

# Inhibition of p21 and Akt Potentiates SU6656-Induced Caspase-Independent Cell Death in FRO Anaplastic Thyroid Carcinoma Cells

## Authors

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## Key words

- anaplastic thyroid carcinoma
- cell death
- SU6656
- p21
- Akt

## Abstract

SU6656 is a small-molecule indolinone that selectively inhibits Src family kinase and induces death of cancer cells. The aim of the present study was to investigate the influence of SU6656 on cell survival and to assess the role of p21 and PI3K/Akt signaling in cell survival resulting from SU6656 treatment in anaplastic thyroid carcinoma (ATC) cells. When 8505C, CAL62, and FRO ATC cells were treated with SU6656, the viability of 8505C and CAL62 ATC cells decreased only after treatment with SU6656 at a dosage of 100  $\mu$ M for 72 h, while the viability of FRO ATC cells decreased after treatment with SU6656 in a concentration- and time-dependent manner. Cell viability was not changed by pretreatment with the broad-spectrum caspase inhibitor

z-VAD-fmk. Phospho-Src protein levels were reduced, and p21 protein levels were elevated. Phospho-ERK1/2 protein levels were multiplied without alteration of total ERK1/2, total Akt, and phospho-Akt protein levels. Regarding FRO ATC cells, the decrement of cell viability, the increment of cleaved PARP-1 protein levels, and the decrement of phospho-Src protein levels were shown in p21 siRNA- or LY294002-pretreated cells compared to SU6656-treated control cells. ERK1/2 siRNA transfection did not affect cell viability and protein levels of cleaved PARP-1, p21, and Akt. In conclusion, these results suggest that SU6656 induces caspase-independent death of FRO ATC cells by overcoming the resistance mechanism involving p21 and Akt. Suppression of p21 and Akt enhances the cytotoxic effect of SU6656 in FRO ATC cells.

received 25.08.2012

accepted 07.01.2013

## Bibliography

**DOI** <http://dx.doi.org/10.1055/s-0033-1333716>  
 Published online:  
 February 5, 2013  
 Horm Metab Res 2013;  
 45: 408–414  
 © Georg Thieme Verlag KG  
 Stuttgart · New York  
 ISSN 0018-5043

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## Introduction

Anaplastic thyroid carcinoma (ATC) is a high-grade malignant neoplasm of the thyroid gland presenting with extrathyroidal invasion and distant metastasis [1–3]. Clinically significant improvement of the survival rate in ATC has not been achieved despite multimodal strategies, and new therapeutic agents are under exploration [1–3]. Src family kinase (SFK) is a multifunctional tyrosine kinase that regulates survival, growth, and proliferation [4–6]. SFK exerts a protumorigenic effect via multiple signalings, and is overexpressed and/or activated in various malignancies [4–8]. Aberrant activation of SFK is associated with thyroid carcinoma invasiveness [9]. SU6656, a small-molecule indolinone, induces cell death in both nonmalignant and malignant cells by selectively inhibiting SFK [10, 11]. SU6656 suppresses growth, motility, invasion, angiogenesis through dual blockade of both SFK and Aurora kinase in synovial sarcoma cells [12].

However, the effect of SU6656 on cell survival in ATC cells has not been evaluated.

p21/WAF1 modulates survival and growth via a p53-dependent or -independent pathway [13]. p21 mediates cell cycle arrest by impediment of p53-dependent cyclin dependent kinases, while it represses cell death by hindrance of p53-dependent or -independent pathway [13]. p21 is present in 40% of patients with papillary thyroid carcinoma (PTC), 7% of patients with poorly differentiated thyroid carcinoma, and 0% of patients with ATC, and its levels are reduced in PTC cells which have a tendency to undergo early metastasis [14, 15]. Even though changes in p21 caused by some agents are related to survival of human cancer cells, the relationship of p21 with cell survival in SU6656-treated ATC cells has not been clarified [16–18].

PI3K/Akt signaling is involved in the control of multiple cellular processes including survival, growth, proliferation, differentiation, and migration [19]. It plays pivotal roles in insulin/IGF-1-

mediated cell cycle progression and cell growth in FRTL5 thyroid cells [20]. PI3K/Akt signaling is deregulated in ATC cells, and Akt1 is activated in most ATC patients [21,22]. We reported recently that CCAAT/enhancer-binding protein-homologous protein augments cytotoxicity in vascular endothelial cell growth factor receptor (VEGFR) inhibitor-treated FRO ATC cells by modulating p21 and PI3K/Akt signaling, suggesting that p21 and Akt are associated with resistance to cell death [23]. SFK regulates PI3K/Akt signaling by altering PTEN [24]. Although SU6656 inhibits Akt activation in human cancer cells, the relevance of PI3K/Akt signaling to cell survival in SU6656-treated ATC cells has not been verified [25].

The aim of the present study was to investigate the influence of SU6656 on cell survival and to assess the role of p21 and PI3K/Akt signaling in cell survival resulting from SU6656 treatment in ATC cells.

## Materials and Methods



### Materials

DMEM medium, RPMI1640 medium, and fetal bovine serum (FBS) were obtained from Life Technologies (Gaithersburg, MD, USA). SU6656 and the broad-spectrum caspase inhibitor z-VAD-fmk were purchased from Sigma (St. Louis, MO, USA), and stored as stock solution with dimethyl sulfoxide (10 mM/ml). Primary antibodies raised against cleaved PARP-1, total Src and phospho-Src (Tyr416), total ERK1/2 and phospho-ERK1/2 (Thr202/Tyr204), and  $\beta$ -actin were obtained from Cell Signaling Biotechnology (Danvers, MA, USA), and primary antibodies raised against p21, total Akt and phospho-Akt (Ser473) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Vector Labs (Burlingame, CA, USA). All other reagents were obtained from Sigma unless otherwise stated.

### Cell culture

8505C ATC cells and CAL62 ATC cells were purchased from DSMZ GmbH (Braunschweig, Germany), and FRO ATC cells were provided by Professor Young Joo Park (Division of Endocrinology and Metabolism, Seoul National University, Republic of Korea). 8505C and CAL62 ATC cells were grown in DMEM medium supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin. FRO ATC cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin. Cells received fresh medium at regular intervals. Treatments and experiments were performed using cells that were confluent at 50–70%.

### CCK-8 assay

The experiment was carried out using CCK-8 Assay Kit (Dojindo laboratories, Kumamoto, Japan). Cells ( $5 \times 10^3/100 \mu\text{l}$ ) in 96-well plates were incubated for overnight, and treated for an additional 4 h at 37 °C. Absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices, Palo Alto, CA, USA). Cell viability (%) was calculated according to the following equation: Cell viability (%) = [OD450 (sample)/OD450 (control)]  $\times$  100. The absorbance of 5 wells for each experiment was averaged. All experiments were performed in triplicate.

### Trypan blue assay

Cells ( $1 \times 10^4/500 \mu\text{l}$ ) in 12-well plates were incubated, and mixed with trypan blue dye at 37 °C. Stained cells were counted using hemocytometer. All experiments were carried out in triplicate.

### Transfection of small interfering RNA (siRNA)

ERK1/2-specific siRNA was purchased from Cell Signaling Biotechnology, and p21-specific siRNA from Bioneer (Daejeon, Republic of Korea). At 50% confluence, cells were transfected with siRNA using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Transfection efficiency was tested by Western blotting.

### Western blotting

Cells were lysed in RIPA buffer containing 1  $\times$  protease inhibitor cocktail and 1  $\times$  phosphatase inhibitor cocktail set V (Calbiochem, La Jolla, CA, USA). Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Equivalent amounts of protein (50  $\mu\text{g}$ ) were separated by 10% SDS-PAGE, and transferred to Immobilon-P Membrane (Millipore, Bedford, MA, USA). Western blotting was performed using specific primary antibodies and horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies. The diluted concentrations of primary antibodies were as follows: cleaved PARP-1 (1:1000), total Src and phospho-Src (1:1000), p21 (1:500), total ERK1/2 and phospho-ERK1/2 (1:1000), total Akt and phospho-Akt (1:1000), and  $\beta$ -actin (1:1000). Bands were detected using ECL or ECL Plus Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, UK).  $\beta$ -Actin was used as positive control. All reactions were carried out in triplicate.

### Statistical analysis

All data are expressed as mean  $\pm$  SE. Data were analyzed by unpaired Student's *t*-test or ANOVA as appropriate. A *p*-value less than 0.05 was considered to be statistically significant. All analyses were performed using SPSS version 10.0 (SPSS, Chicago, IL, USA).

## Results



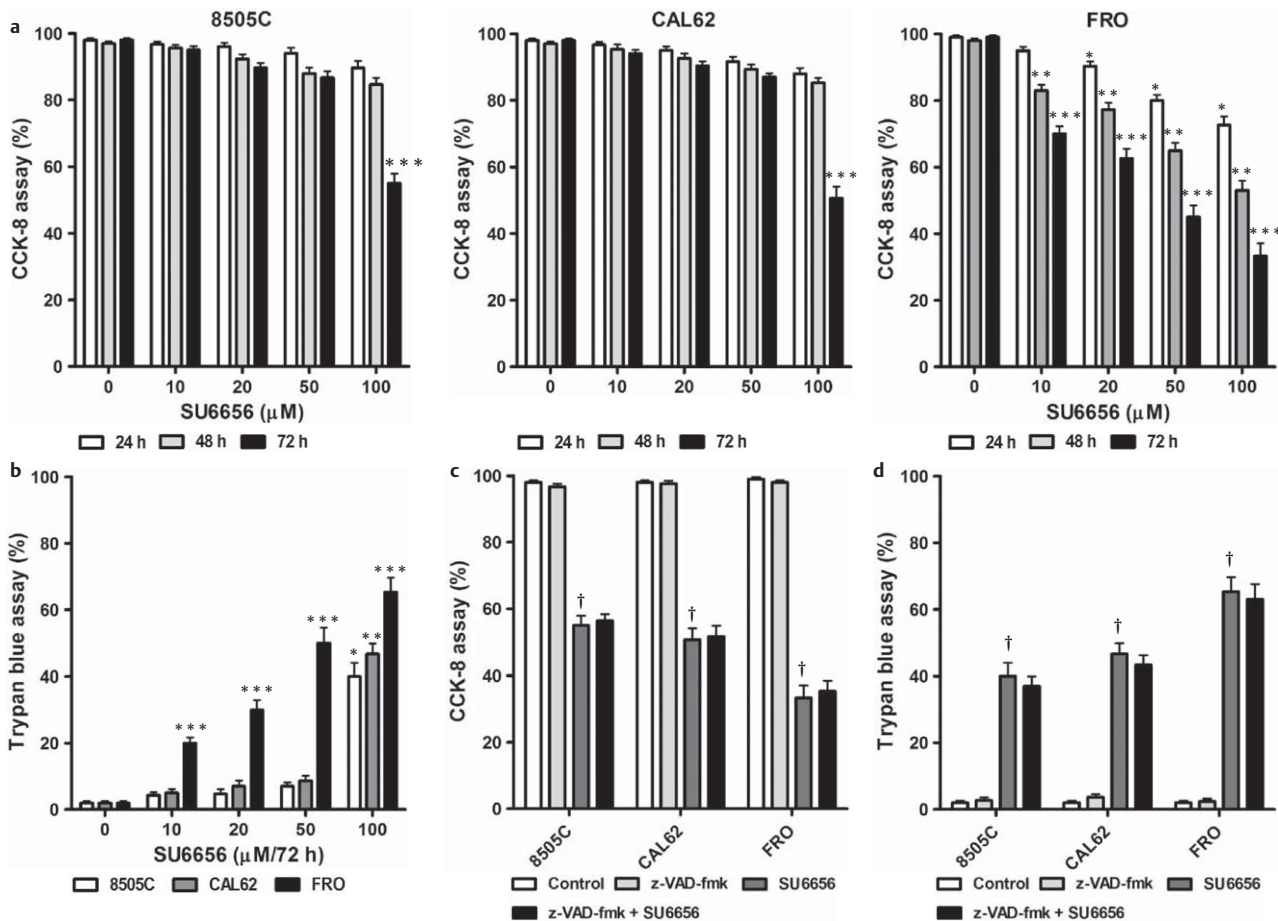
### SU6656 induces caspase-independent cell death in ATC cells

To evaluate the effect of SU6656 on cell survival in 8505C, CAL62, and FRO ATC cells, the cells were treated with SU6656 at a dosage of 10, 20, 50, 100  $\mu\text{M}$  for 24, 48, 72 h, respectively, and cell viability was measured (◻ Fig. 1a, b). The viability of 8505C and CAL62 ATC cells decreased only after treatment with SU6656 at a dosage of 100  $\mu\text{M}$  for 72 h. In contrast, the viability of FRO ATC cells decreased after treatment with SU6656 in a concentration- and time-dependent manner.

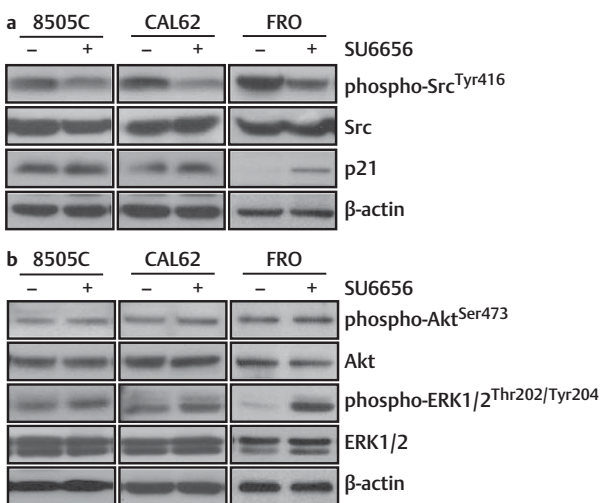
To define the dependency of SU6656-induced cell death on activation of caspases, cells were pretreated with z-VAD-fmk (50  $\mu\text{M}$ , 1 h) followed by treatment with SU6656 at a dosage of 100  $\mu\text{M}$  for 72 h, and cell viability was measured (◻ Fig. 1c, d). The viability of SU6656-treated cells was not affected by pretreatment with z-VAD-fmk.

### SU6656 regulates p21, Akt, and ERK in ATC cells

To investigate the influence of SU6656 on Src in 8505C, CAL62, and FRO ATC cells, the cells were treated with SU6656 at a dos-



**Fig. 1** The effect of SU6656 on cell survival and activation of caspases in 8505C, CAL62, and FRO ATC cells. **a, b** The cells were treated with SU6656 at a dosage of 10, 20, 50, 100  $\mu\text{M}$  for 24, 48, 72 h, respectively. Cell viability was measured using CCK-8 and trypan blue assay. **c, d** Cells were pretreated with z-VAD-fmk (50  $\mu\text{M}$ , 1 h) before SU6656 treatment at a dosage of 100  $\mu\text{M}$  for 72 h. Cell viability was measured using CCK-8 and trypan blue assay. All experiments were performed in triplicate. Data are expressed as mean  $\pm$  SE. \* $p < 0.05$  vs. control at 24 h. \*\* $p < 0.05$  vs. control at 48 h. \*\*\* $p < 0.05$  vs. control at 72 h. † $p < 0.05$  vs. control.

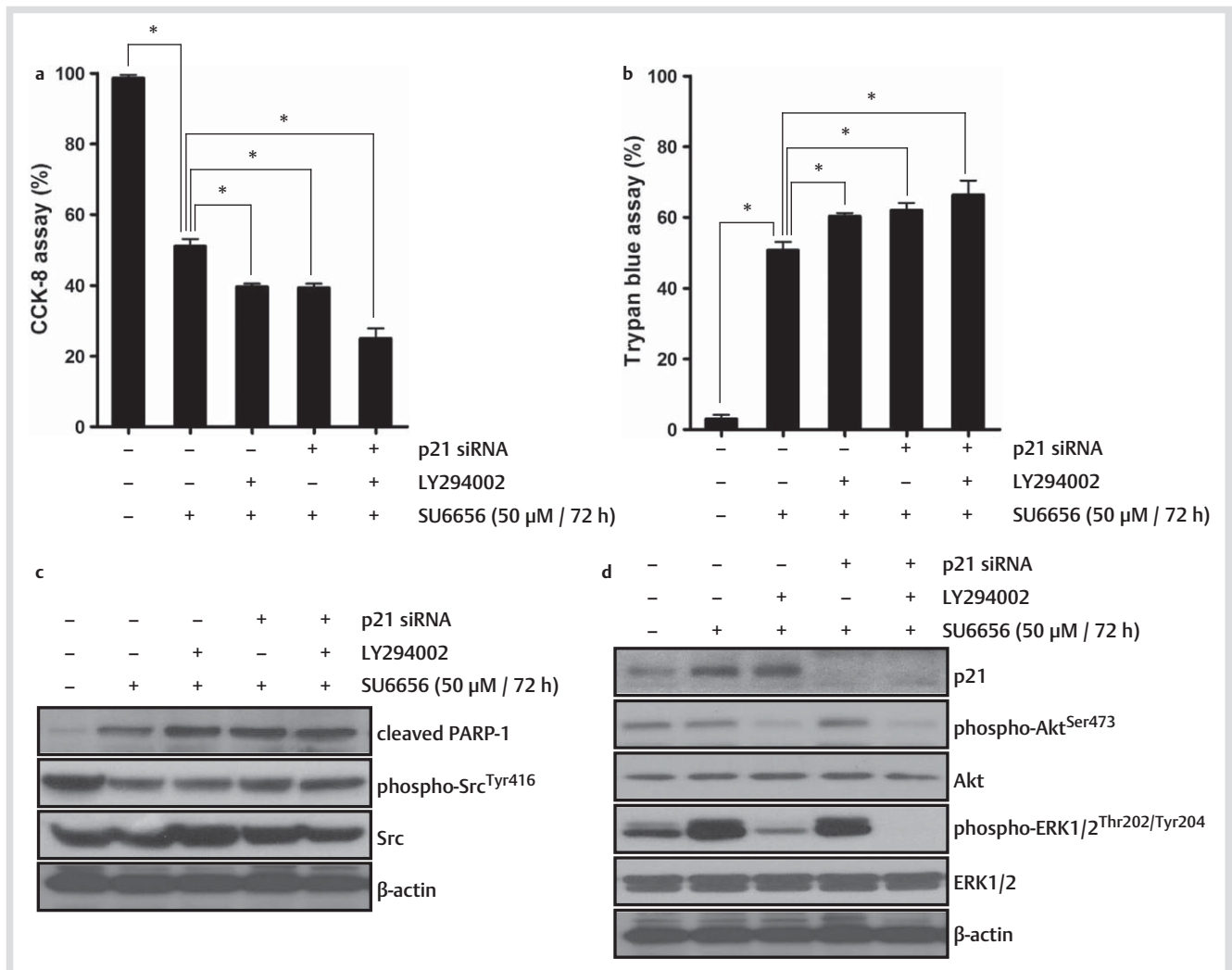


**Fig. 2** The relationship of SU6656 with Src, p21, Akt, ERK in 8505C, CAL62, and FRO ATC cells. The cells were treated with SU6656 at a dosage of 100  $\mu\text{M}$  for 72 h. **a** Src and p21 protein levels were measured. **b** Akt and ERK1/2 protein levels were measured. All experiments were performed in triplicate. The blots are representative of independent experiments.

age of 100  $\mu\text{M}$  for 72 h, and Src protein levels were measured (○ Fig. 2a). As a result of SU6656 treatment, phospho-Src protein levels were reduced, whereas total Src protein levels were unchanged. Next, to clarify the relationship of SU6656 with p21, cells were treated with SU6656 at a dosage of 100  $\mu\text{M}$  for 72 h, and p21 protein levels were measured (○ Fig. 2a). After SU6656 treatment, p21 protein levels were not altered in 8505C and CAL62 ATC cells, while these were elevated in FRO ATC cells. To verify the relevance of SU6656 to Akt and ERK, they were treated with SU6656 at a dosage of 100  $\mu\text{M}$  for 72 h, and Akt and ERK1/2 protein levels were measured (○ Fig. 2b). Under SU6656 treatment, Akt and ERK1/2 protein levels were not varied in 8505C and CAL62 ATC cells, whereas only phospho-ERK1/2 protein levels were multiplied without change of total ERK1/2, total Akt, phospho-Akt protein levels in FRO ATC cells.

#### Suppression of p21 and Akt enhances SU6656-induced cell death in FRO ATC cells

Further experiments in which FRO ATC cells were treated with SU6656 at a dosage of 50  $\mu\text{M}$  for 72 h were performed. To determine whether p21 and Akt are involved in SU6656-induced cell death, p21 siRNA (100 nM, 24 h) and the Akt inhibitor LY294002 (100  $\mu\text{M}$ , 1 h) were pretreated before SU6656 treatment. Cell viability (○ Fig. 3a, b), and cleaved PARP-1 and Src protein levels



**Fig. 3** The involvement of p21 and Akt in SU6656-induced cell death in FRO ATC cells. p21 siRNA (100 nM, 24 h) and LY294002 (100 μM, 1 h) were pre-treated prior to SU6656 treatment at a dosage of 50 μM for 72 h. **a**, **b** Cell viability was measured using CCK-8 and trypan blue assay. **c** Cleaved PARP-1 and Src protein levels were measured. **d** p21, Akt, ERK1/2 protein levels were measured. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean ± SE. \**p* < 0.05.

(**Fig. 3c**) were measured. Cell viability was lower in p21 siRNA- or LY294002-pretreated cells compared to SU6656-treated control cells. The increment of cleaved PARP-1 protein levels and the decrement of phospho-Src protein levels were shown in p21 siRNA- or LY294002-pretreated cells compared to SU6656-treated control cells. Cell viability, and cleaved PARP-1 and phospho-Src protein levels in p21 siRNA- or LY294002-pretreated cells were not different from those in p21 siRNA- and LY294002-pretreated cells.

To assess whether p21 and Akt have an effect on ERK in SU6656-treated cells, cells were pretreated with p21 siRNA and LY294002 prior to SU6656 treatment (**Fig. 3d**). While LY294002 lessened phospho-ERK1/2 protein levels, p21 siRNA had no such effect.

#### ERK knockdown does not affect SU6656-induced cell death in FRO ATC cells

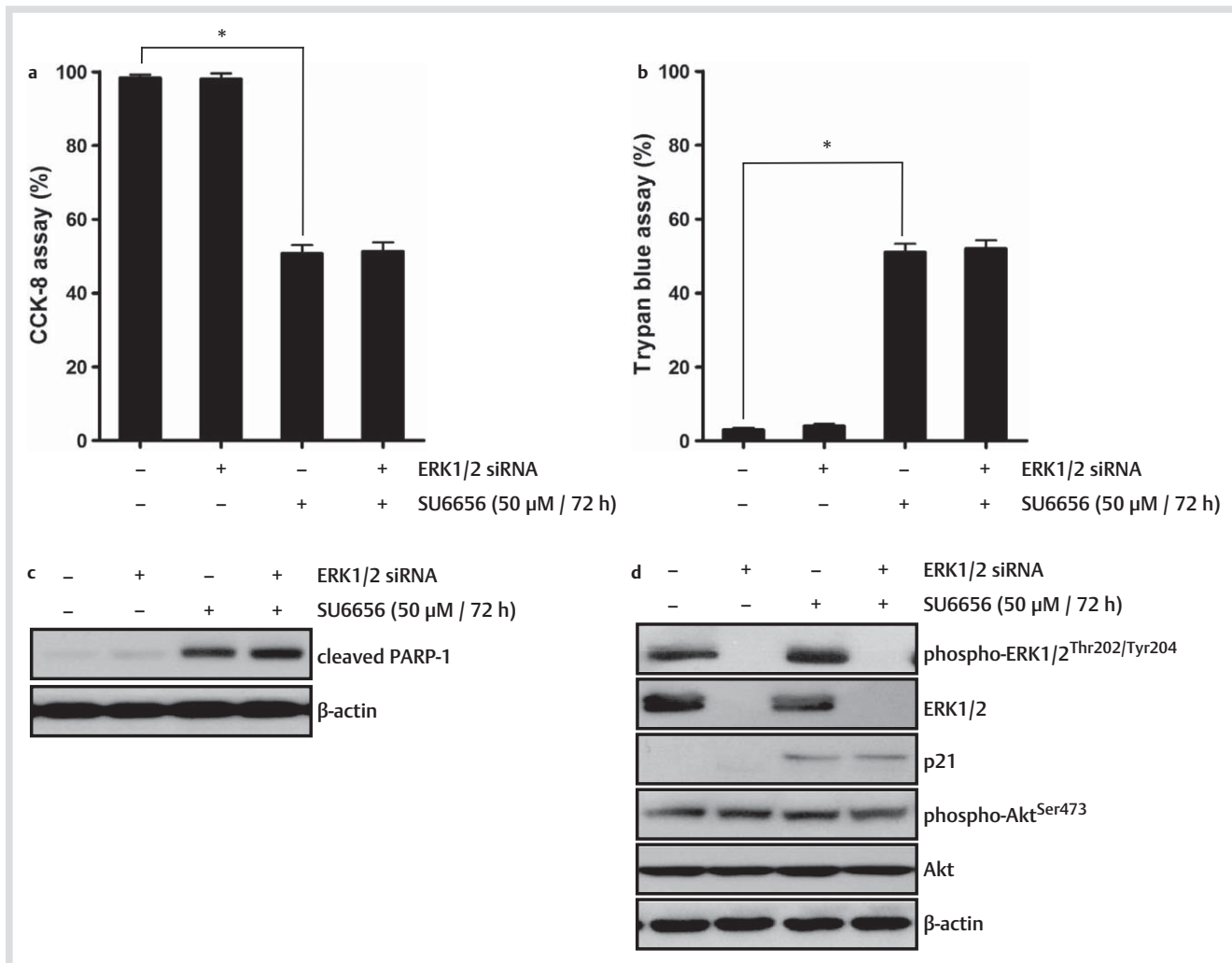
To explore whether ERK participates in SU6656-induced cell death, ERK1/2 siRNA (100 nM, 24 h) was transfected, after which SU6656 was applied. Cell viability (**Fig. 4a, b**) and cleaved PARP-1 protein levels (**Fig. 4c**) were measured. In SU6656-treated cells, cell viability and cleaved PARP-1 protein levels were not affected by ERK1/2 siRNA transfection.

To survey whether ERK has an influence on p21 and Akt in SU6656-treated cells, cells were transfected with ERK1/2 siRNA followed by SU6656 treatment (**Fig. 4d**). ERK1/2 siRNA did not alter p21 and Akt protein levels.

#### Discussion

In thyroid cancer, SFK is related to invasion, and SFK inhibitors suppress growth and metastasis [9,26]. Among the SFK inhibitors, SU6656 induces death of various cell types including irradiated endothelial cells and synovial sarcoma cells by regulating signal proteins [10–12]. However, the effect of SU6656 on survival of thyroid cancer cells including ATC cells has not been evaluated. Our data indicate that SU6656 decreases cell viability and increases cleaved PARP-1 protein levels in ATC cells, suggesting that SU6656 causes cell death in ATC cells (**Fig. 1, 3c, 4c**). To the best of our knowledge, this is the first report to demonstrate that SU6656 leads to cell death in thyroid cancer cells including ATC cells. Intriguingly, total and cleaved caspase-3 protein levels in ATC cells were not varied after treatment with SU6656 even at 200 μM (data not shown). Moreover, viability of





**Fig. 4** The participation of ERK in SU6656-induced cell death in FRO ATC cells. ERK1/2 siRNA (100 nM, 24 h) was transfected followed by SU6656 treatment at a dosage of 50 μM for 72 h. **a, b** Cell viability was measured using CCK-8 and trypan blue assay. **c** Cleaved PARP-1 protein levels were measured. **d** ERK1/2, p21, and Akt protein levels were measured. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean ± SE. \**p* < 0.05.

ATC cells was not affected by pretreatment with the broad-spectrum caspase inhibitor z-VAD-fmk. These results imply that SU6656 results in cell death in ATC cells via a caspase-independent pathway. Given that the minimal effective concentration of SU6656 in FRO ATC cells in the present study was 10 μM, the concentration applied to other cancer cell types in previous studies, our results connote that SU6656 is a candidate therapeutic agent in ATC [12,27]. For clinical application in ATC, the efficacy of SU6656 in vivo models should be obviously elucidated.

p21 inhibits cell death in tunicamycin-treated mouse embryonic fibroblasts and interferon-γ-treated macrophages [28,29]. While suppression of p21 induces death of tunicamycin-treated A549 lung cancer cells, it increases size and invasiveness of intestinal tumor cells [28,30]. p21 contributes to the desensitization of cancer cells to chemotherapeutic agents [31]. Ultimately, the role of p21 in the survival of cancer cells is altered according to the cell type and the chemotherapeutic agent used [31]. There is report that p21 expression decreases in PTC cells with a tendency to undergo early metastasis and another one that p21 expression is not correlated with clinicopathological factors in PTC tissues [15,32]. We found that p21 is associated

with resistance to cell death in VEGFR inhibitor-treated FRO ATC cells [23]. Meanwhile, proteasome inhibitor and epigallocatechin-3-gallate cause cell death in ARO colon cancer cells by increasing p21 levels, and PPARγ agonist exerts an antiproliferative effect in ARO colon cancer cells and DRO melanoma cells by increasing p21 levels [16–18,33]. SU6656 has no effect on p21 in Panc1 pancreatic cancer cells [34]. However, because we previously showed the role of p21 in resistance to cell death in VEGFR inhibitor-treated FRO ATC cells, the relationship of p21 with SU6656-induced cell death in FRO ATC cells was clarified [23]. Our data manifest that SU6656 increases p21 levels concomitantly with cell death and p21 ablation potentiates SU6656-induced cell death in FRO ATC cells. These results denote that activated p21 functions to protect against cell death, rather than as an effector, in SU6656-treated FRO ATC cells.

PI3K/Akt signaling plays crucial roles in insulin/IGF-1-mediated cell cycle progression and cell growth in FRTL5 thyroid cells [20]. We reported that alpha-lipoic acid ameliorates endoplasmic reticulum stress-induced cell death in FRTL5 thyroid cells by activating PI3K/Akt signaling [35]. PI3K/Akt signaling, which promotes cell survival, is deregulated in cancer cells including ATC cells [19,21]. We showed previously that PI3K/Akt signaling

as well as p21 are involved in the resistance of FRO ATC cells to cell death resulting from VEGFR inhibitor treatment [23]. SFK modulates PI3K/Akt signaling by modifying PTEN [24]. SFK inhibitors induce cell death in rat cerebral cortical cells by suppressing Akt phosphorylation [36]. SU6656 causes cell death with suppression of Akt phosphorylation in irradiated human umbilical vein endothelial cells and human cancer cells [11,25]. However, the relevance of PI3K/Akt signaling to cell death in SU6656-treated ATC cells has not been verified. Our data reveal that SU6656 leads to cell death without alteration of Akt phosphorylating status and repression of Akt phosphorylation augments SU6656-induced cell death in FRO ATC cells. These results suggest that maintenance of PI3K/Akt signaling following SU6656 treatment confers on FRO ATC cells resistance to cell death.

In the present study, SU6656 had a tendency to induce greater cytotoxicity in FRO ATC cells with double knockdown of p21 and Akt compared to those with knockdown of either p21 or Akt alone. These results imply that simultaneous inhibition of p21 and PI3K/Akt signaling does not antagonize the effect of SU6656 on FRO ATC cells. Based on our results for p21 and Akt, it is proposed that increased p21 expression and unchanged Akt activity constitute the phenotype of resistance to SU6656-induced cell death in FRO ATC cells. Considering that both SFK inhibition (the present study) and VEGFR inhibition (our previous report) magnify their cytotoxic effects on FRO ATC cells through regulation of p21 and PI3K/Akt signaling, it would seem that manipulation of p21 and PI3K/Akt signaling potentiates the therapeutic efficacy of SFK inhibitors and VEGFR inhibitors in ATC [23].

PI3K/Akt signaling regulates survival in harmony with p21 [37]. However, our data indicate that inhibition of Akt does not induce suppression of p21 and vice versa in SU6656-treated FRO ATC cells. It appears likely that PI3K/Akt signaling is independent of p21 in SU6656-induced cell death in FRO ATC cells.

ERK regulates survival, growth, proliferation, and differentiation, and its downregulation promotes cell death [38]. Epigallocatechin-3-gallate inhibits ERK1/2 phosphorylation, thereby inducing cell death in ARO colon cancer cells [16]. SFK exerts its effect on proliferation via a Ras/ERK/MAPK signaling, while the SFK inhibitor AZD0530 causes growth arrest without change of ERK1/2 activity in PTC and ATC cells [39,40]. Meanwhile, ERK is involved in prosurvival processes by interacting with PI3K/Akt signaling [38,40]. In the present study, because LY294002 decreased both cell viability and phospho-ERK1/2 protein levels in SU6656-treated FRO ATC cells, the participation of ERK in cell death in SU6656-treated FRO ATC cells was explored. Our data demonstrate that decreased phosphorylation of ERK does not lead to cell death in FRO ATC cells. Thus, it may be inferred that ERK is not directly linked to SU6656-induced cell death in FRO ATC cells.

In conclusion, our results suggest that SU6656 induces caspase-independent death of FRO ATC cells by overcoming the resistance mechanism associated with p21 and Akt. Suppression of p21 and PI3K/Akt signaling allows SU6656 to enhance the cytotoxic effect in FRO ATC cells. Thus, this study provides 1) the possibility of SU6656 as a promising compound and 2) the concept of 'resistance' to chemotherapeutic agents in the treatment of ATC patients.

## Acknowledgements

▼ This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (2011-0012965) to S. J. Lee, Republic of Korea.

## Conflict of Interest

▼ The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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