Effects of Voltage-gated K^+ Channel Blockers in Gefitinib-resistant H460 Non-small Cell Lung Cancer Cells

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Abstract. *Voltage-gated K^+ (Kv) channels are known to be associated with the proliferation of several types of cancer cells, including lung adenocarcinoma cells, and certain Kv channel blockers inhibit cancer cell proliferation. In the present study, we investigated the effects of Kv channel blockers in gefitinib-resistant H460 non-small cell lung cancer (NSCLC) cells. Treatment with dendrotoxin-κ (DTX-κ), which is a Kv1.1-specific blocker, reduced H460 cell viability and arrested cells in G_1/S transition during cell-cycle progression. We administered DTX-κ in a xenograft model using nude mice. The tumor volume was reduced by the injection of DTX-κ into the tumor tissues compared to the control group. These results indicate that DTX-κ has antitumor effects in gefitinib-resistant H460 cells through the pathway governing the G_1/S transition both in vitro and in vivo. These findings suggest that Kv1.1 could serve as a novel therapeutic target for gefitinib-resistant NSCLC.*

Voltage-gated K^+ (Kv) channels are a class of ion channels that are expressed in a wide range of cells and tissues (1). They also play important roles in vital cellular signaling processes in both excitable and non-excitable cells (2-5). 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 (6). Kv channels are also important in non-excitable cells, such as various types of cancers, and controlled cellular functions such as hormone secretion, cell volume regulation, and cell proliferation (6, 7). The role of Kv channels in proliferation and tumor growth have been demonstrated for a number of cancer types, including prostate, colon, lung, and breast cancer cells (8-12). In addition, several Kv channel subunits are known to be involved in apoptosis pathways (13), and the blockade of Kv1.1 or Kv1.3 reduces cell proliferation in A549 cells due to cell-cycle arrest (14, 15). Therefore, Kv channels are presented as potential therapeutic targets for various types of cancers (4, 11, 16-20).

Dendrotoxin-κ (DTX-κ) derives from *Dendroaspis polylepis polylepis* and inhibits the Kv1 family of channels in a nanomolar range (21). DTX-κ interacts with Kv1.1 through residues in its *N*-terminus and β-turn (22). DTX-κ inhibits the fast-activating, slowly-inactivating voltage-dependent K^+ current, produced by Kv1.1 (23). Recently, specific channel blockers have been used to investigate the role of Kv channels in cell survival.

Gefitinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (24) and is used in treating patients with advanced non-small cell lung cancer (NSCLC). Gefitinib blocks the binding between ligands, such as epidermal growth factor (EGF) and its receptor EGFR, which inhibits phosphorylation of EGFR by tyrosine kinase activity and downstream signaling related to cell survival (24). Recent studies have found that gefitinib resistance is induced by the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation which is related to persistent activation of EGFR signaling (25, 26). Approximately 15%-30% of lung cancer cases include the *KRAS* mutation, which is detected in codons 12 and 13 and in exon 2 (27, 28). This mutation may be associated with unsatisfactory therapy outcomes (29). H460 cells are gefitinib-resistant NSCLC cells that harbor a mutation in codon 61 of *KRAS* (30). Combinatorial therapy has been suggested as a promising approach to overcome such drug resistance (31, 32). Several combination therapies have been suggested for NSCLC (30, 33, 34).

In the present study, we investigated the possibility of Kv channels as therapeutic targets of NSCLC. In addition, we examined whether combination treatment with Kv channel blockers and gefitinib results in synergistic anticancer effects on gefitinib-resistant NSCLC using H460 and A549 cells.

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Key Words: Dendrotoxin-κ, gefitinib, Kv1.1, non-small cell lung cancer, xenograft, channel blockers.

Materials and Methods

Cell culture. Human lung adenocarcinoma H460 cells (Korean cell line bank, Seoul, Korea) were cultured in RPMI-1640 medium (Welgene, Daegu, Korea) with 10% fetal bovine serum (Welgene) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA). Cells were grown at 37˚C in humidified air, containing 5% CO_2.

RT-PCR. Total RNA was extracted using Trizol (Takara Bio, Otsu, Japan). The purity and concentration of RNA were measured by UV spectrophotometry (Thermo Fisher Scientific Inc, Waltham, MA, USA). cDNA (20 μl) was synthesized by 2 μg of extracted RNA using random primers and an 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'-ACATTGTGGCCATCATTCCT-3', reverse: 5'-GCTCTTCCCCCTC AGTTTCT-3') (15) synthesized at Cosmogenetech (Cosmogenetech Corporation, Busan, Korea) and 1 × GoTaq green master mix (Promega, Madison, WI, USA). The PCR reaction conditions of Kv1.1 were; Initial denaturation at 94˚C for 5 min, cycling (35 cycles) at 94˚C for 40 s, 55˚C for 40 s, and 72˚C for 1 min, with a final extension at 72˚C for 7 min. The PCR product was electrophoresised on 1.5% agarose gel and stained with ethidium bromide.

Western blot. H460 cells were 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 protein 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% skimmed non-fat milk (5% TTBS) (Difco, Sparks, MD, USA) for 1 h at room temperature. After blocking, primary antibodies were bound overnight at 4˚C: Kv1.1 (1:1000) (Abcam, Cambridge, MA, USA), 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, CA, USA) for 1 h. The results were detected using WEST-ZOL® plus Western Blot Detection System (iNtRON Biotechnology, Gyeonggi, Korea).

Cell viability assay. H460 cells were seeded in a 96-well plate with 2×10^3 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 labs, Jerusalem, Israel), various concentration of gefitinib (Santacruz Biotechnology, CA, USA), or the combination of DTX-κ and gefitinib in fresh medium for 24 h. In order to confirm the relative proliferation rate, the drug and medium were removed and 0.5 mg/ml methylthiazoltetrazolium (MTT) solution (Sigma) was put in the 96-well plate for 4 h at 37˚C. When H460 cells 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 cells were treated with 100 nM DTX-κ for 24 h. Cells were harvested and then washed with ice-cold PBS. To fix H460 cells, 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 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).

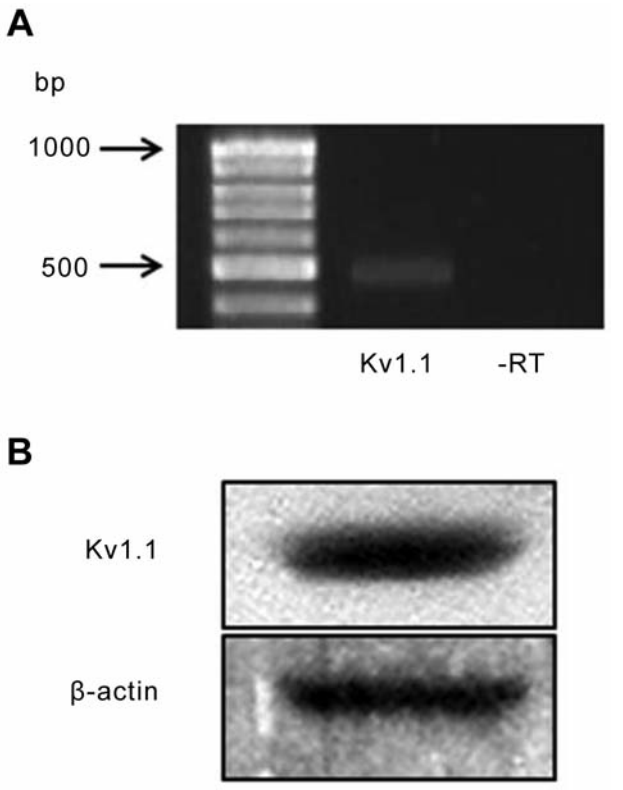

Figure 1. *mRNA and protein expression of Kv1.1 in H460 cells. A: Kv1.1 mRNA was detected in H460 cells. The results were obtained by electrophoresis of polymerase chain reaction products and confirmed by ethidium bromide staining. B: The existence of Kv1.1 protein was identified by western blot analysis in H460 cells.*

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. (Gyeonggi, Korea) and housed in sterile cages with filter lids. H460 cells (1×10^6) were 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 were divided into DTX-κ treatment group and control group. DTX-κ was injected directly into the tumor; the final concentration of DTX-κ was approximately 100 nM in the tumor tissue. Selective Kv1.1 channel blocker injection and tumor size measurement were performed once every other day until 10 days. Tumor tissues were stored in liquid nitrogen after the experiment was finished. Tumor volume was measured using the formula volume = (width × length × depth) × (π/6).

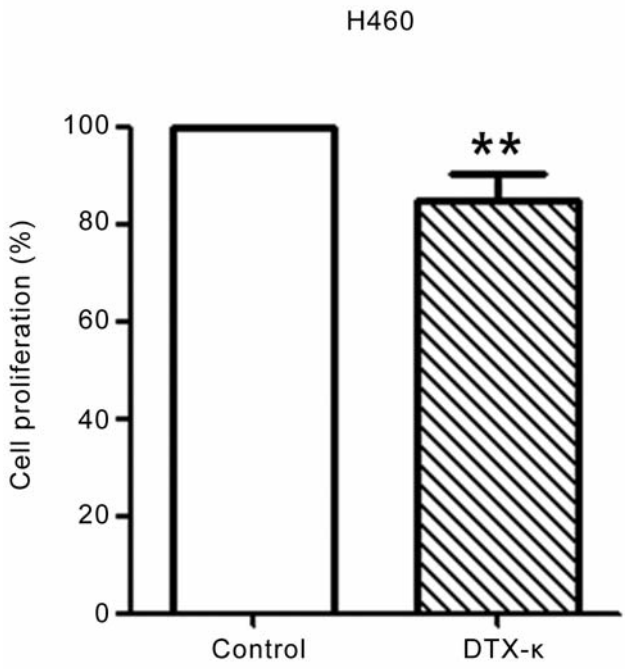

Figure 2. *Inhibition of cell proliferation in H460 cells by dendrotoxin-κ (DTX-κ). Cell proliferation in H460 cells was significantly reduced by DTX-κ (100 nM for 24 h). The data were normalized to control values and are presented as the mean±standard error (control group: n=22; DTX-κ group: n=22) (**p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

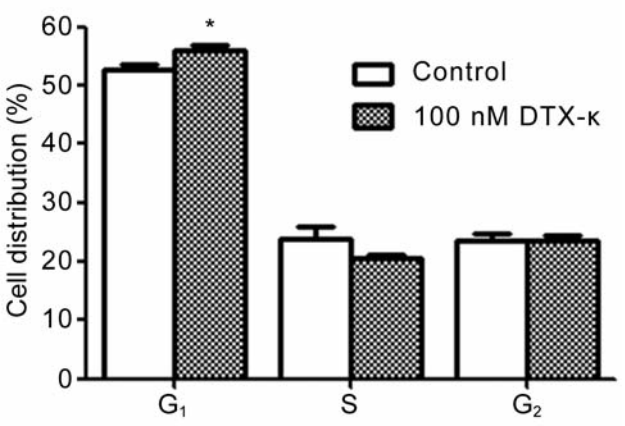

Figure 3. *Change of cell-cycle distribution by blockade of Kv1.1 in H460 cells. The cell-cycle distribution was changed by the blockade of Kv1.1 in H460 cells. The results show the effect of 100 nM DTX-κ on inhibiting cells in the G_1/S phase. Data were measured by flow cytometry and are presented as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

Statistical analysis. Data are presented as means±standard error. Statistical significance was determined by the *t*-test and Mann-Whitney *U*-test using the OriginPro 8 program (OriginLab Corporation, Northampton, MA, USA). Using the 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}))), where X is the logarithm of concentration and Y is the response .

Results

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

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

Change of cell-cycle distribution by specific blockade of Kv1.1 in H460 cells. In order to confirm whether DTX-κ changes the cell-cycle distribution, we performed cell-cycle analysis using flow cytometry. Figure 3 shows the alteration of the cell-cycle distribution after Kv1.1 blockade 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 3). These results show that the blockade of Kv1.1 by DTX-κ induces cell-cycle arrest of H460 cells during the G_1/S phase transition.

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 4A). After treatment of 1 nM DTX-κ for six days, the difference in tumor volume between the experimental and control groups gradually increased by DTX-κ (Figure 4B) (control group: n=6; DTX-κ group: n=6).

Synergistic effect of Kv1.1 blocker, DTX-κ, and gefitinib in H460 and A549 cells. 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 cells, which are known to be gefitinib-resistant cells, was reduced compared to the groups treated with gefitinib-only (Figure 5A and B). The IC_{50}

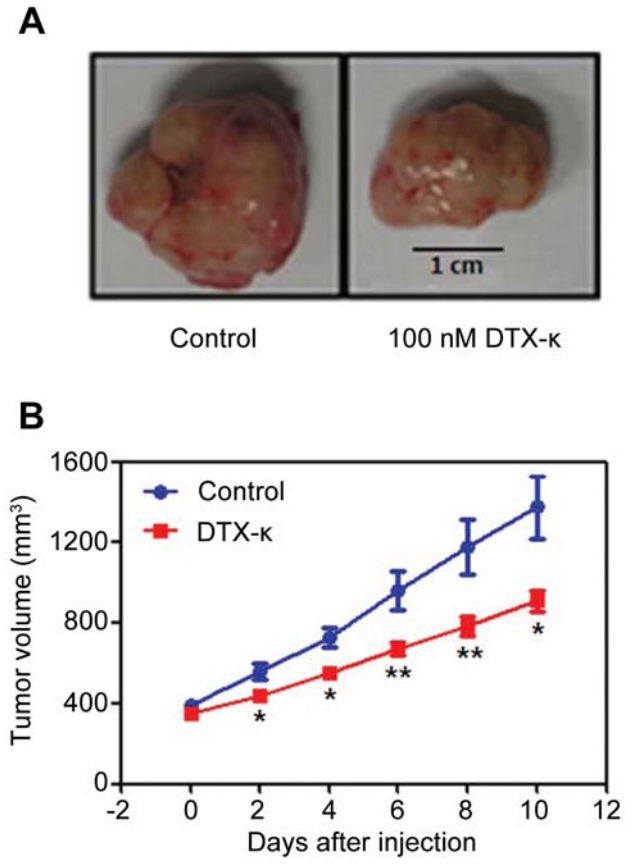

Figure 4. *Inhibition of tumor growth by dendrotoxin-κ (DTX-κ) in a xenograft model. A: Representative image of tumor tissue in each group. The image shows the antitumor effect of DTX-κ compared to control (scale bar: 1 cm). B: The graph shows the tumor size changed after treatment with 100 nM DTX-κ 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).*

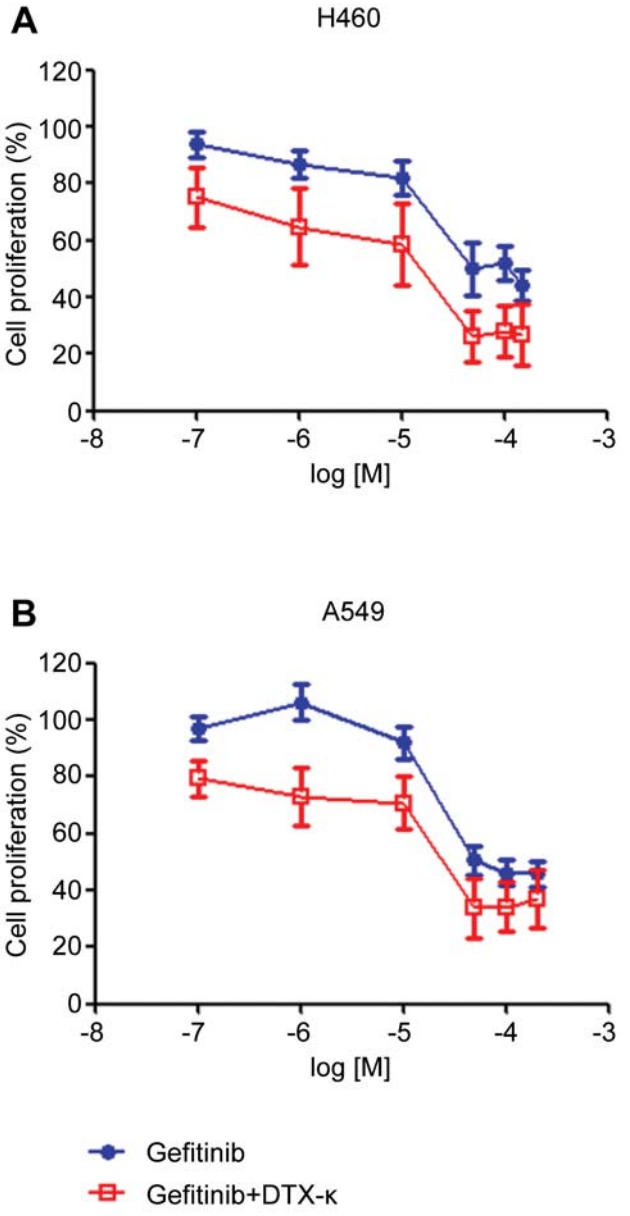

Figure 5. *Effects of combination treatment of dendrotoxin-κ (DTX-κ) and gefitinib on non-small cell lung cancer cells. Combination treatment with DTX-κ and gefitinib provided synergistic anticancer effects compared to each drug alone in H460 (A) and A549 (B) lung cancer cells. Data were normalized by 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).*

values for gefitinib in H460 and A549 cells were 82.1 μM and 89.7 μM, respectively. However, when the H460 and A549 cells 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.

Discussion

In the present study, we investigated the effects of DTX-κ, a selective Kv1.1 blocker, on gefitinib-resistant H460 NSCLC cells. H460 cell viability was inhibited by DTX-κ and reduced cell viability was due to the arrest of the G_1/S transition during the cell-cycle progression. The effects of DTX-κ were also confirmed *in vivo*. Moreover, synergistic effects were induced in H460 and A549 cells with a combination treatment of DTX-κ and gefitinib.

Kv channels have been demonstrated to be related to cell proliferation in breast (18, 19, 35), gastric (20), and lung (14, 15) cancer cells. It has been demonstrated that the blockade of Kv channels, including Kv1.1 and Kv1.3, inhibits cell proliferation due to cell-cycle arrest accompanied by the

alteration of proteins related to the G_1/S phase, such as $p21^{WAF1/CIP1}$ and cyclin D3, in human lung adenocarcinoma A549 cells (14, 15, 35). Similarly to previous studies, the blockade of Kv1.1 by DTX-κ, inhibited cell proliferation and induced cell-cycle arrest in the G_1/S phase in H460 cells.

Gefitinib is a well-known EGFR tyrosine kinase inhibitor (24) and is generally used in second- or third-line treatment of patients with advanced NSCLC (36). However, gefitinib resistance has been reported. *EGFR* and *KRAS* mutation, have been suggested as mechanisms for this resistance (36, 37). *KRAS* mutation has been shown to be associated with worse clinical outcomes when patients are treated with gefitinib or erlotinib (38, 39).

In order to overcome the resistance of tyrosine kinase inhibitors, the molecular mechanisms of resistance have been investigated and many candidate targets have been suggested (40). In addition, multi-target drugs in cancer therapy are known to have advantages in overcoming drug resistance (41-43). As demonstrated in the present study, Kv1.1 could be one of multiple molecular targets to overcome tyrosine kinase inhibitor resistance in NSCLC. The specific mechanisms underlying the synergic effects between tyrosine kinase inhibitor and Kv1.1 blockers need to be determined. It has been recently demonstrated that K^+ channels are regulated by EGFR (44, 45). Therefore, it would be interesting to study the signaling pathways between K^+ channels and EGFR.

At present, combination treatment with Kv channel blockers and other anticancer drugs in anticancer drug-resistant cell lines has not been reported. We, therefore, suggest Kv1.1 as a possible therapeutic target in the development of anticancer agents for gefitinib-resistant NSCLC.

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