수의학석사학위논문

게피티니브 내성 H460 폐암세포주에서 전압의존성 포타슘 채널 차단제 Dendrotoxin-κ와 Margatoxin의 항암효과

Anti-cancer Effects of Voltage-gated K^+ Channel Blockers, Dendrotoxin-κ and Margatoxin, in Gefitinib-resistant H460 Lung Cancer Cell Line

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수의학과 수의생명과학 전공

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전 원 일

Master's Thesis

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이 논문을 수의학 석사학위 논문으로 제출함

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전 원 일

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ABSTRACT

Membrane ion channels are well known to be associated with various cellular functions in most types of cells. Especially, voltage-gated K^+ (Kv) channels are related to the proliferation of several types of cancer cell lines, including lung adenocarcinoma cell line, and certain Kv channel blockers inhibit cancer cell proliferation. Therefore, Kv channels have received attention due to their possibility as promising targets to develop novel anti-cancer therapy. In the present study, we investigated the effects of Kv channel blockers in gefitinib-resistant H460 lung cancer cell line. 1) The mRNA and protein of both Kv1.1 and Kv1.3 were detected in H460 lung cancer cell line. 2) Treatment of Kv1.1 specific blocker, dendrotoxin-κ (DTX-κ), and Kv1.3 specific blockers, margatoxin (MgTX) and 5-(4-phenoxybutoxy) psoralen (PAP-1), reduced H460 cell viability through not apoptosis but cell cycle arrest in G_1/S transition during cell cycle progression. 3) Anticancer effects of Kv channel blockers were also shown in xenograft model using nude mice. Tumor volume was reduced by the regular injection of DTX-κ or MgTX into the tumor tissues compared to the control groups. 4) Furthermore, combination treatment of gefitinib with DTX-κ or MgTX leads to synergistic anticancer effects in H460 cell line. These results indicate DTX-κ and MgTX have anticancer effects in gefitinib-resistant H460 cell line through the pathway governing the G_1/S transition both *in vitro* and *in vivo*. The relation between cell viability and Kv channels, Kv1.1 and Kv1.3, suggests that these Kv channels would serve as novel therapeutic targets for gefitinib-resistant lung cancer cell lines.

Keywords: Kv1.1, Kv1.3, channel blocker, cell cycle arrest, apoptosis, xenograft

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ABBREVIATIONS

Kv channels	voltage-gated K^+ channels
NSCLC	non-small cell lung cancer
DTX-κ	dendrotoxin-κ
MgTX	margatoxin
PAP-1	5-(4-phenoxybutoxy) psoralen
EGFR	epidermal growth factor receptor
TKI	tyrosine kinase inhibitor
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MTT	methylthiazoltetrazolium
IC_{50}	the half maximal inhibitory concentration
EGF	epidermal growth factor

INTRODUCTION

Effect of voltage-gated potassium channels in cancer

Voltage-gated K^+ (Kv) channels are well known as a class of ion channels that are expressed in a wide range of cells and tissues (Miller, 2000). They also play important roles in vital cellular signaling processes in both excitable and non-excitable cells. Kv channels are related to many different cellular processes, including the regulation of action potentials, cardiac pacemaking, signal integration, and neurotransmitter release in excitable cells. Kv channels are also important in non-excitable cells, such as various cancers and controlled cellular functions, including hormone secretion, cell volume regulation, and cell proliferation (Edwards and Weston, 1995; O'Grady and Lee, 2005; Pongs, 1999; Shieh et al., 2000). Kv channels are known to be involved in signaling pathways related to cell survival and cell death; their roles in proliferation and tumor growth have been demonstrated in numerous cancer cell lines, including prostate, colon, lung, and breast cancer (Camacho, 2006; Kunzelmann, 2005; Pardo, 2004; Pardo et al., 2005; Wonderlin and Strobl, 1996). For instance, certain kinds of Kv channels, such as Kv11.1, are involved in regulating apoptosis and proliferation (Wang et al., 2002), and other Kv channels, including Kv1.1 and Kv1.3, have demonstrated anticancer effects in the A549 lung cancer cell line through cell cycle arrest (Jang et al., 2011a; Jang et al., 2011b). Consequently, Kv channels are presented as potential therapeutic targets for various types of cancer (Jang et al., 2009a; Jang et al., 2009b; Kim et al., 2010; Rouzaire-Dubois and Dubois, 1998;

Zhou et al., 2003).

Application of potassium channel blockers in cancer therapy

Potassium channels are essential for cell proliferation and appear to have an important role in the development of several types of cancer cell lines. This has led to certain potassium channel blockers being suggested as novel drugs for cancer treatment through the induction of apoptosis or cell cycle arrest. For instance, clofilium is a blocker of delayed rectifier K^+ channels and exerts growth inhibitory and antiproliferation effects in the HL-60 human promyelocytic leukemia cell line by inducing apoptosis (Choi et al., 1999). Ca^{2+}-activated K^+ channel, on the other hand, is related to cell cycle control in the MCF7 breast cancer cell line, and clotrimazole, Ca^{2+}-activated K^+ channel blocker, could affect viability by arresting cell cycle progression at the end of the G_1 and S phases (Ouadid-Ahidouch et al., 2004). Recently, specific channel blockers, DTX-κ and MgTX, have been used to investigate the role of particular Kv subunit types Kv1.1 and Kv1.3 in cell survival and cell death in various cancer cell lines (Jang et al., 2011a; Jang et al., 2009b; Jang et al., 2011b). DTX-κ is derived from *Dendroaspis polylepis polylepis* and inhibits the Kv1 family of channels in a nanomolar range (Harvey, 1997). DTX-κ interacts with Kv1.1 through residues in its N-terminus and β-turn (Smith et al., 1997), which inhibits the fast-activating and slowly-inactivating voltage-dependent K^+ current produced by Kv1.1 (Wang et al., 1999). MgTX is isolated from venom of *Centruroides margaritatus* and the three C-terminal residues of MgTX are important for efficient toxin binding to Kv1.3

(Bednarek et al., 1994). MgTX significantly reduces outward currents of Kv1.3 channels and depolarized resting membrane potential (Cheong et al., 2011). PAP-1 is also a potent small molecule blocker of the Kv1. 3 channel and has from 33- to 125-fold selectivity over other Kv1 family channels (Schmitz et al., 2005).

Epidermal growth factor receptor and gefitinib resistance

Epidermal growth factor receptor (EGFR) is a member of the EGFR family, which consists of EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4), and is related to the signaling pathway that affects cell proliferation and cell survival (Yarden, 2001). Gefitinib is an EGFR-tyrosine kinase inhibitor (TKI) and is used in treating patients with advanced non-small cell lung cancer (NSCLC) (Fukuoka et al., 2003). Gefitinib inhibits phosphorylation of EGFR by tyrosine kinase activity and downstream signaling related to cell survival (Ono and Kuwano, 2006). Recent, studies have focused on EGFR as a target in the treatment of certain breast, lung, and other cancers (Gravalos and Jimeno, 2008; Maemondo et al., 2010; Siziopikou and Cobleigh, 2007). However, resistance to TKI, such as gefitinib or imatinib, has been reported (Kosaka et al., 2006). Gefitinib resistance is induced by the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation that is related to persistent activation of EGFR signaling (Pao et al., 2005). Approximately 15–30% of lung cancer cases include *KRAS* mutation, which is detected in codons 12 and 13 and in exon 2 (Bos, 1989). The H460 cell line is a gefitinib-resistant NSCLC cell line that harbors a mutation in codon 61 of *KRAS*, which is associated with unsatisfactory therapy

outcomes (Janmaat et al., 2006).

Purpose of the present study

Recent studies have revealed that several Kv channels play crucial roles connected with cell proliferation in cancer cells, and it is expected that Kv channels would become apposite targets to overcome resistance to anticancer agents. In general, it is well known that inhibition of Kv channels induces suppression of cell proliferation, which leads to various pharmacological approaches using certain Kv channel blockers in diverse cancer cell lines (Pardo, 2004). In other studies, resistance to gefitinib has become a concern in cancer research because of its restriction in anticancer efficacy. Consequently, development of new therapeutic targets or combination therapy with gefitinib and various anticancer drugs have been attempted to surmount the obstacle of gefitinib resistance to NSCLC (Bill et al., 2012; Janmaat et al., 2006; Park et al., 2010). In the present study we investigated the possibility of Kv channels, Kv1.1 and Kv1.3, as therapeutic targets of NSCLC. In addition, we examined whether combination treatment of gefitinib with Kv channel blockers DTX-κ or MgTX leads to synergistic anticancer effects in the gefitinib-resistant NSCLC cell lines H460 and A549.

MARTERIALS AND METHODS

Cell culture

H460 and A549 (Korean cell line bank, Seoul, Korea), human NSCLC cell lines, were cultured in RPMI 1640 medium (Welgene, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (Welgene, Daegu, Korea) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA). Cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

Total RNA extraction and RT-PCR

Total RNA was isolated from the H460 cell line by using Trizol (Takara Bio, Otsu, Japan). After DNase I (Takara) treatment to eliminate genomic DNA, the concentration of RNA was measured by UV spectrophotometry (Thermo Fisher Scientific Inc., Waltham, MA, USA). Extracted RNA was reverse transcribed into cDNA using random primers and a Moloney murine leukemia virus (MMLV) reverse transcription kit (Promega, Madison, WI, USA). Synthesized cDNA was amplified by PCR reaction to confirm the target genes using specific primers (Kv1.1 forward: 5'-ACATTGT GGCCATCATTCCT-3', reverse: 5'-GCTCTTCCCCCTCAGTTTCT-3'; Kv1.3 forward: 5'-GTACTTCTTCGACCGCAACC-3', reverse: 5'-ACCAGCAGTTCGAAGGAGAA-3') (Jang et al., 2011a; Jang et al., 2011b) synthesized at Cosmogenetech (Cosmogenetech Corporation, Busan, Korea) and 1 × GoTaq green master mix (Promega). The PCR reaction conditions of Kv1.1 were

initial denaturation at 94℃ for 5 min, cycling (35 cycles) at 94℃ for 40 s, 55℃ (Kv1.1) or 60℃ (Kv1.3) for 40 s, and 72℃ for 1 min, with a final extension at 72℃ for 7 min. The PCR product was electrophoresised on 1.5% agarose gel and stained with ethidium bromide.

Western blot analysis

H460 cell line was lysised by 1 × passive lysis buffer (Promega) and quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Approximately 25 μg of proteins were used for electrophoresis on 10% polyacrylamide gel and transferred to a 0.45 μm polyvinylidene fluoride (PVDF) membrane (Pall Corporation, Port Washington, NY, USA). Transferred membranes were blocked by 1 × TBS-Tween 20 containing 5% skim nonfat milk (5% TTBS) (Difco, Sparks, MD, USA) for 1 h at room temperature. After blocking, primary antibodies, Kv1.1 (1:1000) (Abcam, Cambridge, MA, USA) and Kv1.3 (1:500) (Alomone laboratories, Jerusalem, Israel), were bound overnight at 4°C, and on the second day, the membrane was probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (1:5000) (Santacruz Biotechnology, Santa Cruz, CA, USA) for 1 h. The results were detected using WEST-ZOL® plus Western Blot Detection System (Intron Biotechnology, Seongnam-Si, Gyeonggi-do, Korea).

Cell viability assay

H460 cell line was seeded in a 96-well plate with 5×10^3 cells/well in RPMI1640 medium with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. The 96-well plate was incubated at 37°C in humidified air containing 5% CO_2 for 24 h. The next day, cells were treated with 100 nM DTX-κ (Alomone laboratories), 1 nM MgTX (Alomone laboratories), two concentrations of 5-(4-Phenoxybutoxy) psoralen (PAP-1) (sigma), and various concentration of gefitinib (Santacruz Biotechnology) in fresh medium for 24 h. To confirm the relative proliferation rate, the drug and medium were removed and 0.5 mg/ml methylthiazol tetrazolium (MTT) solution (Sigma) was put in the 96-well plate for 4 h at 37°C. When H460 cell line formed formazan, the formazan was dissolved using dimethyl sulfoxide (Sigma) and the absorbance was measured using Infinite® F50/Robotic absorbance microplate readers (Tecan, Männedorf, Switzerland).

Analysis of cell cycle by flow cytometry

H460 cell line was treated with 100 nM DTX-κ or 1 nM MgTX for 24 h. Cell line was harvested and then washed with ice-cold PBS. To fix H460 cell line, samples were suspended in 70% ethanol for at least 40 min at −20°C. After fixation, the samples were incubated with 5 μl/ml of RNase A solution (Amresco, Solon, OH, USA) for 30 min at 37°C. Cell cycle arrest was measured using BD FACSCalibur™ (BD Biosciences, San Jose, CA, USA) staining cells with 40 μl/ml of propodium iodide (Sigma).

Xenograft model and injection of channel blockers

In vivo experiments were performed using five-week-old male CAnN.Cg Foxn1^{nu}/CrljOri nude mice. Nude mice were purchased from Orient Bio Inc. (Seongnam, Gyeonggi, Korea) and housed in sterile cages with filter lids. H460 cell line (1×10^6) was suspended in 100 μl 10% RPMI medium and injected subcutaneously into the skin of the back of nude mice. When the tumor volume increased to approximately 300-400 cm^3, the mice was randomly divided into experimental and control groups. DTX-κ and MgTX were injected directly into the tumor of the each experimental group, and the tumor size was measured once every other day. The final concentrations of channel blockers were 100 nM DTX-κ and 1 nM MgTX in the tumor tissue and tumor volume was measured using the formula volume = (width × length × depth) × (π/6) (Bakker et al., 1999). Tumor tissues were stored in liquid nitrogen after the experiment was finished.

Statistical analysis

Data are shown as means±standard error. Statistical significance was determined by *t*-test and Mann-Whitney U-test using the OriginPro 8 program (OriginLab Corporation, Northampton, MA, USA). Using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA), the half maximal inhibitory concentration (IC_{50}) values were calculated from log (inhibitor) *vs.* response curves. (Y=minimum response+(maximum response−minimum response)/(1+10^((X−logIC_{50}))); X=the logarithm of concentration; Y=the response)

RESULTS

Ⅰ. *Properties of Kv1.1 related to cell viability in the human NSCLC cell lines*

Kv1.1 mRNA and protein expression in H460 cell line

PCR and western blot analysis were performed to identify the expression of Kv1.1 mRNA and protein in H460 cell line. Kv1.1 mRNA was detected using RT-PCR analysis with the predicted mRNA size (498 bp) in H460 cell line (Figure 1A). The protein expression of Kv1.1 in H460 cell line was confirmed by western blot analysis with approximately 56 kDa (Figure 1B).

Inhibition of H460 cell viability by DTX-κ

To determine if the specific Kv1.1 blocker inhibits H460 cell viability, we performed an MTT assay. After treating H460 cell line with 100 nM DTX-κ for 24 h, cell viability was suppressed by 17% compared to the control (Figure 2). These results suggest that blockade of Kv1.1 inhibits cell viability of H460 cell line.

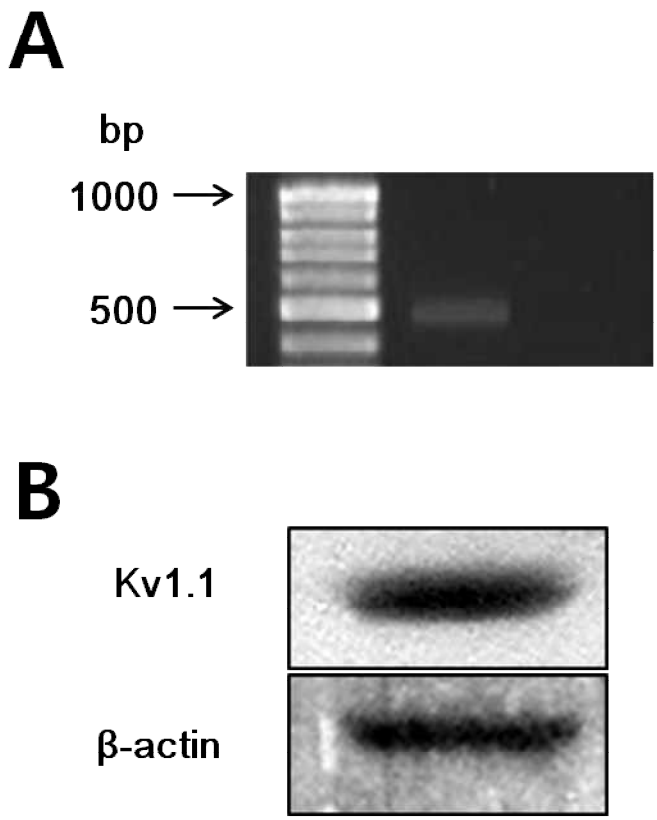

Figure 1. mRNA and protein expression of Kv1.1 in H460 cell line. (A) Kv1.1 mRNA was detected in H460 cell line (size of 498 base pairs). The results were obtained by electrophoresis of PCR products and confirmed by ethidium bromide staining. (B) The existence of Kv1.1 protein was identified by western blot analysis in H460 cell line.

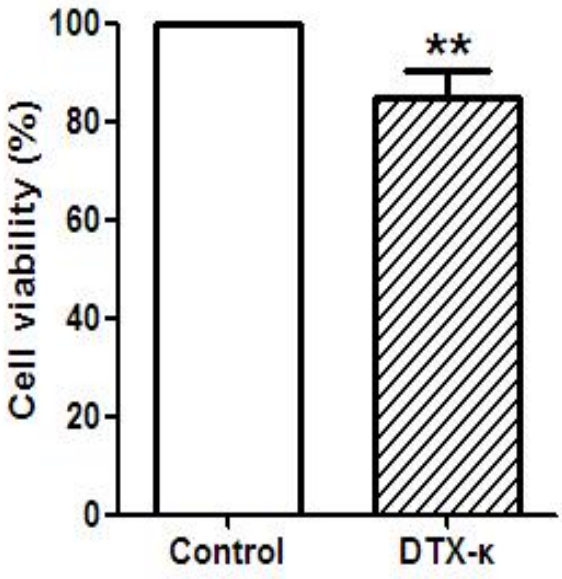

Figure 2. Inhibition of cell viability in H460 cell line by DTX-κ. H460 cell viability was significantly reduced by DTX-κ (100 nM for 24 h). The data were normalized to control values and are presented as the mean±standard error (control group: n=22; DTX-κ group: n=22; ** $p<0.01$).

Blockade of Kv1.1 unrelated to apoptosis

Apoptosis analysis was performed to confirm whether the blockade of Kv1.1 induced apoptosis. After treating with 100 nM DTX-κ for 24 h, proportion of apoptotic cells was measured by using flow cytometry. Percentage of apoptosis was not significantly changed (control: 0.94%; DTX-κ: 1.30%; Figure 3). These data suggested that blockade of Kv1.1 using DTX-κ was not affected apoptosis in H460 cell line.

Change of cell cycle distribution by blockade of Kv1.1 in H460 cell line

In order to confirm whether DTX-κ changes the cell cycle distribution, we performed cell cycle analysis using flow cytometry. Figure 4A shows the alteration of the cell cycle distribution after Kv1.1 blocker treatment for 24 h. The G_1 phase was significantly increased by 100 nM DTX-κ from 52.8±1.3% to 56±1.2% compared to the control. DTX-κ also reduced the proportion of S-phase cells from 23.9±3.2% to 20.7±0.7% (Figure 4B). These results show that the blockade of Kv1.1 by DTX-κ induces cell cycle arrest of H460 cell line during the G_1/S phase transition.

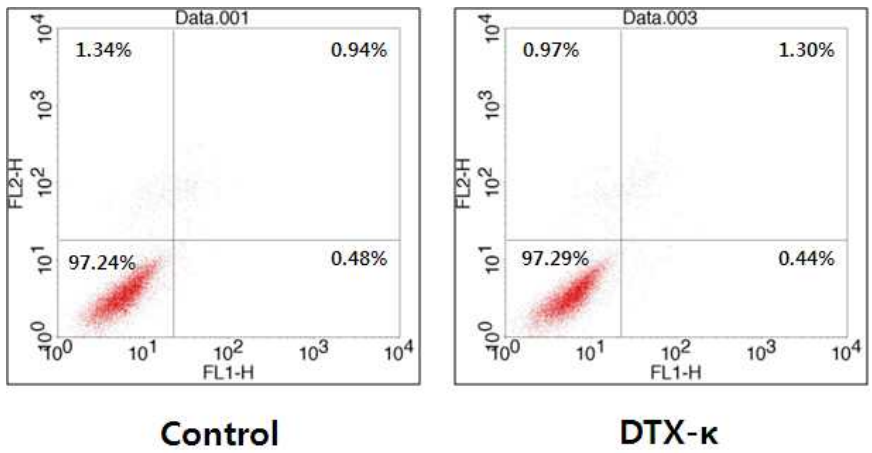

Figure 3. Blockade of Kv1.1 by DTX-κ did not induce apoptosis in H460 cell line. H460 cell line was analyzed by flow cytometry to confirm apoptosis after treatment of 100 nM DTX-κ for 24 h. Blockade of Kv1.3 by DTX-κ did not induce apoptosis in H460 cell line.

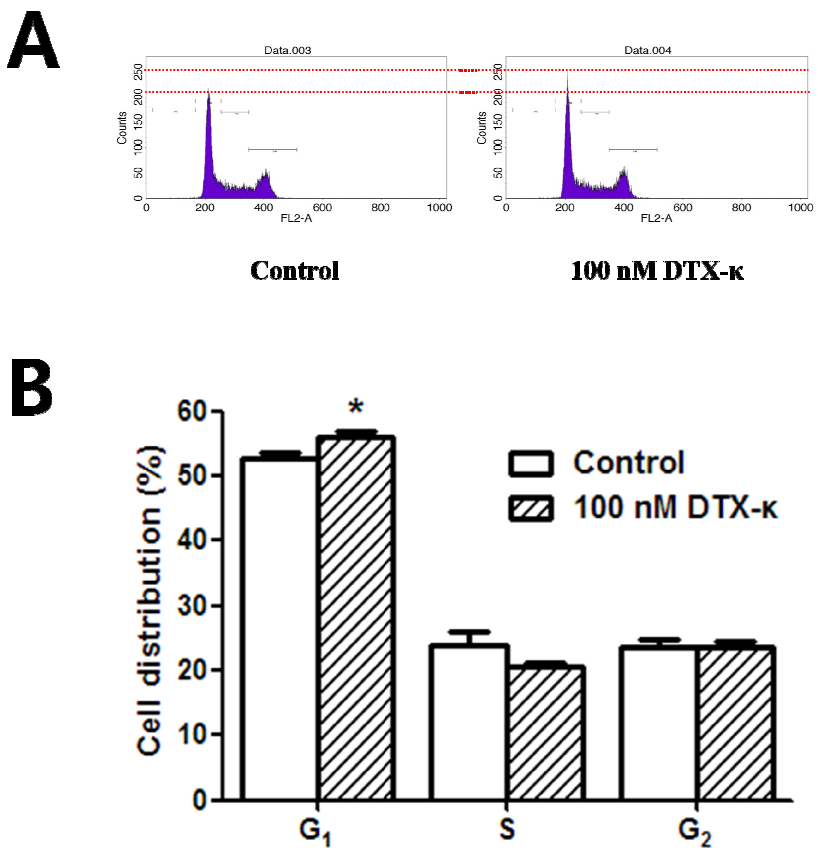

Figure 4. Change of cell cycle distribution by blockade of Kv1.1 in H460 cell line.

(A) The cell cycle distribution was changed by the blockade of Kv1.1 in H460 cell line. (B) Flow cytometry analysis demonstrated the effect of 100 nM DTX-κ on inhibiting cells in the G_1/S phase. Data were measured by flow cytometry and are presented as the mean±standard error (control group: n=3; DTX-κ group: n=3; * p<0.05).

Inhibition of tumor growth by DTX-κ in a xenograft model

We investigated whether DTX-κ has an anticancer effect on tumor tissues. We found that the relative tumor volume in mice was significantly reduced by DTX-κ compared to the control group (Figure 5A). After treatment of 1 nM DTX-κ for six days, the difference in tumor volume between the experimental and control groups were gradually increased by DTX-κ (Figure 5B). These results indicate that anticancer effects of DTX-κ were detected *in vivo* as well as *in vitro* (control group: n = 6; DTX-κ group: n = 6).

Synergistic effect of Kv1.1 blocker, DTX-κ, and gefitinib in H460 and A549 cell line

To evaluate whether the selective Kv1.1 blocker, DTX-κ, has synergistic effects with gefitinib, we performed an MTT assay. When 100 nM DTX-κ was combined with different concentrations of gefitinib, the viability of H460 and A549 cell lines, which are known to be gefitinib-resistant cell lines, was reduced compared to the groups treated with gefitinib only (Figure 6A and B). The IC_{50} values for gefitinib in H460 and A549 cell lines were 82.1 μM and 89.7 μM respectively. When the H460 and A549 cell lines were treated with a combination of DTX-κ and gefitinib, the IC_{50} values for gefitinib changed to 18.4 μM and 37.2 μM respectively.

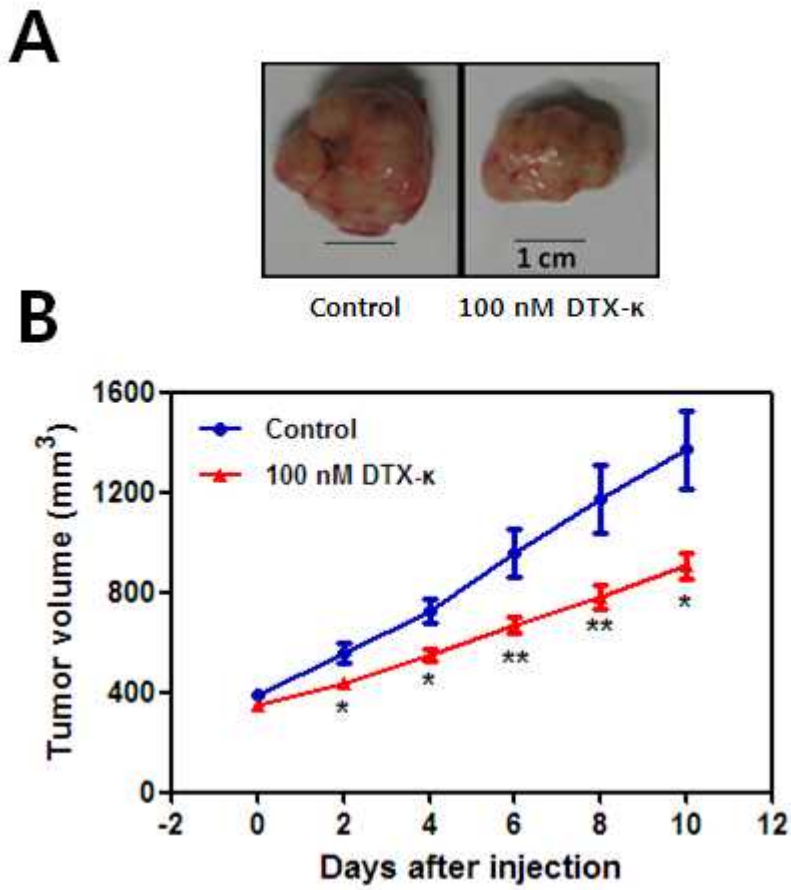

Figure 5. Inhibition of tumor growth by DTX-κ in a xenograft model. (A) Representative image of tumor tissue in each group. The image shows the anticancer effect of DTX-κ compared to the control (scale bar: 1 cm). (B) The graph shows the tumor size changed after a treatment of 100 nM DTX-κ compared to the untreated control. DTX-κ suppressed tumor growth in the xenograft model. Tumor volume was measured once every other day (control group: n=6; DTX-κ group: n=6; * $p<0.05$; ** $p<0.01$)

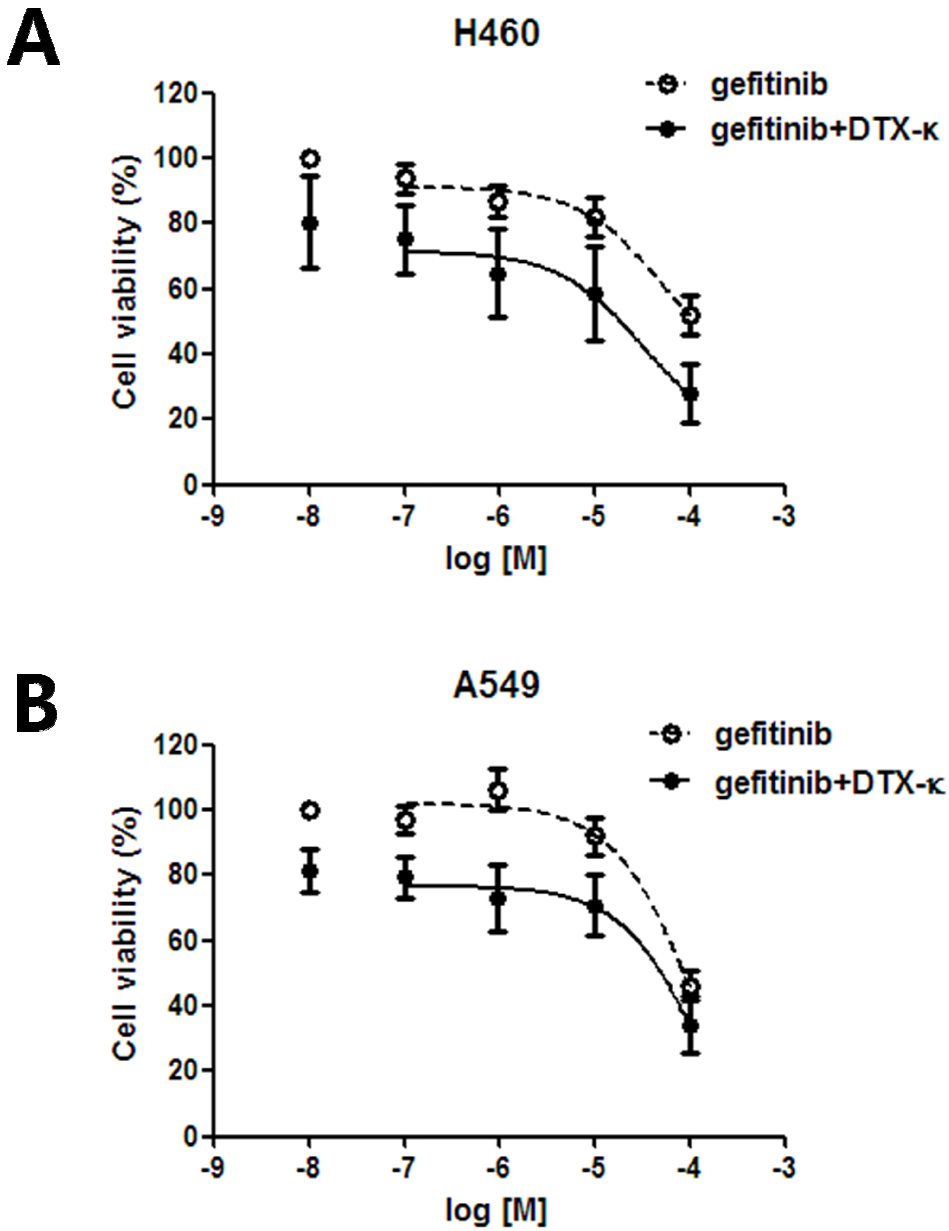
A
H460
gefitinib
gefitinib+DTX-κ
Cell viability (%)
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
-3
log [M]
B
A549
gefitinib
gefitinib+DTX-κ
Cell viability (%)
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
-3
log [M]

Figure 6. Effects of combination treatment using gefitinib with DTX-κ in H460 and A549 cell lines. Combination treatment with 100 nM DTX-κ and gefitinib provided synergistic anticancer effects compared to each drug alone in H460 (A) and A549 (B) lung cancer cell lines. These data were normalized by these of the control group and are presented as the mean±standard error (H460: gefitinib group: n=19 and gefitinib+DTX-κ group: n=7; A549: gefitinib group: n=22 and gefitinib+DTX-κ group: n=8).

Ⅱ. *Properties of Kv1.3 related to cell viability in the human NSCLC cell lines*

Expression of Kv1.3 mRNA and protein in H460 cell line

Kv1.3 mRNA and protein was detected using RT-PCR and western blot analysis in H460 cell line. Kv1.3 mRNA was confirmed with the expected product size of 498 base pairs in H460 cell line (Figure 7A). Kv1.3 protein was also found by western blot analysis with approximately 43 kDa (Figure 7B).

Inhibition of H460 viability by blockade of Kv1.3

To demonstrate whether the blockade of Kv 1.3 affected H460 cell viability, we performed an MTT assay. The treatment of 1 nM MgTx for 24 h induced suppression of H460 cell viability by 10% (Figure 8A). In addition, H460 cell viability was gradually decreased by treating 100 pM and 100 nM PAP-1 for 24 h (Figure 8B). These results demonstrate that Kv1.3 was related to the cell proliferation. Also, H460 cell viability was decreased by blockade of Kv1.3 in H460 cell line.

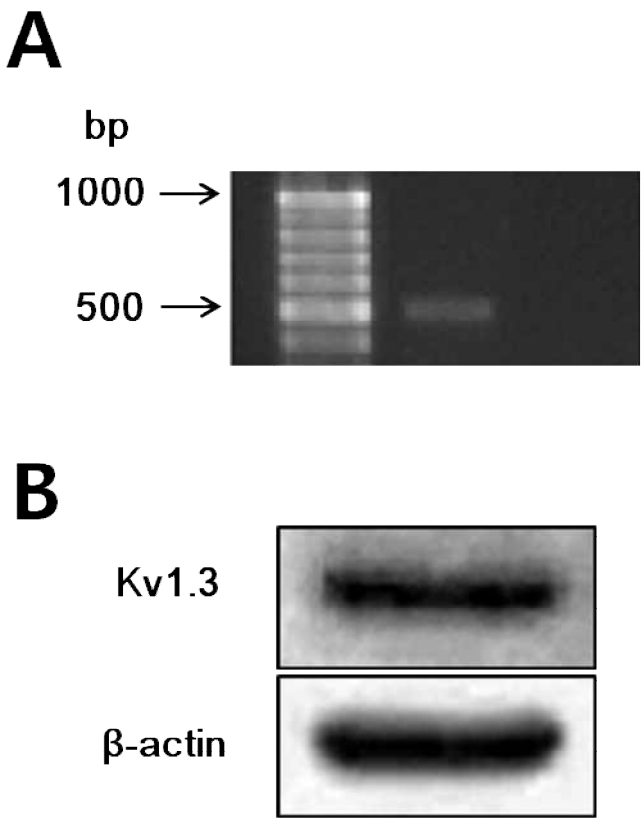

Figure 7. mRNA and protein expression of Kv1.3 in H460 cell line. (A) The expression of Kv1.3 mRNA was identified in H460 cell line (size of 498 base pairs). PCR products were confirmed by electrophoresis on 1.6% agarose gel and ethidium bromide stain. (B) The protein of Kv1.3 in H460 cell line was detected by western blot analysis.

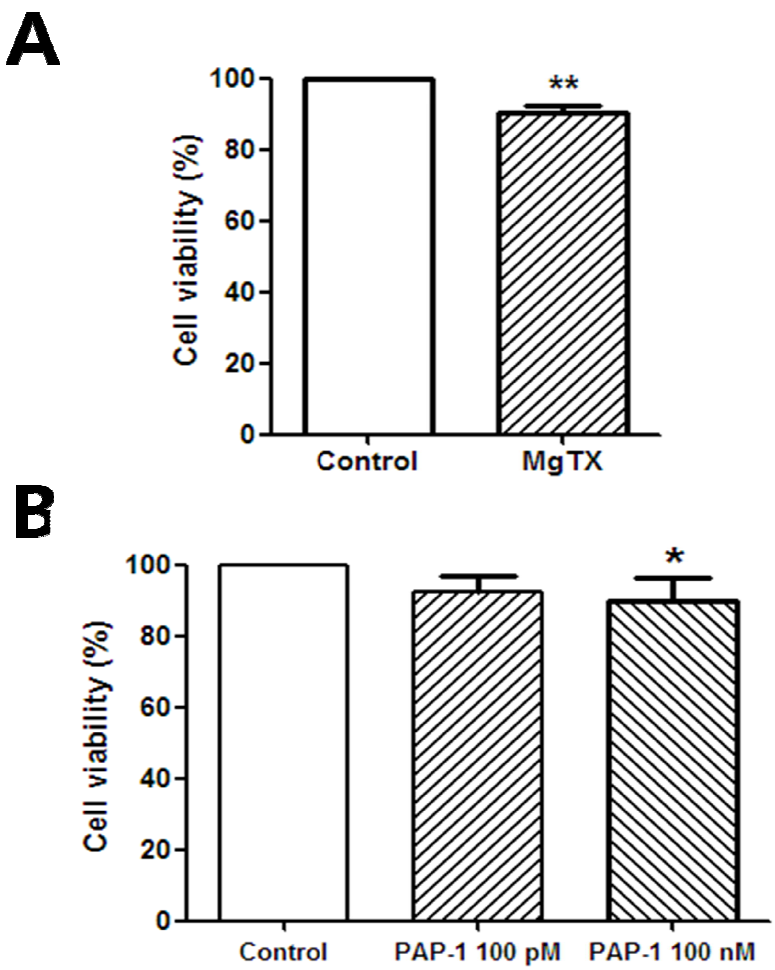

Figure 8. Suppression of cell viability by treating MgTX and PAP-1 in H460 cell line. H460 cell line was treated with 1 nM MgTX (A), 100 pM and 100 nM PAP-1 (B) for 24 h. The data were normalized to control values and standard error obtained in multiple independent MTT assay. The results are presented as the mean±standard error (control group: n=18; MgTX group: n=18; PAP-1: n=12; * $p<0.05$; ** $p<0.01$)

Blockade of Kv1.3 unrelated to apoptosis

In order to confirm whether MgTX induces the apoptosis, we performed apoptosis analysis using flow cytometry. Figure 9 shows blockade of Kv1.3 using 1 nM MgTX for 24 h was not related to apoptosis in H460 cell line. Percentage of apoptotic cells was not statistically different compared with MgTX groups (control: 0.94%; DTX-κ: 1.10%; Figure 9). These results show that the blockade of Kv1.3 by MgTX was not associated with apoptosis in H460 cell line.

Cell cycle arrest by blockade of Kv1.3 in H460 cell line

Cell cycle analysis was performed to evaluate whether the Kv1.3 blockade affected cell cycle regulation. After treating with 1 nM MgTX for 24 h, changes in cell cycles were measured by using flow cytometry. The results of the flow cytometry demonstrated that the G_1 phase was significantly increased (control: 52.7±0.7%; MgTX: 56.6±0.4%). MgTX also induced the diminishment of cells in the S-phase (control: 23.9±1.9%; MgTX: 20.7±0.1%; Figure 10A and B). These data suggested that Kv1.3 blockade using MgTX decreased cell viability through G_1-S transition.

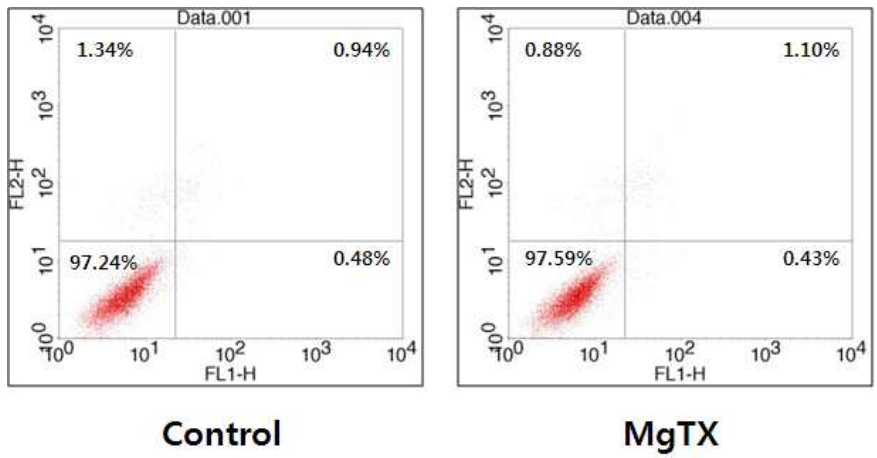

Figure 9. Blockade of Kv1.3 by MgTX did not induce apoptosis in H460 cell line. Apoptosis analysis was performed by flow cytometry to confirm apoptotic effects of the blockade of Kv1.3. Apoptosis was not induced by treating 1 nM MgTX for 24 h in H460 cell line.

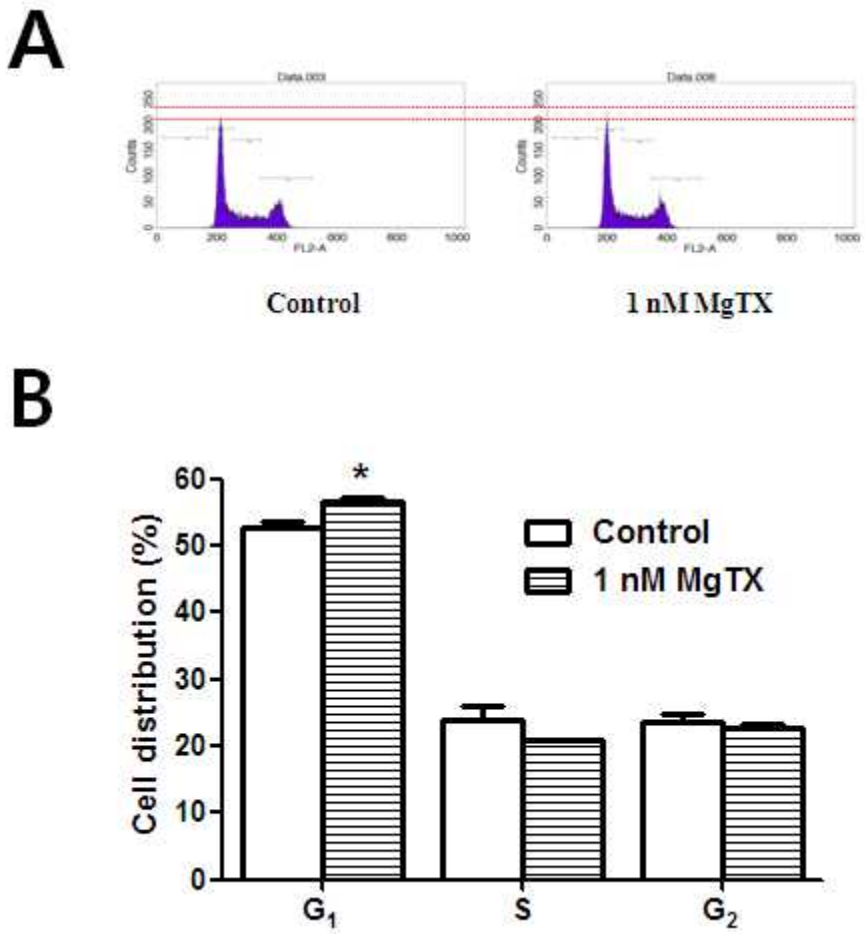

Figure 10. Cell cycle arrest induced by blockade of Kv1.3 in H460 cell line. (A) H460 cell line was treated with 1nM MgTX for 24 h, and analyzed by flow cytometry. (B) Blockade of Kv1.3 by MgTX was also induced inhibition of cell cycle in the G_1/S phase. The results are presented as the mean±standard error (control group: n=3; MgTX group: n=3; * p<0.05).

Anticancer effects of MgTX against H460 xenograft model

In order to determine whether the blockade of Kv1.3 induced suppression of tumor growth *in vivo,* we performed an experiment using a xenograft model. When MgTX was injected regularly into tumor tissues, the relative tumor volume in xenograft was significantly reduced compared to the control (Figure 11A and B). These data indicated that MgTX has anticancer effects on H460 cells *in vivo*.

Synergistic effect of MgTX with gefitinib in H460 cell line

We performed an MTT assay to investigate whether MgTX shows the synergistic effects with gefitinib. When 1 nM MgTX was treated with various concentration of gefitinib in H460 cell line, the combination treatment with MgTX and gefitinib revealed synergisitic effects compared to MgTX or gefitinib groups in the low gefitinib concentration range (Figure 12). These results suggest that MgTX has the possibility as additional option to overcome gefitinib-resistant in H460 cell line by treating with gefitinib.

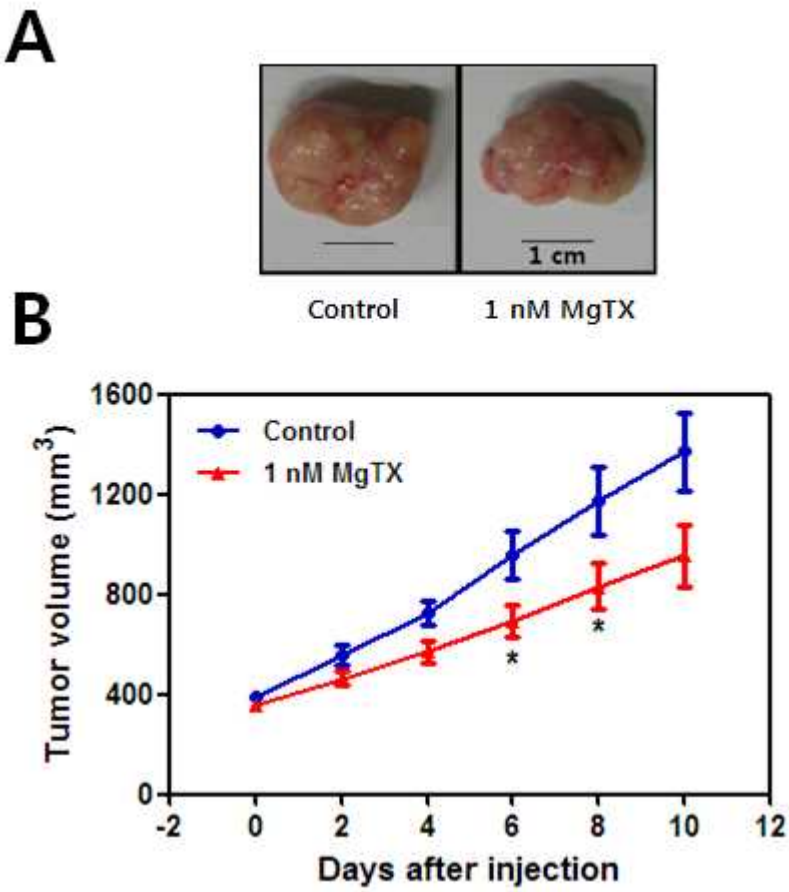

Figure 11. Anticancer effects of MgTX in a xenograft model. (A) The image shows the tumor tissue of each group and the inhibition of tumor growth by treating MgTX compared to the control (scale bar: 1 cm). (B) The graph indicates change of tumor size resulted from treatment of 1 nM MgTX compared to the untreated group in the nude mice. Tumor size was measured once every other day (control group: n=6; MgTX group: n=6; * p<0.05).

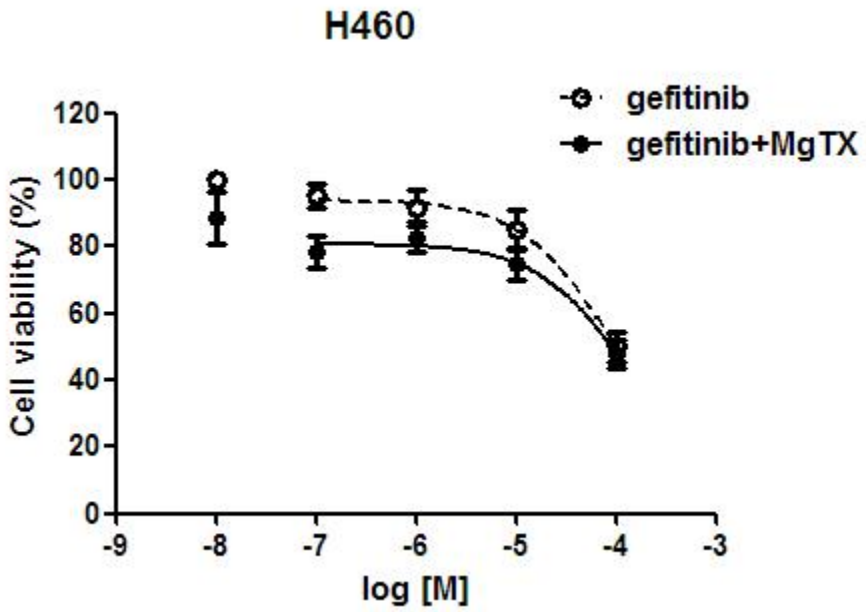

Figure 12. Synergistic effects of combination treatment with MgTX and gefitinib in H460 cell line. Additional treatment of MgTX with gefitinib signified synergistic anticancer effects compared to the gefitinib group in H460 NSCLC. The result was normalized by the control group and presented as the mean±standard error (gefitinib group: n=27 and gefitinib+MgTX group: n=7; * $p<0.05$).

DISCUSSION

The results of this study revealed that mRNA and protein of the Kv channels Kv1.1 and Kv1.3 were expressed in the gefitinib-resistant H460 NSCLC cell line. The blockade of Kv1.1 or Kv1.3 by treating each channel blocker (Kv1.1, DTX-κ; Kv1.3, MgTX, or PAP-1) could suppress viability of the H460 cell line though cell cycle arrest at the G_1/S transition. The anticancer effects of both DTX-κ and MgTX were also detected in xenograft models using nude mice. Moreover, we verified that combination treatment of gefitinib with DTX-κ or MgTX led to synergistic anticancer effects in the gefitinib-resistant H460 and A549 NSCLC cell lines.

Kv channels as a therapeutic target of cancer

We revealed that Kv1.1 and Kv1.3 were related to the viability of the gefitinib-resistant H460 NSCLC cell line. Kv channels and their role in cell proliferation have been continually investigated in cancer research, and many studies have demonstrated that various types of Kv channel are involved in the cell proliferation in breast (Jang et al., 2009a; Jang et al., 2009b; Ouadid-Ahidouch et al., 2000), gastric (Kim et al., 2010), and lung (Jang et al., 2011a; Jang et al., 2011b) cancer cell lines. Furthermore, Kv10.1 and Kv11.1 were reported to be highly overexpressed in several cancers and were considered as potential cancer biomarkers in diagnosis and prognosis (Pardo, 2004). Also, altered expression of Kv1.3 and Kv1.5

channels were found in several types of cancer cell lines (Comes et al., 2013; Jang et al., 2009b). In addition, Kv1.1, Kv1.3, Kv1.5, and Kv11.1 have contributed to apoptosis in various cell lines, and it has been demonstrated that expression of Kv1.1 and Kv1.3 represents sensitization of various cancer cell lines to cytotoxins (Leanza et al., 2013). Taken together, it is commonly accepted that blockade of Kv channels leads to the suppression of cell viability, making Kv channels promising targets for cancer therapy. Therefore, experiments regarding the role of Kv channels in cancer cell lines are exploited for pharmacological approaches to promote effects of chemotherapy.

Cell cycle arrest induced by blocking Kv channels

We confirmed that suppression of cell viability resulted from alteration of cell distribution by the blockade of Kv1.1 and Kv1.3 in the H460 cell line. Wonderlin and Strobl (1996) demonstrated that alteration of K^+ channel activity could influence various signaling due to the change in membrane potential, which modifies checkpoint control of the cell cycle through the G_1 phase. Cells need Kv channels to maintain the cell cycle, and blockade of Kv channels has been shown to be anti-proliferative in various types of cancer cell lines (Pardo, 2004). For instance, inhibition of human *ether-a-go-go*-related gene (*hERG*) potassium channel, Kv11.1, causes cell cycle arrest at the G_0/G_1 phase in the C-33A uterine cancer cell line (Suzuki and Takimoto, 2004) and blockade of Kv4.1 induced cell cycle arrest at the G_1/S transition in MKN-45 and SNU-638 (Kim et al., 2010). In addition, when Kv1.1 and Kv1.3 were blocked by DTX-κ and MgTX in the A549 cell line, cell viability decreased and the expression

level of proteins related to the G_1/S phase, such as $p15^{INK4B}$, $P21^{WAF1/CIP1}$, $p27^{KIP1}$, Cdk4, and cyclin D3, was altered (Jang et al., 2011a; Jang et al., 2011b). Several ion channels participate in the processes related to G_2/M phase transition of the cell cycle. For example, electrophysiological properties of Kv10.1 were altered with cell cycle progression, and currents of Kv10.1 were blocked during the G_2/M transition in Chinese hamster ovary mammalian somatic cell line (Camacho et al., 2000). Moreover, transient receptor potential channel6 (TRPC6) was highly expressed in gastric cancer epithelial cell lines compared to normal gastric epithelial cell lines, and blockade of TRPC6 using SKF96365 induced cell cycle arrest in the G_2/M phase and suppression of cell growth in gastric cell lines (Cai et al., 2009).

Possible mechanisms of Kv channels related to cell proliferation

Alteration of cell volume, effects of growth factor, and control of membrane potential on Kv channels have been postulated as possible mechanisms of Kv channels in regulating cell proliferation (Pardo, 2004).

Many studies have demonstrated that the alteration of cell volume is connected with cell proliferation (Dubois and Rouzaire-Dubois, 2004; Lang et al., 2000; Rehberg et al., 2013). K^+ channel blockers can alter the K^+ ion concentration and affect cell volume, resulting in inhibition of proliferation (Wonderlin and Strobl, 1996).

Kv channels are also important for pathways connected with various growth factors. Upregulation of delayed rectifier K^+ currents and increments of Kv1.3 mRNA expression were detected after treatment of transforming growth factor-β in microglia

(Schilling et al., 2000). In addition, Kv1.3 is modulated by epidermal growth factor (EGF), and treatment of EGF induced suppression of the Kv1.3 currents in the HEK 293 cell line co-expressing Kv1.3 and EGFR (Bowlby et al., 1997). Also, blockade of K^+ channel by 4-aminopyridine suppressed the activity of extracellular signal-regulated protein kinase 2 that resulted from interaction between EGF and EGFR in the human myeloblastic leukemia ML-1 cell line (Xu et al., 1999).

Membrane voltage is important in cell cycle progression (Sachs et al., 1974). It has been demonstrated that K^+ channels play important roles in regulating cell cycle progression (Ouadid-Ahidouch and Ahidouch, 2013). Because hyperpolarization is required for proper cell cycle at the early G_1 phase, blockade of Kv channels using their blockers leads to prevention of G_1–S progression by depolarizing the membrane potential of cells (Pardo, 2004).

None of the hypotheses suitably explains the role of Kv channels in cell proliferation. Further studies are warranted to verify the specific mechanisms related to cell proliferation regulation by Kv channel blockers.

EGFR signaling and the mechanism of gefitinib

EGFR plays critical roles in the operation of signaling networks affecting cell proliferation, inhibition of apoptosis, invasion and metastasis, and tumor-induced angiogenesis in common solid tumors (Ono and Kuwano, 2006). In general, high EGFR activity is detected in several tumor types, including NSCLC. Activation of

EGFR by binding specific ligands, such as EGF and transforming growth factor (TGF), induced initiation of downstream signaling pathways, including the Ras/MAPK and Akt/mTOR kinase cascades, which regulate transcription factors and other proteins involved in cell proliferation, survival, motility, and differentiation (Normanno et al., 2006). Gefitinib is a well-known EGFR-TKI (Armour and Watkins, 2010). Gefitinib inhibits the autophosphorylation of EGF-stimulated EGFR in EGFR-expressing cancer cell lines; this blocks the signal transduction pathway implicated in cell proliferation and survival. Therapeutic effects of gefitinib are continually being investigated in various cancer types, including breast, gastric, and prostate cancers (Baselga et al., 2005; Kris et al., 2003; Rojo et al., 2006). For this reason, EGFR using gefitinib is an attractive target of anticancer therapy in a wide range of cancer cell lines.

Gefitinib resistance and combination treatment

Gefitinib resistance has been reported in several lung cancers, and *EGFR* and *KRAS* mutations have been suggested as major mechanisms of gefitinib resistance (Kobayashi et al., 2005; Pao et al., 2005). When patients receive treatments using gefitinib for cancer, gefitinib resistance has been shown to be associated with worse clinical outcomes. To surmount the resistance of TKI, the molecular mechanisms of resistance have been investigated and many candidate targets have been suggested (Giaccone and Wang, 2011). Combination therapy has been considered as a possible alternative to improve treatment efficiency in drug-resistant cancer cell lines. Combination treatment with gefitinib and various anticancer drugs have been tried to

overcome gefitinib resistance in gefitinib-resistant NSCLC (Ciardiello et al., 2000; Sirotnak et al., 2000). Although the specific mechanisms remain to be determined, the results of that study suggest that combination treatment of Kv channel blockers and gefitinib is a possible option to overcome resistance of gefitinib in a gefitinib-resistant NSCLC cell lines.

CONCLUSIONS

We investigated the effects of DTX-κ (Kv1.1 specific blocker) and MgTX and PAP-1 (Kv1.3 specific blockers) in gefitinib-resistance H460 NSCLC. We found Kv1.1 and Kv1.3 to be expressed in the H460 cell line, and treatment of DTX-κ, MgTX, and PAP-1 induced inhibition of H460 cell viability. Cell cycle analysis by using flow cytometry demonstrated that inhibitory effects of the Kv channel blockers DTX-κ and MgTX resulted from cell cycle arrest at the G_1/S transition. Moreover, anticancer effects of DTX-κ and MgTX were detected in *in vivo* studies using a xenograft model, indicating that DTX-κ and MgTX have anticancer effects both *in vitro* and *in vivo*. In addition, combination treatment of gefitinib with DTX-κ or MgTX demonstrated synergistic effects by combining different effects of each drug in Kv1.1- and Kv1.3-expressing NSCLC H460 and A549 cell lines. In conclusion, this study provides the possibility of using Kv channels Kv1.1 and Kv1.3 as therapeutic targets to increase the therapeutic effects in gefitinib-resistant NSCLC. In addition, certain Kv channel blockers could be used as possible substitutes for anticancer drugs that are ineffective in drug-resistant cancer cell lines.

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국문초록

게피티니브 내성 H460 폐암세포주에서 전압의존성 포타슘 채널 차단제 Dendrotoxin-κ 와 Margatoxin의 항암효과

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세포막에 존재하는 이온 채널은 다양한 세포 내 기능과 관련이 있다고 잘 알려져 있다. 특히, 전압 의존성 포타슘 (Kv) 채널은 폐암세포주를 포함한 여러 종류의 암세포주 생존에 영향을 미치며 특정 전압 의존성 포타슘 채널의 차단제는 암세포주의 증식을 억제시키는 것으로 확인되고 있다. 따라서 전압 의존성 포타슘 채널은 새로운 항암치료법 개발에 있어서 유망한 치료 대상으로서의 가능성으로 관심을 받고 있다. 본 연구는 게피티니브에 내성을 보이는 H460 폐암세포주에서 전압 의존성 포타슘 채널 차단제들의 효과에 대해 연구하였다. 1) H460 폐암세포주에서 Kv1.1과 Kv1.3의 mRNA와 단백질이 발현됨을 확인하였고 2) Kv1.1의 특이적 차단제인 dendrotoxin-κ (DTX-κ) 또는 Kv1.3의 특이적 차단제인 margatoxin (MgTX)과 5-(4-phenoxybutoxy) psoralen (PAP-1)을 처리하였을 경우 세포자살이 아닌 세포주기 중 G_1기에서

S기로의 진행 과정이 억제됨에 따라 H460 세포의 생존능력이 줄어들었다. 3) 또한, 전압의존성 포타슘 채널 차단제의 항암 효과는 누드마우스를 이용한 이종이식 모델에서도 동일하게 나타났으며 DTX-κ 또는 MgTX을 종양 조직에 투여했을 때 종양의 성장이 유의적으로 억제되었고 4) 게피티니브와 DTX-κ 또는 MgTX의 병용 처치가 H460 세포주에서 상승적인 항암 효과를 보임을 MTT assay를 통해 관찰하였다. 위와 같은 연구 결과는 DTX-κ와 MgTX이 게피티니브에 내성을 보이는 H460 세포주에서 항암 효과를 갖는다는 사실을 *in vitro*와 *in vivo*를 통해 보여주고 있다. 세포 생존능력에 대한 Kv1.1과 Kv1.3의 관련성은 이들 채널들이 게피티니브 내성 폐암세포주의 새로운 치료제 개발을 위한 분자 타켓이 될 수 있다는 사실을 보여준다.

주요어: Kv1.1, Kv1.3, 채널 차단제, 세포주기억제, 세포자살, 이종이식
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