

## Original Article

# Dendrotoxin- $\kappa$ suppresses tumor growth induced by human lung adenocarcinoma A549 cells in nude mice

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Voltage-gated K<sup>+</sup> (Kv) channels have been considered to be a regulator of membrane potential and neuronal excitability. Recently, accumulated evidence has indicated that several Kv channel subtypes contribute to the control of cell proliferation in various types of cells and are worth noting as potential emerging molecular targets of cancer therapy. In the present study, we investigated the effects of the Kv1.1-specific blocker, dendrotoxin- $\kappa$  (DTX- $\kappa$ ), on tumor formation induced by the human lung adenocarcinoma cell line A549 in a xenograft model. Kv1.1 mRNA and protein was expressed in A549 cells and the blockade of Kv1.1 by DTX- $\kappa$ , reduced tumor formation in nude mice. Furthermore, treatment with DTX- $\kappa$  significantly increased protein expression of p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, and p15<sup>INK4B</sup> and significantly decreased protein expression of cyclin D3 in tumor tissues compared to the control. These results suggest that DTX- $\kappa$  has anti-tumor effects in A549 cells through the pathway governing G1-S transition.

**Keywords:** G1-S transition, Kv1.1, lung cancer, voltage gated K<sup>+</sup> channels, xenograft

## Introduction

It is widely known that voltage-gated K<sup>+</sup> (Kv) channels play a vital role in processes of excitable cells such as regulation of membrane potential and neuronal activity [10]. However, Kv channels are also found in non-excitabile cells including immune cells [25,30], alveolar epithelial cells [2,19] and various types of cancer cells [23]. Several subtypes of Kv channels are overexpressed when the cells become cancerous and selective knock-down of Kv channels inhibit cell proliferation [1,13,14, 21]. Therefore, Kv channels may be involved in the proliferation of cancer cells even though the mechanism is

not yet clearly understood. Recently, Kv channels have been suggested as emerging molecular targets for the treatment of various cancers [6,7,13,14,18,24,31,36].

Dendrotoxin- $\kappa$  (DTX- $\kappa$ ), isolated from *Dentropaipis polylepsis*, is known to inhibit the Kv1 family of channels in a nanomolar range [9]. It is known that DTX- $\kappa$  has a much higher selectivity for Kv1.1 than  $\alpha$ DTX because several residues in the N-terminus and  $\beta$ -turn of DTX- $\kappa$  are critical for interaction with Kv channels whereas  $\alpha$ DTX bind to targets through its N-terminus [29,34].

It has been proven that cell growth is controlled by several cell cycle-related proteins known as cyclins. Most cyclins have partners called cyclin-dependent kinases (Cdk) and regulation of the cell cycle is determined by the activity of Cdks and their inhibitors such as p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, and p15<sup>INK4B</sup> [15,20]. Cyclin D1-3 is an important factor in the regulation of G1 progression and activation of Cdk4 and Cdk6 [20]. In addition, both p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> are able to inhibit multiple cyclin-Cdk complexes and p15<sup>INK4B</sup> binds to Cdk4 and Cdk6 [15,20]. Both p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> are involved in G1-S transition of the cell cycle phase and play an important role in the determination of cell cycle progression [16].

In the present study, we investigated the effects of DTX- $\kappa$ , a selective Kv1.1 blocker, on tumor growth induced by human lung adenocarcinoma A549 cells in nude mice. In addition, we explored the mechanisms of anti-proliferative effects induced by DTX- $\kappa$  in nude mice.

## Materials and Methods

### Cell culture

Human lung adenocarcinoma A549 cells and MRC-5 cells derived from normal fetal lung tissue [12] were grown in RPMI 1640 (Welgene, Korea) and MEM (ATCC, USA) medium with 10% fetal bovine serum (Welgene, Korea) and a 1% antibiotic-antimycotic solution (Sigma, USA) in a humidified 95% air / 5% CO<sub>2</sub> at 37°C. The medium was changed every other day.

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## Chemicals

DTX- $\kappa$  was purchased from the Alomone Labs (Israel) and dissolved in distilled water. The concentration of the stock solution was 10  $\mu$ M and diluted to the appropriate concentration before the experiment.

## Total RNA extraction and RT-PCR

Cultured MRC-5 and A549 cells were collected and total RNA was extracted using RNAiso Plus (Takara Bio, Japan) following the manufacturer's instructions. The concentration and purity of total RNA was measured at 260 and 280 nm by UV spectrophotometer (Thermo Fisher Scientific, USA). Total RNA (2  $\mu$ g) was reverse transcribed using a random hexamer and M-MLV reverse transcription kit (Promega, USA) in a 20  $\mu$ L volume. Freshly synthesized cDNA was used for the PCR reaction using 1 $\times$  GoTaq green master mix (Promega, USA) with the following conditions: initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 40 sec, 55°C for 40 sec, and 72°C for 1 min; and a final extension at 72°C for 7 min. The primers used for the amplification of Kv1.1 mRNA were 5'-ACATTGTGGCCATCATTCT-3' and 5'-GCTCTTCCCCTCAGTTTCT-3' and synthesized by Bioneer (Bioneer, Korea). A negative control was generated by replacing the cDNA with distilled water.

## Cell proliferation assay

Both MRC-5 and A549 cells ( $6 \times 10^4$  cells/mL, 200  $\mu$ L/well) were prepared in a 96-well plate with 10% FBS and 1% antibiotic-antimycotic solution. After 24 h, DTX- $\kappa$  diluted in PBS was added to the cells for 24 h and 72 h; the medium and DTX- $\kappa$  was changed every other day. To measure cell viability, a 0.5 mg/mL methylthiazolotetrazolium solution (Sigma, USA) was added, and the plate was incubated for 3 h at 37°C to allow the formation of formazan. The formazan was dissolved in dimethyl sulfoxide (Sigma, USA) and the absorbance was determined at 490 nm using a microplate reader (Emax; Molecular Devices, USA).

## Western blot analysis

All proteins were lysed with 1 $\times$  passive lysis buffer (Promega, USA) and quantified by a BCA protein assay kit (Pierce, USA). Approximately 50  $\mu$ g of protein were electrophoresed on a 10~15% polyacrylamide gel and transferred to a 0.45  $\mu$ m PVDF membrane (Pall Corporation, USA). The blots were blocked with 1 $\times$  TBS-Tween 20 containing 5% nonfat milk (5% TTBS; Difco, USA) and were incubated with primary antibodies against Kv1.1 (1 : 500; Millipore, USA), p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, p15<sup>INK4B</sup> and cyclin D3 (1 : 1,000; Cell Signaling Technology, USA) in 5% TTBS. The next day, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (1 : 6,000; Cell Signaling Technology, USA). An enhanced chemiluminescent detection kit (iNtRon Biotechnology, Korea) was used to visualize

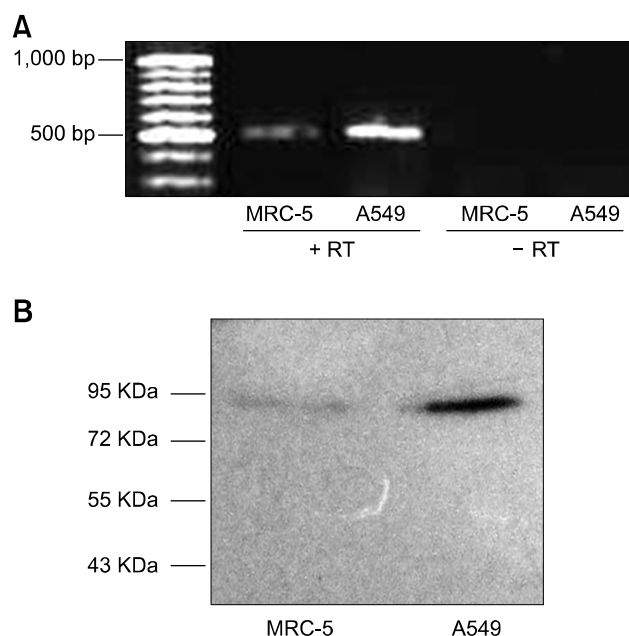
antibody binding and the intensity of the bands was measured using Image J software 1.42q (NIH, USA).

## Xenograft model and DTX- $\kappa$ injection

CAnN.Cg-Foxn1<sup>nu</sup>/CrijOri 6-week old male nude mice were obtained from Orient Bio (Orient Bio, Korea) and housed according to the guidelines of the Institute of Laboratory Animal Resources, Seoul National University. The mice were anesthetized with 25 mg/kg zoletil 50 (Virbac Laboratory, France) and 2% rompun (Bayer, Germany) in a 2 : 1 ratio (1 mL/kg) and inoculated subcutaneously into the right flank with  $5 \times 10^5$  cells suspended in 50  $\mu$ L 10% RPMI medium. Four weeks after inoculation, the mice were randomly divided into control and treatment groups. For the treatment group, 30  $\mu$ L of 5 nM DTX- $\kappa$  was injected intratumorally daily for about 1 week and the same volume of distilled water was injected into the control group. Tumor size was measured every 2 days and tumor tissues were collected immediately after the mice were sacrificed. The tissues were stored in liquid nitrogen until they were used. Tumor volume was determined by the formula: volume = (width  $\times$  length  $\times$  depth)  $\times$  ( $\pi/6$ ).

## Statistical analysis

All values are presented as means  $\pm$  SE. Statistical significance was determined by a *t*-test or Mann-Whitney U test using the statistical program (SAS version 9.1; SAS, USA).



**Fig. 1.** mRNA and protein expression of Kv1.1 in MRC-5 and A549 cells. (A) The mRNA of Kv1.1 was detected with the predicted product size in MRC-5 and A549 cells. The PCR product was electrophoresed on 1.5% agarose gel and visualized by ethidium bromide. (B) Kv1.1 protein was found with the expected molecular weight in A549 cells.

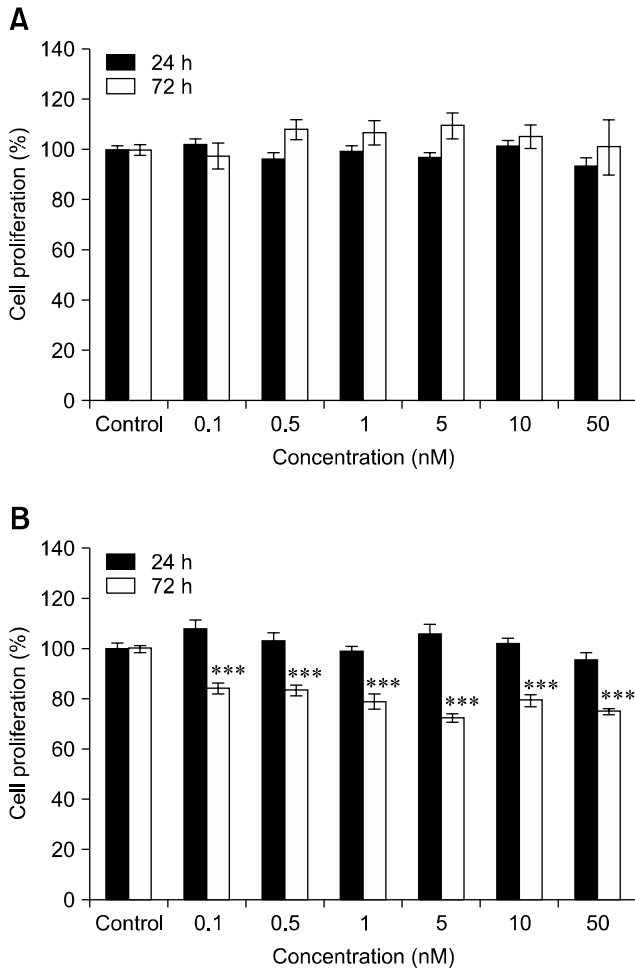
## Results

### Expression of Kv1.1 mRNA and protein in A549 cells

PCR and Western blot analysis were performed to detect Kv1.1 mRNA and protein obtained from the non-cancerous MRC-5 cell line and adenocarcinoma A549 cells. Using gene-specific primers, Kv1.1 mRNA was detected with a product size of 498 base pairs in A549 cells. A negative control experiment was carried out to exclude PCR reagent contamination (Fig. 1A). In addition, the protein expression of Kv1.1 was observed in A549 cells as shown in Fig. 1B. On the other hand, very low expression of Kv1.1 mRNA and protein was observed in MRC-5 cells (Fig. 1).

### Effect of DTX- $\kappa$ on proliferation in MRC-5 and A549 cells

To evaluate whether the selective blocker of Kv1.1,



**Fig. 2.** Inhibition of cell proliferation by DTX- $\kappa$  in MRC-5 and A549 cells. (A) Addition of DTX- $\kappa$  to MRC-5 cells did not affect cell proliferation. (B) The proliferation of A549 cells was significantly reduced by incubation with DTX- $\kappa$  for 72 h compared to the control. Data are expressed as the mean  $\pm$  SE of three or five independent experiments (\*\*\*)  $p < 0.0001$ .

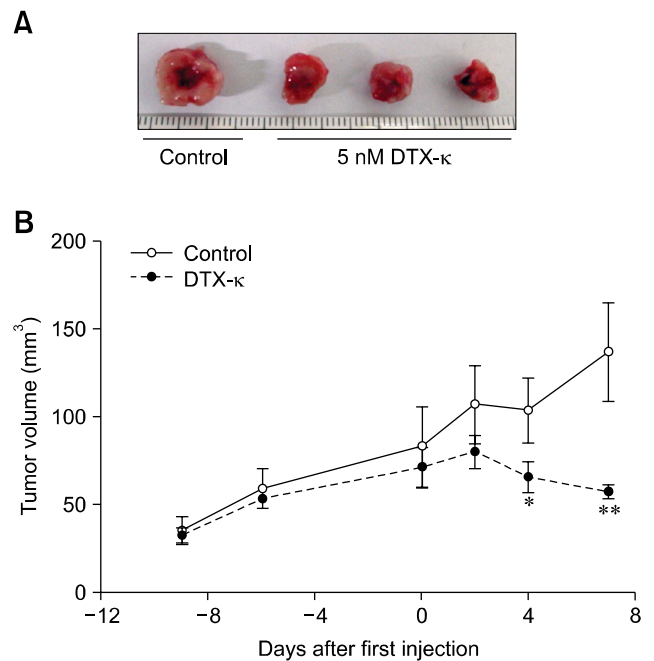
DTX- $\kappa$ , induces the inhibition of proliferation in MRC-5 and A549 cells, an MTT assay was carried out. After treatment with DTX- $\kappa$  at various concentrations, the proliferation of A549 cells was significantly inhibited by 72%~84% at 72 h compared to the control (Fig. 2B). After 24 h of DTX- $\kappa$  treatment, the growth of A549 cells was not significantly altered. On the other hand, the proliferation of non-cancerous MRC-5 cells was not affected by treatment with DTX- $\kappa$  for 72 h even at a high concentration (Fig. 2A). This result suggests that Kv1.1 is specifically involved in cancer cell proliferation.

### Inhibition of tumor growth by treatment with DTX- $\kappa$

In order to investigate whether DTX- $\kappa$  affects tumor growth induced by A549 cells, an *in vivo* experiment using a xenograft model was performed. After treatment with 5 nM DTX- $\kappa$  for 7 days, the tumor volume was significantly reduced compared to the water-injected control (control group:  $n = 2$ , DTX- $\kappa$  group:  $n = 5$ ) (Fig. 3). There was no difference in body weight between the control group and DTX- $\kappa$  treatment group (data not shown).

### Up- or down-regulation of p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, p15<sup>INK4B</sup>, and cyclin D3 proteins in tumor tissues

To identify the cell signaling molecules involved in the



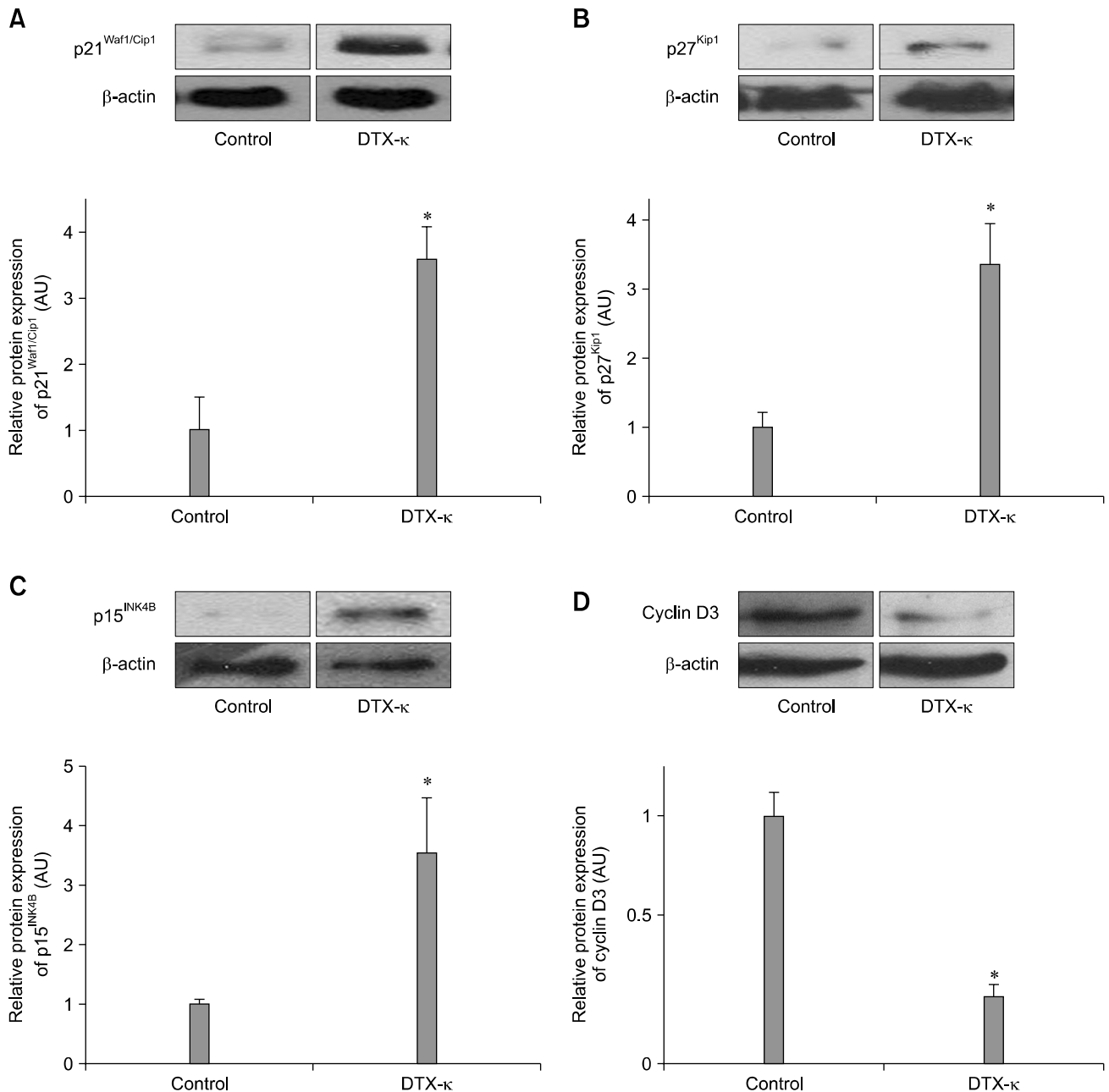
**Fig. 3.** Suppression of tumor growth in the xenograft model by DTX- $\kappa$  treatment. (A) Representative image of tumor tissue in the nude mice control group and DTX- $\kappa$  treatment group taken 7 days after DTX- $\kappa$  treatment. (B) Inhibition of Kv1.1 using DTX- $\kappa$  led to the suppression of tumor growth compared to the control. Solid and dashed lines represent the control group and DTX- $\kappa$  group, respectively. Data are expressed as the means  $\pm$  SE (control group:  $n = 2$ , DTX- $\kappa$  group:  $n = 5$ ; \* $p < 0.05$ , \*\* $p < 0.01$ ).

anti-tumor effect of DTX- $\kappa$ , expression of p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, p15<sup>INK4B</sup> and cyclin D3 protein were examined by Western blot analysis. As shown in Fig. 4, intratumoral injection of 5 nM DTX- $\kappa$  significantly increased protein expression of p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, and p15<sup>INK4B</sup> by approximately 3.6-fold, 3.4-fold, and 3.5-fold, respectively, compared to the control. In contrast, protein expression of

cyclin D3 significantly decreased by 0.3-fold in tumor tissues of nude mice compared to the control (control group: n = 2, DTX- $\kappa$  group: n = 6).

## Discussion

In the present study, we investigated the anti-tumor



**Fig. 4.** Increased protein expression of p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, and p15<sup>INK4B</sup> and decreased protein expression of cyclin D3 upon treatment with DTX- $\kappa$ . This figure shows a representative image from Western blot analysis and relative protein expression levels of p21<sup>Waf1/Cip1</sup> (A), p27<sup>Kip1</sup> (B), p15<sup>INK4B</sup> (C), and cyclin D3 (D). The protein expression levels were normalized to that of  $\beta$ -actin. Furthermore, data were normalized to the values obtained for the control group and presented as means  $\pm$  SE (control group: n = 2, DTX- $\kappa$  group: n = 6; \* $p < 0.05$ ).

effects of DTX- $\kappa$ , a selective blocker of Kv1.1, using human lung adenocarcinoma cell lines. Intratumoral treatment with DTX- $\kappa$  inhibited the tumor growth induced by A549 cells. In addition, protein expression of cyclin dependent kinase inhibitors (CDKIs), p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, and p15<sup>INK4B</sup> was significantly increased while expression of cyclin D3 was significantly decreased.

The effect of Kv1.1 on cell proliferation was previously reported in a human breast cancer cell line and gastric epithelial cell line *in vitro* [22,35]. Treatment with 1 and 10 nM  $\alpha$ -DTX prevented the proliferation of a human breast cancer cell line by 20% and 30%, respectively [22], and the specific blockade of Kv1.1 using siRNA reduced the proliferation of a gastric epithelial cell line [35]. However, there has been no report showing the effects of DTX- $\kappa$  *in vivo*.

Recently, it has been consistently shown that G1-S progression is inhibited by blocking Kv channels [3,5,21,26,32,39]. For example, blocking human ether  $\alpha$  go-go K<sup>+</sup> channels inhibits the proliferation of human breast cancer cells. This is accompanied by G1-S transition [3,21] and increases protein levels of p21<sup>Waf1/Cip1</sup> [21]. The use of a selective blocker of Kv1.3 reduced the proliferation of oligodendrocyte progenitor cells by inducing G1 phase arrest and down-regulation of cyclin D protein levels [5]. Blocking of Kv1.5 [32] and Kv7.5 [26] are also involved in inhibition of skeletal muscle cell proliferation through accumulation of p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> and reduction of cyclin A and cyclin D1 protein expression during G1-S transition [32]. Similar to the previous reports, our results also support the hypothesis that the inhibition of tumor growth by DTX- $\kappa$  in A549 cells occurs through the G1-S transition pathway.

At the present time, the exact mechanism responsible for cell cycle regulation via Kv1.1 is unknown. One possible mechanism is that CDKIs such as p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup>, and p15<sup>INK4B</sup> and cyclins may be able to bind directly to Kv1.1. It has been demonstrated that the pro-apoptotic Bcl-2 family member Bax directly interacts with Kv1.3 K<sup>+</sup> channels [8]. Alternatively, adaptor proteins may be involved in the interaction between Kv1.1 and CDKIs (or cyclins) that regulates cell cycle progression. Further understanding of the regulation mechanism of Kv1.1 is required. Recently, it has been shown that TRPM2 channel, a member of the transient receptor potential protein family and TREK-1 [33], two pore domain K<sup>+</sup> channel, are expressed in the nucleus region of prostate cancer cell lines. Moreover, the distribution of TRPM2 channels was in the plasma membrane and cytoplasm of non-cancerous prostate cells [38]. Therefore, Kv1.1 in A549 cells may not be localized in the plasma membrane and could be distributed in intracellular regions including nucleus, similar to TRPM2 and TREK-1 channels. Further studies are required to determine the localization of Kv1.1 in A549 cells.

Recently, several papers have been published showing *in*

*in vivo* anti-tumor effects associated with blocking ion channels [4,11,17,27,28,37,40]. For example, the specific blockade of Kv1.5 or human ether  $\alpha$  go-go-related gene using siRNA or shRNA reduces tumor growth in human gastric cancer cells [17,27,40]. Blocking not only Kv channels but also transient receptor potential channel and ATP-sensitive potassium channels prevents tumor progression in several types of cancer *in vivo* [11,28,37,41]. Taken together, these results demonstrate that the selective inhibition of Kv1.1 is able to suppress the tumor growth of A549 cells in a xenograft model. This is the first evidence of Kv1.1 involvement in proliferation of human lung adenocarcinoma A549 cells. Based on our results, selective blockers of Kv1.1 including DTX- $\kappa$  are potential therapeutic candidates for the treatment of human lung cancer.

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