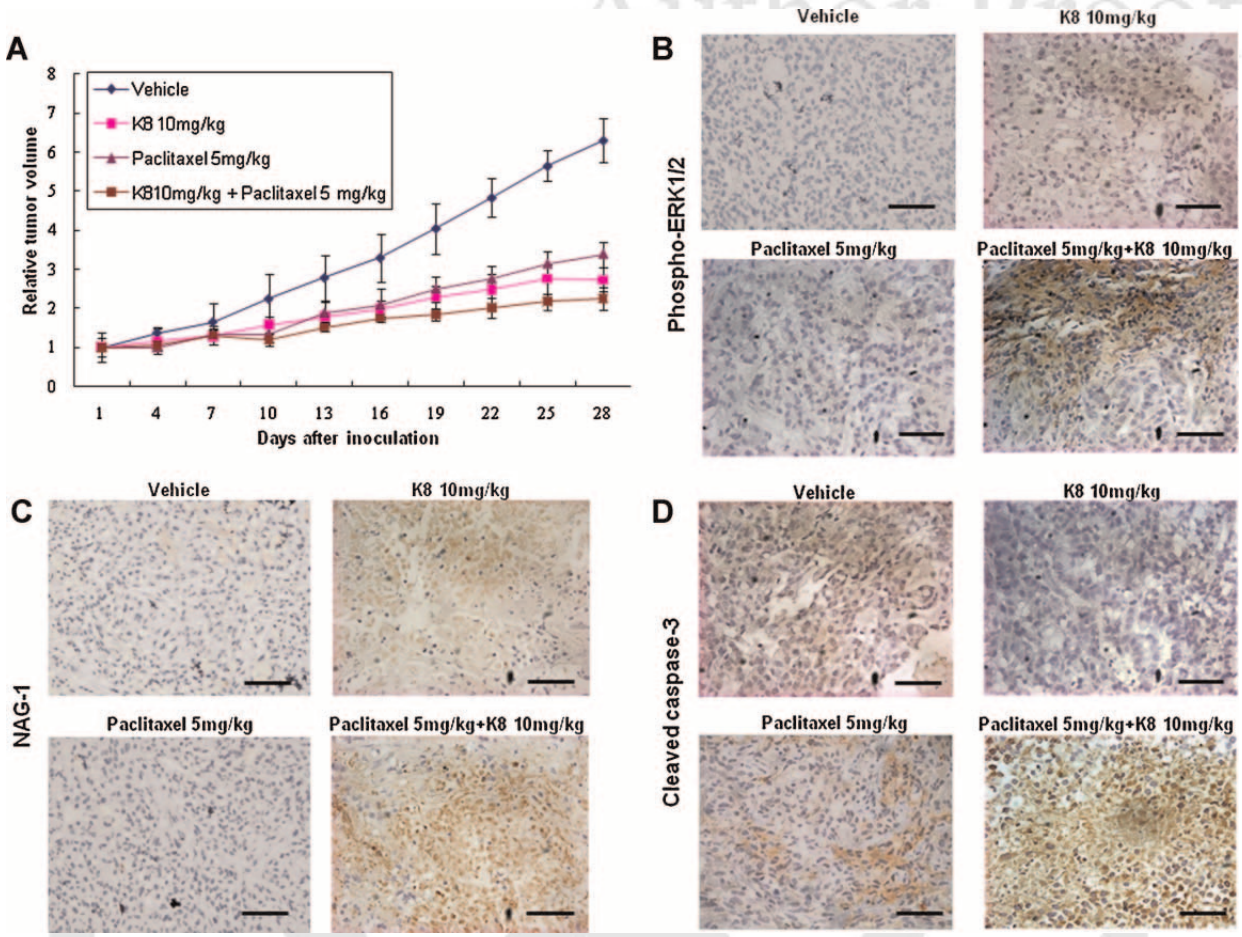

Fig. 6. Enhancement of efficacy of K8 combined with paclitaxel in human lung cancer cells xenografts in Balb/c nude mice. Nude mice were injected with approximately 5×10^6 A549 cells into the dorsal subcutaneous tissue. (A) When the tumor volumes reached 100–250 mm^3, A549 tumor-bearing mice were administered subcutaneously with vehicle control, 10 mg/kg K8, 5 mg/kg paclitaxel, and 10 mg/kg K8 combined with 5 mg/kg paclitaxel on days 0–4 for 5 days. The relative tumor volumes in the control and therapeutic groups are shown. (B), (C), (D) Expression of phospho-ERK1/2, NAG-1, and cleaved caspase-3 in A549 xenografts. A549 xenografts were generated and mice were treated as described above. Five days after treatment, representative sections of A549 tumors in the control group and treatment groups were immunohistochemically stained with (B) phospho-ERK1/2, (C) NAG-1, or (D) cleaved caspase-3 rabbit polyclonal antibodies. The cells positive for phospho-ERK1/2, NAG-1, or cleaved caspase-3 are stained brown. Scale bars, 100 μm.

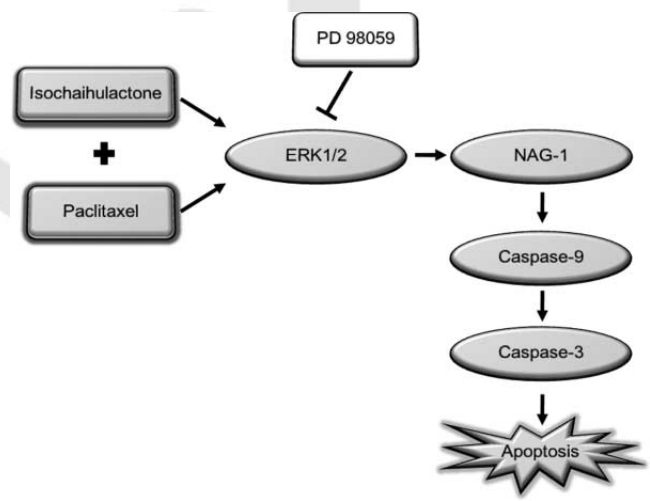

Fig. 7. The schematic illustration summarizes the synergistic apoptotic effect caused by the combination of K8 and paclitaxel in human lung cancer cells via enhancing ERK1/2 and NAG-1 signaling.

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