

# Study On the Antitumor Bioactivity of Koningic Acid in Thyroid Cancer in Vivo and in Vitro

**Changxin Jing**

The First Hospital of Yulin

**Yanyan Li**

The First Hospital of Yulin

**Zhifei Gao**

The First Hospital of Yulin

**Peng Hou**

The First Affiliated Hospital of Xi'an Jiaotong University

**Rong Wang** (✉ [ronger\\_0516@163.com](mailto:ronger_0516@163.com))

The First Hospital of Yulin <https://orcid.org/0000-0001-6553-8416>

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## Research Article

**Keywords:** Koningic acid, thyroid cancer, aerobic glycolysis, antitumor effects

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Study on the antitumor bioactivity of Koningic acid in thyroid cancer *in vivo* and *in vitro*

Changxin Jing<sup>1</sup>, Yanyan Li<sup>1</sup>, Zhifei Gao<sup>1</sup>, Peng Hou<sup>2,3</sup>, Rong Wang<sup>1,2</sup>

<sup>1</sup>Department of Endocrinology, The First Hospital of Yulin, Yulin 719000, People's Republic of China; <sup>2</sup>Department of Endocrinology, <sup>3</sup>Key Laboratory for Tumor Precision Medicine of Shaanxi Province, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, People's Republic of China. People's Republic of China.

**Abstract**

**Purpose** Koningic acid (KA), a sesquiterpene lactone, has been identified as an antimicrobial agent. Recent studies have revealed KA's antitumor activities in colorectal cancer, leukemia, and lung cancer. However, its antitumor effect in thyroid cancer remains largely unknown. The aim of this study is to test the therapeutic potential of KA in thyroid cancer and explore the mechanisms underlying antitumor effects.

**Methods** We examined the effects of KA on proliferation, colony formation, apoptosis, ATP deprivation, and xenograft tumor growth in thyroid cancer cells.

**Results** KA inhibited thyroid cancer cell proliferation, colony formation, and induced cell apoptosis in a dose and time-dependent manner. Our data also showed that KA caused a rapid, extensive decrease of ATP levels in thyroid cancer cells. Growth of xenograft tumor derived from the thyroid cancer cell line C643 in nude mice was significantly inhibited by KA. Importantly, KA treatment did not cause significant liver and kidney damage in mice compared with the control group.

**Conclusion** KA may be used as an effective and safe agent for thyroid cancer treatment.

**Keywords:** Koningic acid, thyroid cancer, aerobic glycolysis, antitumor effects

**Correspondence to:** Rong Wang, email: ronger\_0516@163.com

**Introduction**

Thyroid cancer is a common endocrine malignancy that has rapidly increased in global incidence in recent decades. The global incidence rate of thyroid cancer in women is 10.2 per 100,000, which is 3 times higher than in men [1]. Thyroid cancer is classified into differentiated and undifferentiated thyroid cancer. The former consists of papillary thyroid cancer and follicular thyroid cancer, which account for the vast majority of thyroid cancers [2]. The mortality rates of thyroid cancer are low, with rates from 0.4 to 0.5 in men and women, respectively; and its prognosis is

good. However, the rate of thyroid cancer recurrence or persistence is high and there is risk of developing into anaplastic thyroid cancer (ATC). Although ATC is a rare type of undifferentiated thyroid cancer that makes up approximately 1 % of thyroid cancer cases, it is one of the deadliest malignant tumors in human being [3–5]. Conventional surgical thyroidectomy with adjuvant ablation by radioiodine treatment has been the mainstay of thyroid cancer treatment. However, it is often not curative for patients diagnosed with ATC. ATC is one of the most aggressive and deadly human cancers due to its invasive growth behavior and a high propensity for distant metastasis [6]. Therefore, improved therapeutic strategies against thyroid cancer, especially ATC, are urgently needed.

One hallmark of rapidly proliferating tumor cells is the shift from mitochondrial respiration to aerobic glycolysis (Warburg effect) [7]. Although aerobic glycolysis is inefficient from an energetic perspective, it can provide the required biomass for the rapid proliferation [8]. The distinct metabolism of tumor cells makes targeting of metabolic pathways as a promising approach for therapeutic interventions.

Koningic acid, also known as heptelidic acid, is a sesquiterpene lactone initially identified as an antimicrobial agent, active against anaerobic bacteria and also displays antiparasitic properties [9-10]. Earlier studies have confirmed that KA is an effective and specific inhibitor of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is known to be a glycolytic catalytic enzyme and affects the synthesis of ATP[11]. KA irreversibly inactivates GAPDH via covalent binding to a cysteine residue in the active site of the enzyme [12–14]. It has been demonstrated that KA selectively kills high glycolytic cells through glucose-dependent active ATP deprivation [15]. This mechanism may provide an effective treatment for cancer that relies on high glucose metabolism. However, until today, it has not been reported that the inhibition of KA on thyroid cancer. In this paper, we are going to test the antitumor activity of KA against ATC through a series of in vitro and in vivo studies.

## **Materials and methods**

### **Thyroid cancer cell lines**

Thyroid cancer cell lines C643, 8305C and 8505C were provided by Dr. Haixia Guan (The First Affiliated Hospital of China Medical University, Shenyang, P. R. China) and Dr.

Lei Ye (Ruijin Hospital, Shanghai, P. R. China), respectively. Cells were routinely cultured at 37°C in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Invitrogen Technologies, Inc., CA). Cells were treated with KA at the indicated concentrations and time points. KA were purchased from Cayman Chemical (Ann Arbor, MI, USA). KA was dissolved in dimethyl sulfoxide (DMSO), aliquoted and stored at -20°C until further use. The same volume of DMSO was used as the vehicle control.

### **Cell proliferation assay**

Cells (2000/well) were seeded into 96-well plates and treated with various concentrations of KA for 96 h. Cell proliferation was evaluated by 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. IC<sub>50</sub> values were calculated by using the Reed-Muench method. After drug treatment at the indicated time points, 10µL of 5mg/mL MTT (Sigma, Saint Louis, MO) was added to the medium and incubated for 4h, followed by addition of 150µL of DMSO for 15min. The plates were then read on a microplate reader using a test wavelength of 570nm and a reference wavelength of 670nm. Triplicates were performed for each data point.

### **Colony formation assay**

Colony formation assay was performed using monolayer culture. Cells (2000/well) were seeded into 12-well plates and treated with the indicated concentrations of KA and the medium was refreshed every 2 days. After 12 days of culture, surviving colonies (≥50 cells per colony) were fixed with methanol and stained with 0.5 % crystal violet, and the colonies were then counted. Each experiment was performed in triplicate.

### **Apoptosis assay**

8505C, TPC1, K1 and C643 cells were treated with the indicated concentrations of KA for 48 h. Cells were then harvested, washed with PBS, and subjected to sequential staining with Annexin V-FITC/PI Detection Kit (Roche Applied Science, Penzberg, Germany) by flow cytometer according to the manufacturer's protocol. Early apoptotic cells show Annexin V-FITC+/PI- staining patterns, whereas late apoptotic cells exhibit Annexin V-FITC+/PI+ staining patterns. They were collectively called apoptotic cells. Each experiment was performed in triplicate.

### **ATP Determination**

For ATP measurement, a commercially available firefly luciferase assay kit (Beyotime Institute of Biotechnology, China) was used. Briefly, 8505C, TPC1, K1 and C643 cells were incubated with indicated concentrations of KA for indicated hours. After a single wash with ice-cold PBS, cells were lysed with the ATP-releasing reagent provided by the kit. Then, Luciferin substrate and luciferase enzyme were added and bioluminescence was assessed by a fluorescence spectrophotometer. The level of cellular ATP was converted to percentage of control.

### **Xenograft tumor assay in nude mice**

Female athymic nude mice were purchased from SLAC laboratory Animal Co., Ltd. (Shanghai, PR. China) and housed in a specific pathogen-free (SPF) environment. C643 cells ( $3 \times 10^6$ ) were injected subcutaneously into the flanks of mice at the age of 5 weeks. When tumors grew to 5 mm in diameter, mice were divided into two groups (five mice per group) and administered the following treatments: vehicle control (DMSO) or KA, 1 mg/kg through intraperitoneal injection daily for five days. Tumor volume was measured every 2 days during the course of the therapy, and was calculated by the formula  $(\text{width})^2 \times \text{length}/2$ . After treatment for 7 days, tumors were harvested and weighted. Tumor tissues were embedded in paraffin, sectioned at 4  $\mu\text{m}$ , and stained with hematoxylin and eosin (H&E). Cell proliferation ability was assessed by quantification of Ki-67 staining (percentage of positive cells). In brief, anti-human Ki-67 antibody (BD Pharmingen) was 1:150 diluted and immunostaining was done according to a standard protocol using DAB Substrate Kit (ZSGB-BIO). At least 1000 Ki-67- positive cells were scored by visual examination of 5 randomly selected fields ( $\times 200$  magnification). In addition, to evaluate the effect of different treatments on animal hepatocytes and kidney, we performed H&E staining of liver and kidney sections.

## **Results**

### **KA inhibits thyroid cancer cell proliferation**

The dose and time course of the effect of KA on thyroid cell proliferation was evaluated by MTT assay. 7 thyroid cancer cell lines and an immortalized thyroid epithelial cell HTori-3 were examined. As shown in Figure 1, we found that KA significantly inhibited cell proliferation in a dose-dependent manner, with  $\text{IC}_{50}$  values ranging from 1.35  $\mu\text{M}$  to 79.69  $\mu\text{M}$ . Moreover, our data showed that C643, K1,

TPC1 and 8505C cells were more sensitive to KA than other cancer cell lines (Figure 1A). Furthermore, we analyzed the time-dependent response of thyroid cancer cell lines. As shown in Figure 1B, 3.0  $\mu$ M KA significantly inhibited proliferation of TPC1 cells ( $P < 0.01$ ), and 4.0  $\mu$ M KA significantly inhibited proliferation of 8505C cells at 72 hours ( $P < 0.01$ ). The 2.0  $\mu$ M KA significantly inhibited proliferation of K1 cells ( $P < 0.05$ ) and C643 cells ( $P < 0.05$ ) at 72 hours.

### **KA inhibits colony formation in thyroid cancer cells**

We tested the effect of different concentrations of KA on colony formation in 4 thyroid cancer cell lines, including 8505C, TPC1, K1 and C643 cells. As shown in Figure 2, KA at the indicated concentrations significantly inhibited colony forming ability in monolayer culture as compared to the control. In addition, with the increase of KA concentration, the inhibition of clone formation became more significant.

### **KA induces thyroid cancer cells apoptosis**

We detected the effect of KA on the apoptosis in thyroid cancer cell lines, including 8505C, TPC1, K1 and C643 cells. As shown in Figure 3, thyroid cancer cells treated with the indicated concentrations of KA for 48 hours showed a dramatic increase in both early and late apoptosis in comparison with controls. Furthermore, we found that apoptosis in cells treated with 2  $\mu$ M KA was more significant than cells treated with 1.5  $\mu$ M KA.

### **KA affected ATP levels in thyroid cancer cells**

We tested the effect of different concentrations of KA on ATP levels in 4 thyroid cancer cell lines, including 8505C, TPC1, K1 and C643 cells. As shown in Figure 4, at the indicated concentrations, KA significantly caused a rapid, extensive decrease in ATP levels. In addition, at higher KA concentrations, the ATP deprivation became more significant.

### **KA inhibits xenograft tumor growth**

Given the potent inhibitory effects of KA on thyroid cancer cell growth *in vitro*, it is reasonable to assume that KA would be effective in treating thyroid cancers *in vivo*. Thus, we tested the effect of KA on the growth of xenograft thyroid tumors in nude mice. As shown in Figure 5A, C643 cell-derived xenograft tumors grew progressively

in the control group, whereas the tumors were slow-growing in the KA-treated group. Compared with the control mice, tumor volume was significantly lower in KA-treated mice ( $P<0.01$ ). At the end of experiments, tumors were isolated and weighted. The mean of tumor weight was significantly less in KA-treated mice compared with the control mice ( $P<0.01$ ) (Figure 5B). To compare the proliferation index of tumors treated with KA quantitatively, tumor sections were stained for Ki-67 expression. As shown in Figure 5C, nude mice treated with KA had significantly decreased number of Ki-67-positive cells in tumors compared with the controls ( $P<0.001$ ).

Fortunately, histopathological evidence demonstrated that both control (DMSO) and KA-treated hepatic tissues showed large normal polygonal cells with prominent round nuclei and eosinophilic cytoplasm as well as a few spaced hepatic sinusoids arranged between the hepatic cords. No obvious proliferation of mesangial cells or severe inflammatory cell infiltration was observed in renal tissues for both groups by HE staining (Figure 5D). Unfortunately, one mouse's kidney showed mild mesangial cell necrosis or glomerular atrophy. Before and after treatment, there was no significant weight loss in all mice. In summary, our data show that KA did not cause significant liver and kidney injury in mice, suggesting that it may be a safe and effective antitumor agent for thyroid cancer, at least in xenograft tumor models.

## **Discussion**

Although the mortality of thyroid cancer is relatively low and the prognosis is good, there is a risk it develops into anaplastic thyroid cancer (ATC). ATC is a rare undifferentiated thyroid cancer that accounts for about 1 % of thyroid cancer cases; besides, it is arguably the deadliest malignancy in humans [3–5]. At present, conventional surgical thyroidectomy with adjuvant ablation by radioiodine treatment has been the mainstay of thyroid cancer treatment, but the clinical prognosis of differentiated thyroid cancer as well as ATC with metastasis and recurrence are still not optimistic. Until now, the use of multimodality strategies has failed to improve clinical outcomes significantly in those patients. Therefore, it is urgent to find an effective treatment for them. Thanks to the molecular characterization of those tumors recently, which has facilitated a better understanding of the molecular pathogenesis and helped to explore new potential treatments for this disease.

The distinct metabolism of tumor cells with Warburg effect makes targeting of

metabolic pathways a promising approach for therapeutic interventions. Previous studies have shown that there are differences in the expression of glycolytic related proteins in different subtypes of thyroid cancer. Compared with the other cancer subtypes, the glucose transporter 1 and tumoral monocarboxylate transporter 4 were highly expressed in ATC tumor tissues, while the glucose transporter 1, tumoral and stromal monocarboxylate transporter 4 were highly expressed in papillary thyroid cancer tumor tissues. The glucose transporter 1, hexokinase II, carbonic Anhydrase IX, and tumoral monocarboxylate transporter 4 expression levels were higher in PTC tissue with BRAF V600E mutation [16]. We hypothesized that thyroid tumor is also one of the glycolysis - dependent tumors. Hence, we sought to evaluate an alternative strategy which has received increasing attention in recent years: anti-metabolic targeting therapy in thyroid cancer.

Koningic acid, also known as heptelidic acid, is a sesquiterpene lactone which initially identified as an antimicrobial agent, i.e. antiparasitic properties and anaerobic bacteria resistant bioactivities [9-11]. It is known that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a key enzyme in the glycolysis pathway, and it catalyzes the conversion of glyceraldehyde 3-phosphate to 3-phosphoglycerate [17]. KA can selectively kill hyper-glycolytic cells through glucose-dependent active ATP deprivation, while tumor cells are hyper-glycolytic cells, so it is expected to be an effective anti-tumor drug. It has been reported that KA is only effective in high glycolytic cells otherwise alternate starvation-induced metabolic pathways may be initiated to prolong tumor cell life [15]. Studies have confirmed the anti-tumor proliferation activity of KA in many tumor cell lines, such as A549 (lung cancer), HCT116 (colorectal cancer), KG1 (leukemia) and A375 (melanoma) [11], but it was I research about KA's inhibition on thyroid cancer.

In this study, we demonstrated the anti-thyroid tumor bioactivity of KA for the first time through *in vivo* and *in vitro* experiments. The experiments *in vitro* showed that KA can inhibit the proliferation, colony formation, and induce the apoptosis in different thyroid cancer cell lines. The experiments *in vivo* confirmed that KA inhibited the xenograft tumor growth without significant liver and kidney injury. Further mechanism studies revealed that the anti-tumor bioactivity of KA might be related to ATP deprivation, and the ATP level of thyroid tumor cells treated with KA was significantly reduced. Other studies have also shown that KA selectively kills high-glycolytic cells through glucose-dependent active ATP deprivation, and cell

growth inhibition is not associated with caspase-3 but with severe depletion of ATP promoted by glucose phosphorylation [15].

In summary, both *in vivo* and *in vitro* experiments confirmed that KA has significant anti-thyroid cancer bioactivity without severe adverse drug reactions. These encouraging preliminary results suggested that KA may be a promising therapeutic agent for thyroid cancer.

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** Animal experiments were approved by the Institutional Review Board of Xi'an Jiaotong University Health Science Center.

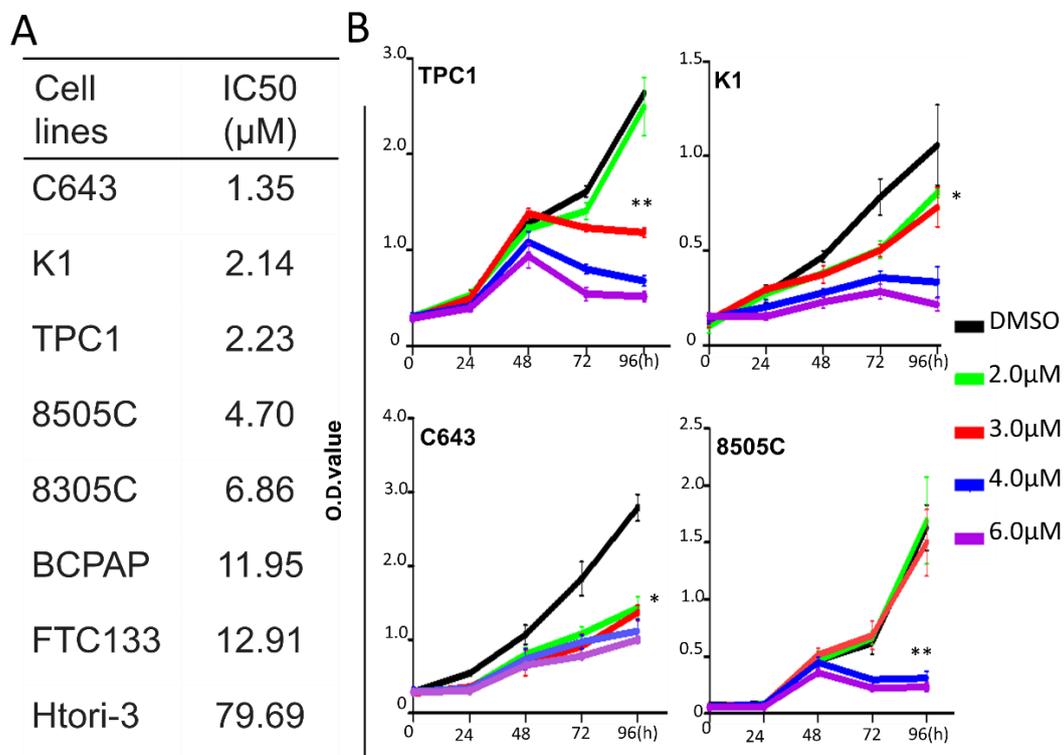


Figure 1. Proliferation-inhibitory of KA on thyroid cancer cell lines. (A) Thyroid cancer cell lines were treated with the indicated concentrations of KA for 24 hours, followed by MTT assay to evaluate cell growth. IC50 values were calculated using the Reed-Muench method. (B) Selected 4 thyroid cancer cell lines were treated with the indicated concentrations of KA or vehicle control (DMSO) at the indicated time point, followed by MTT assay to evaluate the time course of cell proliferation. Statistically significant differences are indicated: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

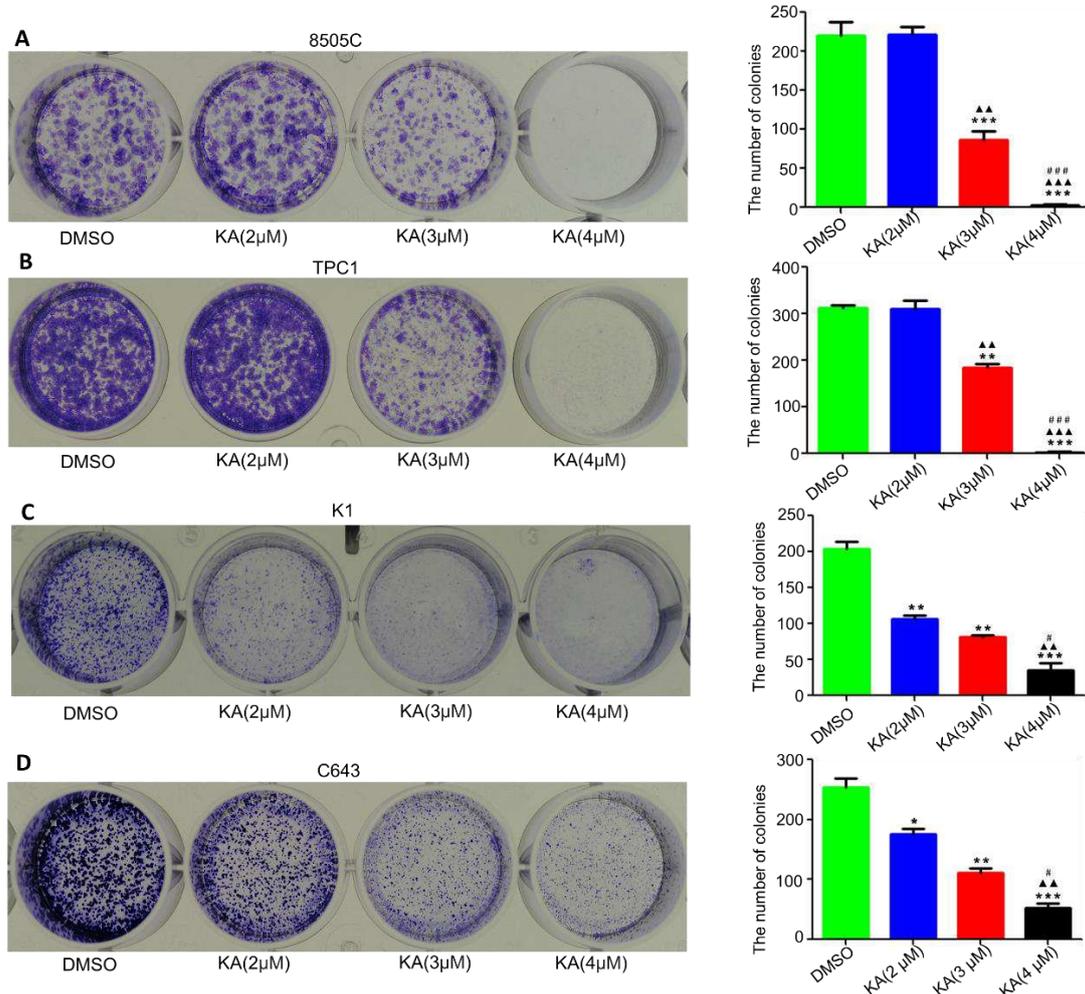


Figure 2. Inhibition of colony formation of thyroid cancer cell lines by KA. Representative images of colony formation in 8505C, TPC1, K1 and C643 cells treated with vehicle control (DMSO) or KA at the indicated concentrations are shown individually. Quantitative analysis of colony numbers is shown in right panel. Data are presented as mean  $\pm$  SD of values from three different measurements. Statistically significant differences are indicated: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  for comparison with control; ▲▲,  $P < 0.01$ ; ▲▲▲,  $P < 0.001$  for comparison with KA in 2 µM concentration; #  $P < 0.05$ ; ###,  $P < 0.01$ ; ####,  $P < 0.001$  for comparison with KA in 3 µM concentration.

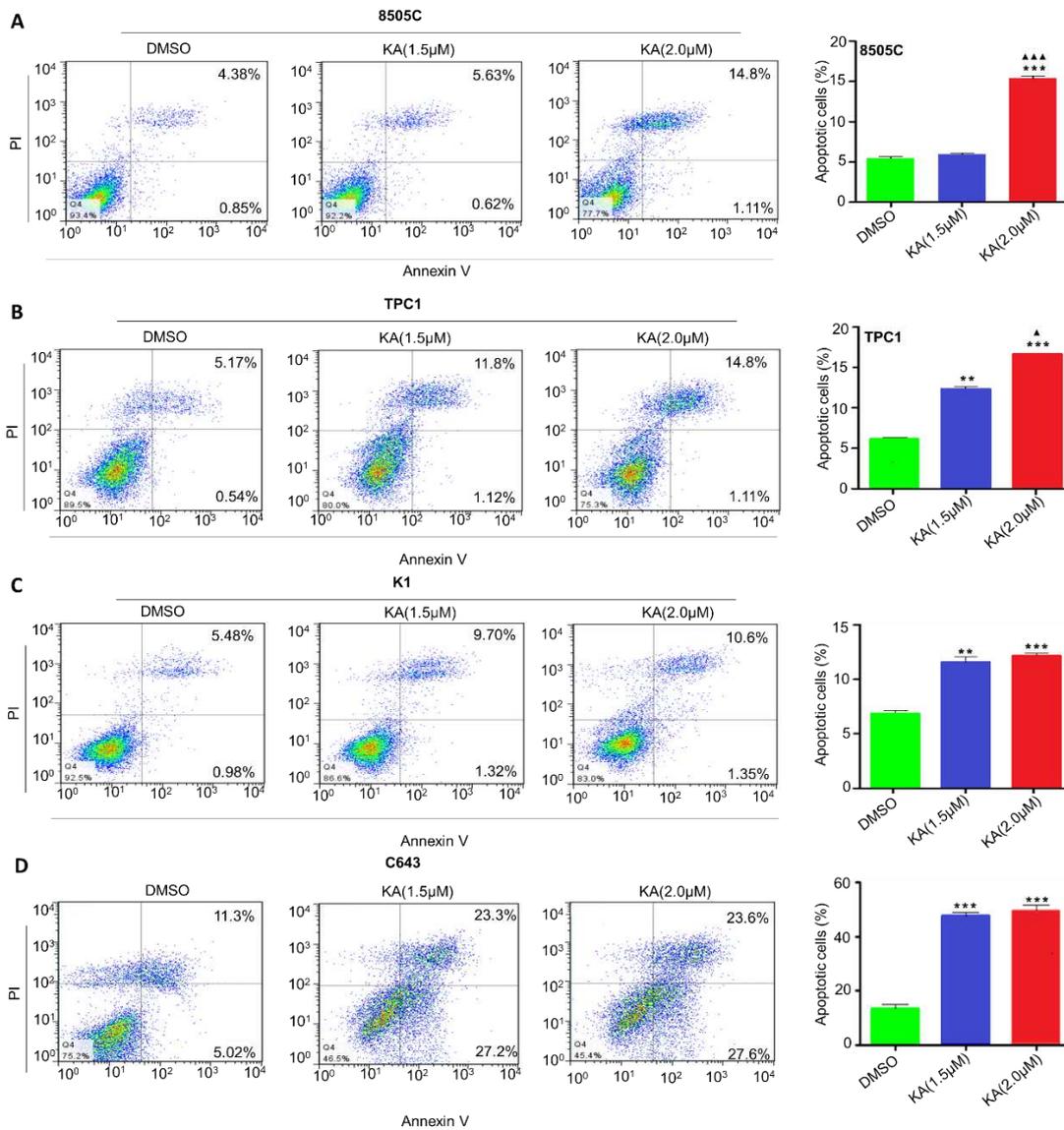


Figure 3. Induction of apoptosis by KA in thyroid cancer cell lines. 8505C (A), TPC1 (B), K1 (C) and C643 (D) cells were treated with vehicle control (DMSO) or KA at the indicated concentrations for 48h. The percentage of early apoptotic (bottom right quarter) and late apoptotic (top right) cells is presented in the figures (left panel). The data are presented as mean  $\pm$  SD of values from three independent experiments in the right panel. Statistically significant differences are indicated: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  for comparison with control.  $\blacktriangle$ ,  $P < 0.01$ ;  $\blacktriangle\blacktriangle$ ,  $P < 0.001$  for comparison with KA in 1.5  $\mu$ M concentration.

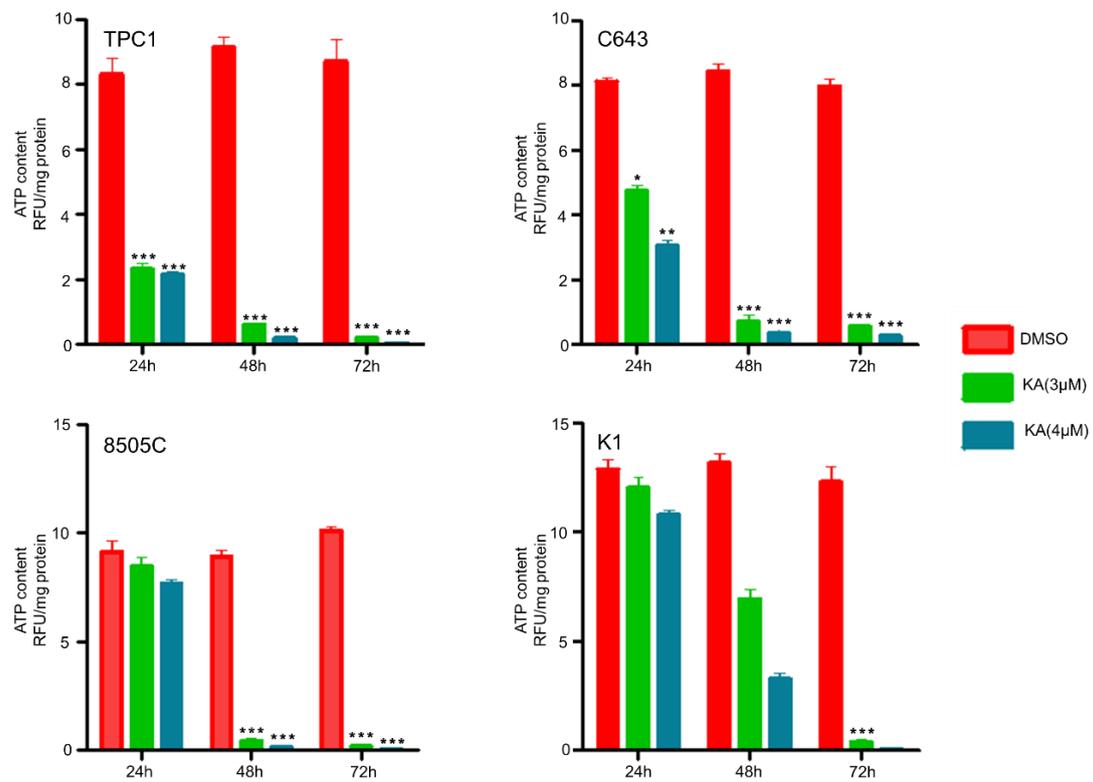


Figure 4. The effect of KA on ATP generation in thyroid cancer cell lines. ATP levels in 8505C, TPC1, K1 and C643 cells were determined after treated with KA at the indicated concentrations for the indicated times. Data are presented as mean  $\pm$  SD of values from three different measurements. Statistically significant differences are indicated: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  for comparison with control.

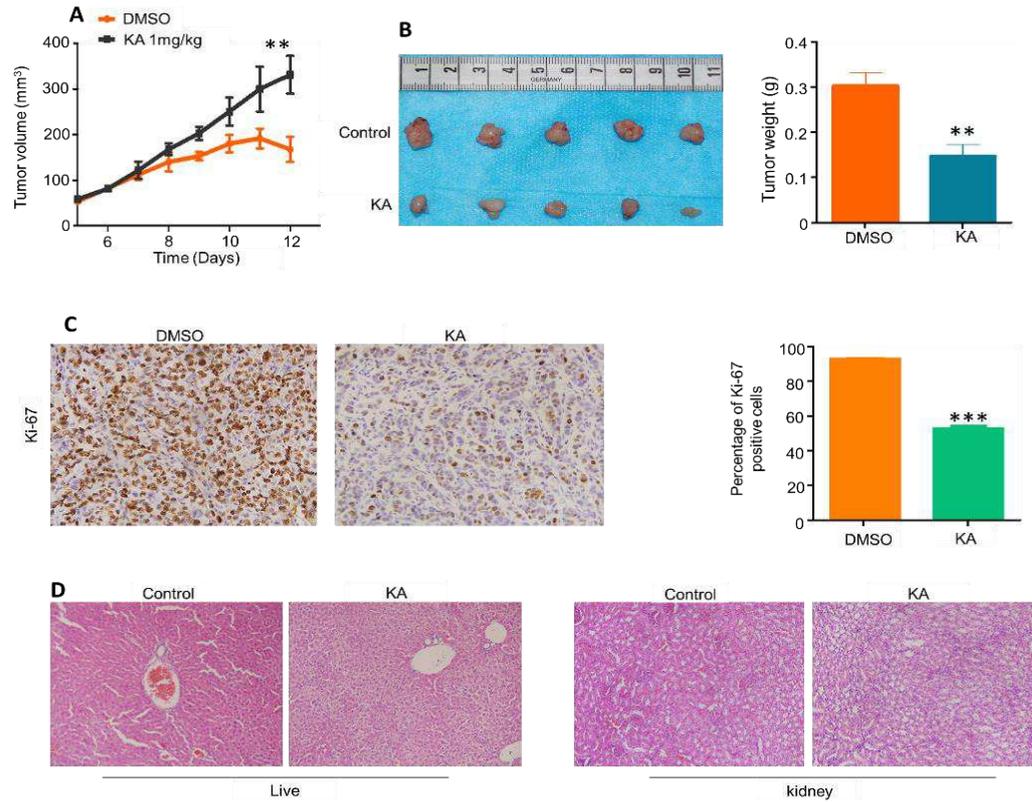


Figure 5. Inhibition of the growth of C643-derived xenograft tumor by KA. Time course of tumor growth and animal weight was measured in each group at the indicated time points of various treatments. Data are presented as mean  $\pm$  SD (n=5/group). **A.** Time course of tumor growth was measured in each group at the indicated time points of various treatments. **B.** Pictures were animal weight at the end time points of different treatments. Bar graphs represents mean tumor weight from mice with the indicated treatments. **C.** Representative Ki-67 stained sections of xenograft tumors. Bar graphs represent mean  $\pm$  SD of the numbers of Ki-67-positive cells from 5 microscopic fields in each group (right panel). **D.** Representative H&E stained liver and kidney sections from the indicated mice. Statistically significant differences are indicated: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  for comparison with control.

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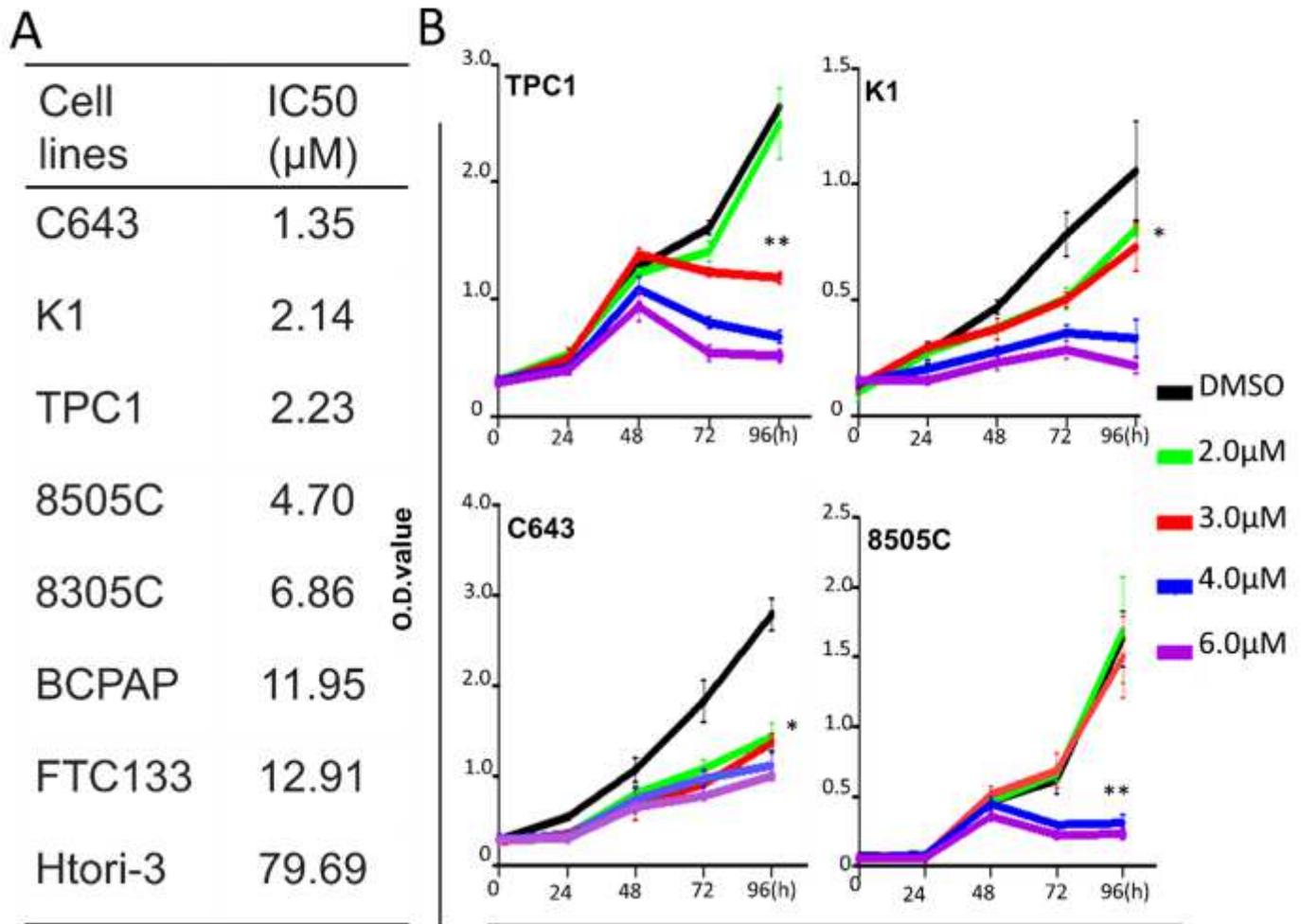
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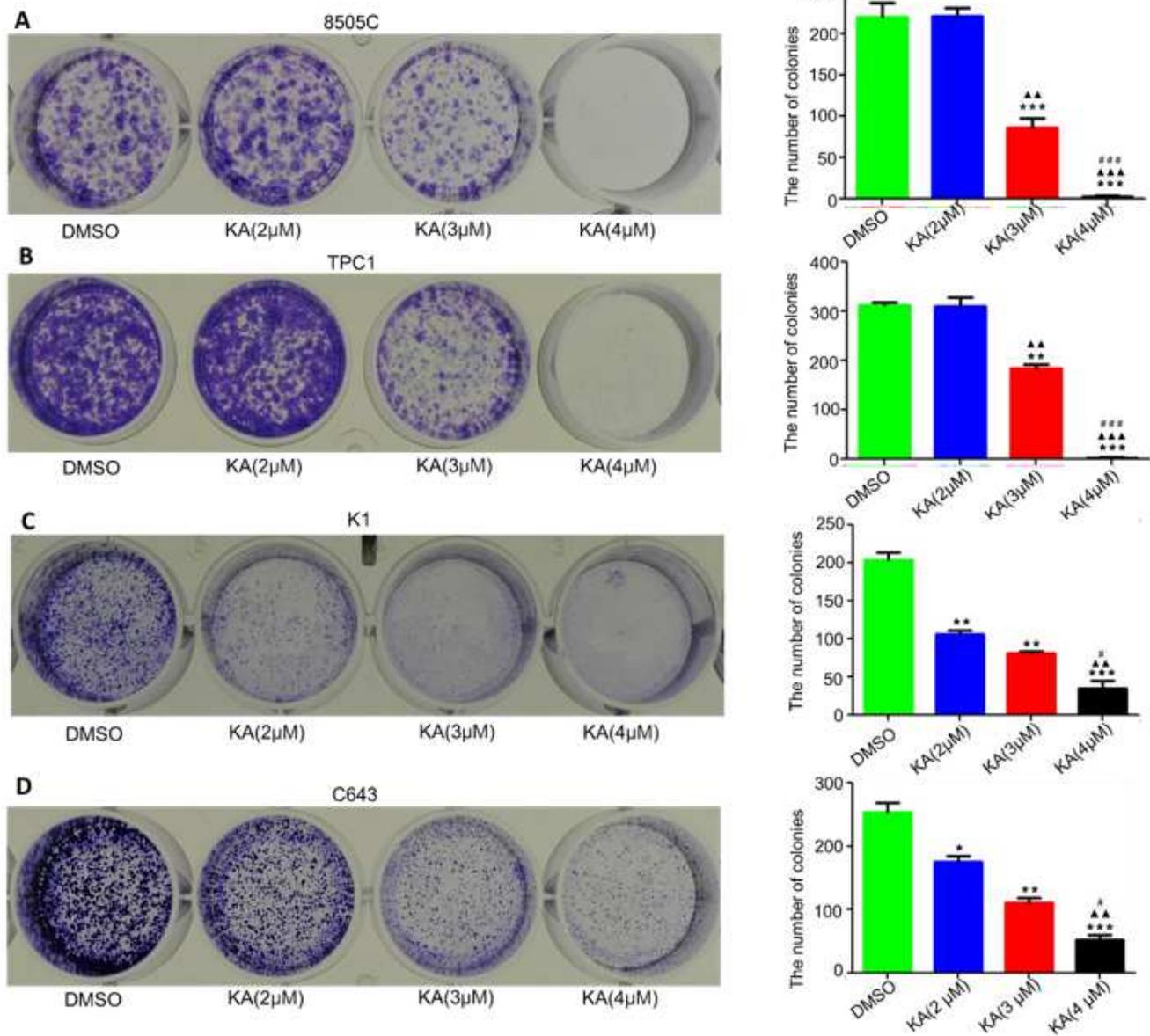
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# Figures



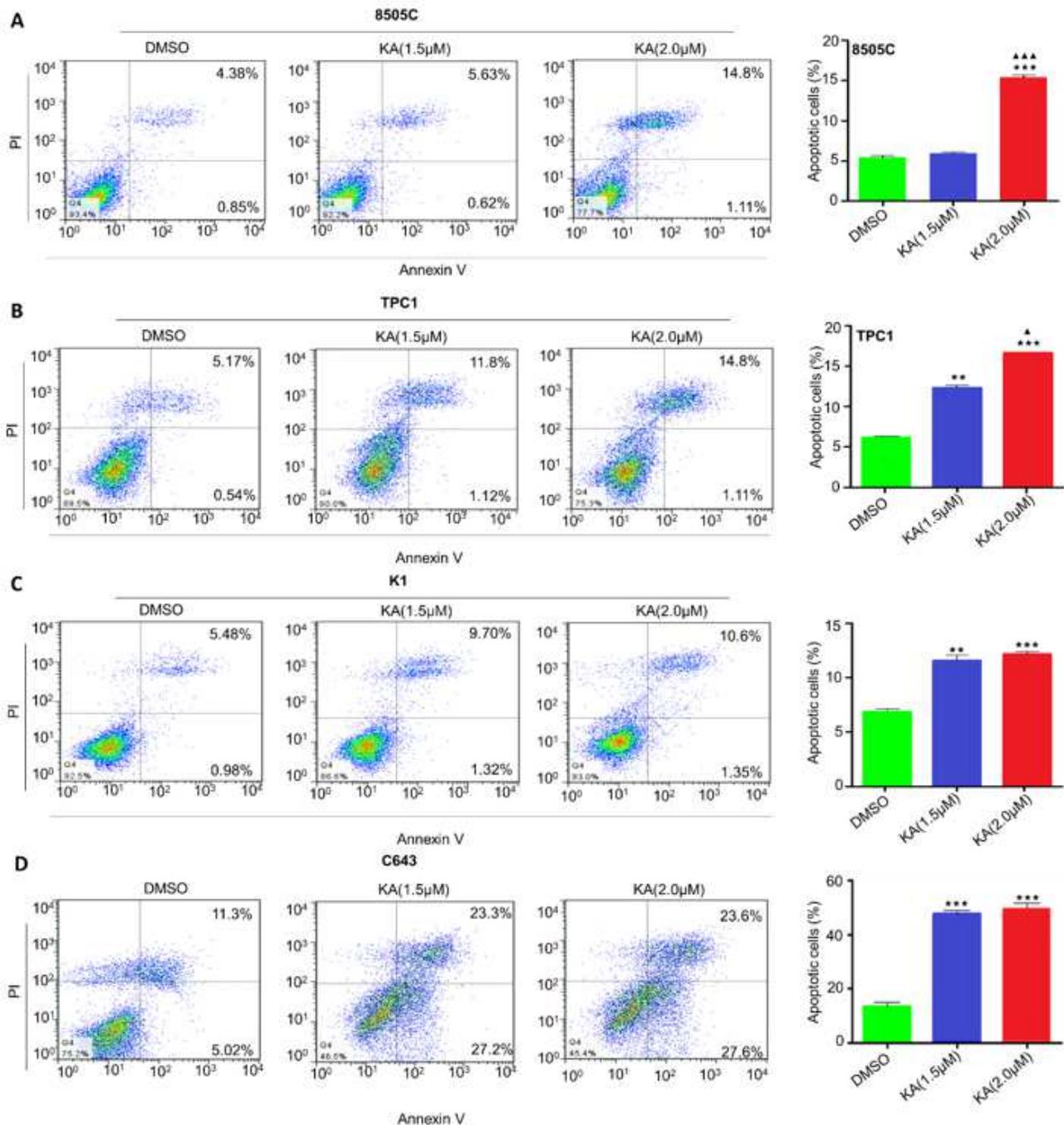
**Figure 1**

Proliferation-inhibitory of KA on thyroid cancer cell lines. (A) Thyroid cancer cell lines were treated with the indicated concentrations of KA for 24 hours, followed by MTT assay to evaluate cell growth. IC50 values were calculated using the Reed-Muench method. (B) Selected 4 thyroid cancer cell lines were treated with the indicated concentrations of KA or vehicle control (DMSO) at the indicated time point, followed by MTT assay to evaluate the time course of cell proliferation. Statistically significant differences are indicated: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



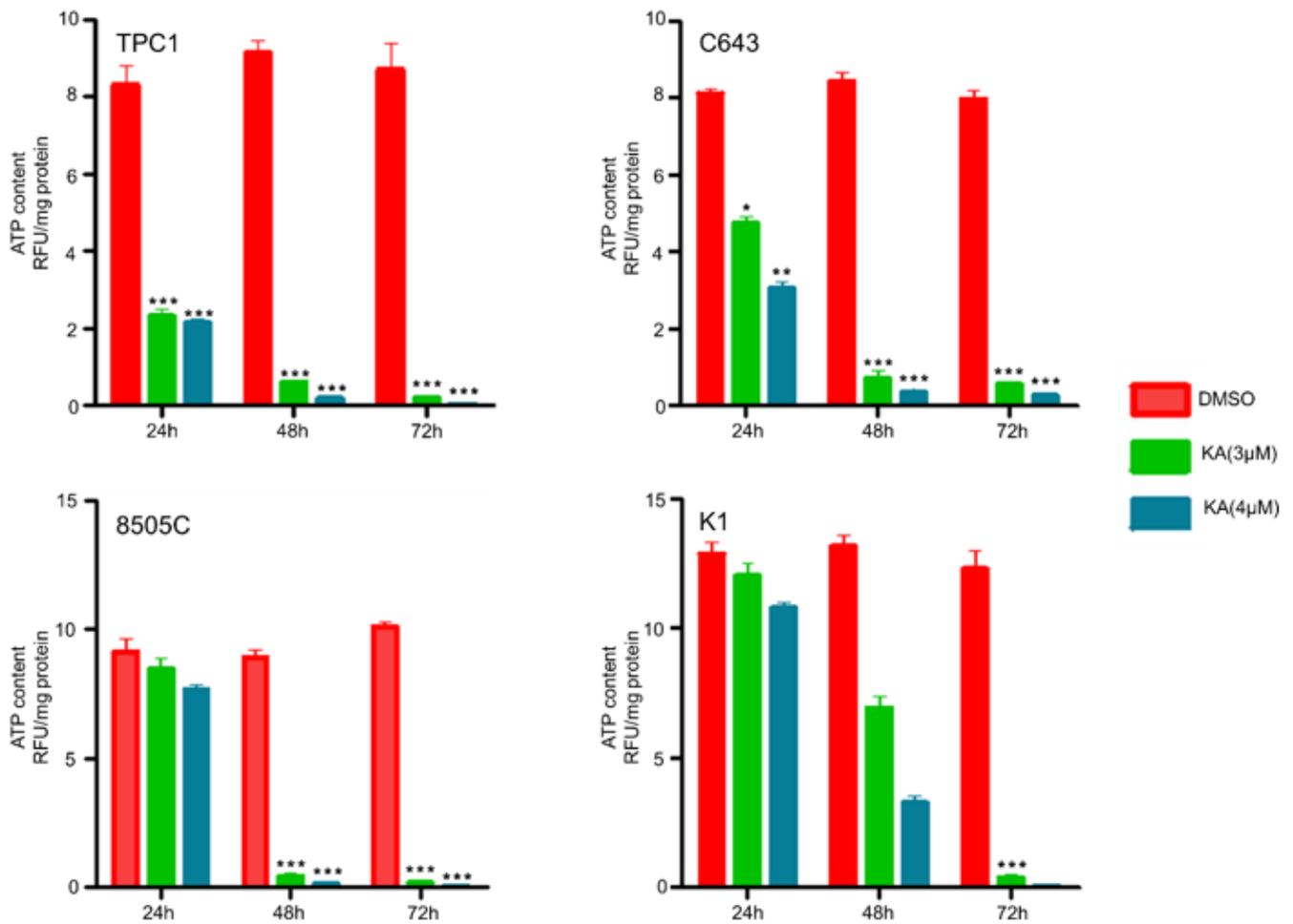
**Figure 2**

Inhibition of colony formation of thyroid cancer cell lines by KA. Representative images of colony formation in 8505C, TPC1, K1 and C643 cells treated with vehicle control (DMSO) or KA at the indicated concentrations are shown individually. Quantitative analysis of colony numbers is shown in right panel. Data are presented as mean  $\pm$  SD of values from three different measurements. Statistically significant differences are indicated: \*, $P < 0.05$ ; \*\*, $P < 0.01$ ; \*\*\*, $P < 0.001$  for comparison with control;  $\square\square$ , $P < 0.01$ ;  $\square\square\square$ , $P < 0.001$  for comparison with KA in 2  $\mu\text{M}$  concentration; # $P < 0.05$ ; ##,  $P < 0.01$ ; ###,  $P < 0.001$  for comparison with KA in 3  $\mu\text{M}$  concentration.



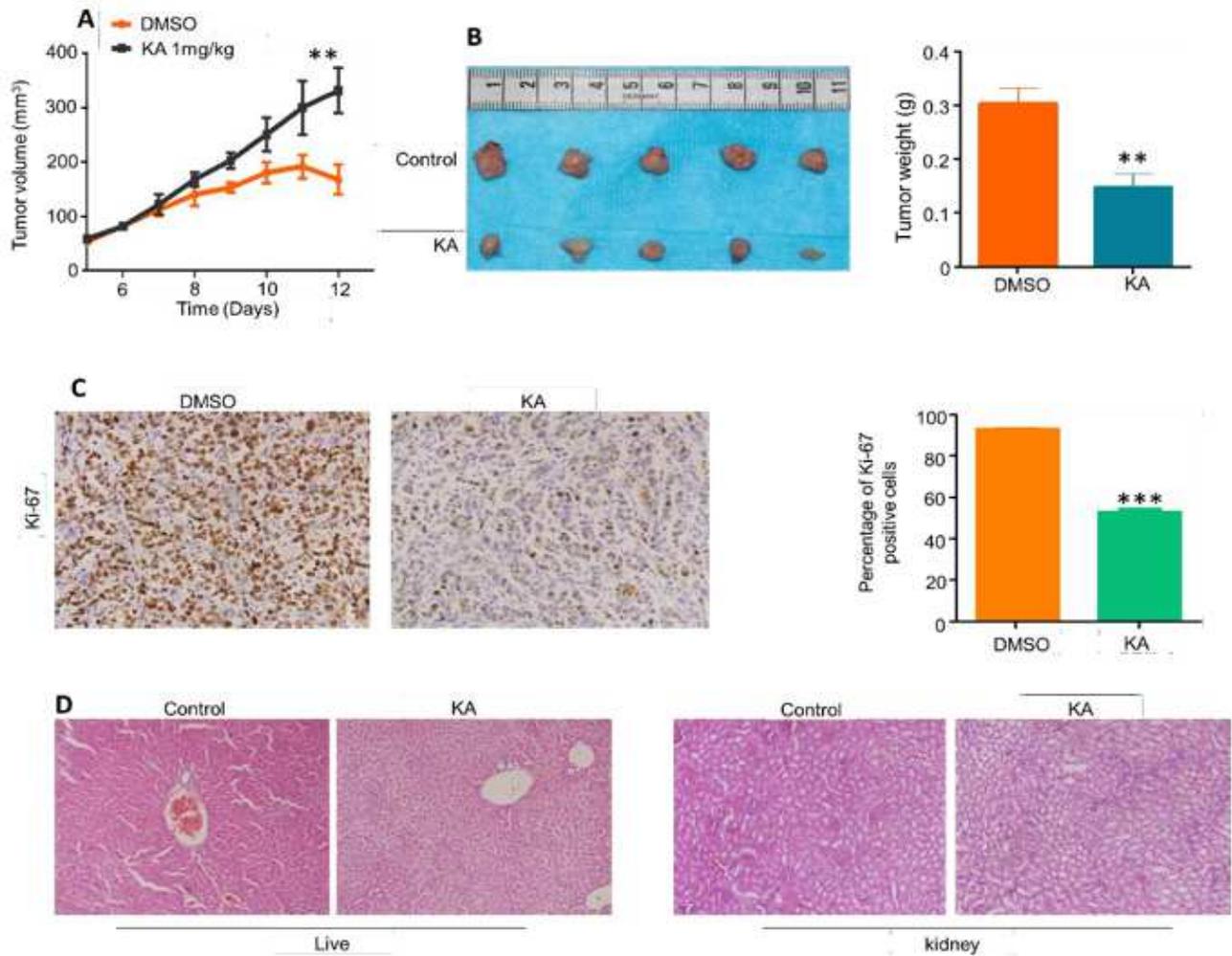
**Figure 3**

Induction of apoptosis by KA in thyroid cancer cell lines. 8505C (A), TPC1 (B), K1 (C) and C643 (D) cells were treated with vehicle control (DMSO) or KA at the indicated concentrations for 48h. The percentage of early apoptotic (bottom right quarter) and late apoptotic (top right) cells is resented in the figures (left panel). The data are presented as mean  $\pm$  SD of values from three independent experiments in the right panel. Statistically significant differences are indicated: \*, $P < 0.05$ ; \*\*, $P < 0.01$ ; \*\*\*, $P < 0.001$  for comparison with control. ☒☒, $P < 0.01$ ; ☒☒☒, $P < 0.001$  for comparison with KA in 1.5  $\mu$ M concentration.



**Figure 4**

The effect of KA on ATP generation in thyroid cancer cell lines. ATP levels in 8505C, TPC1, K1 and C643 cells were determined after treated with KA at the indicated concentrations for the indicated times. Data are presented as mean  $\pm$  SD of values from three different measurements. Statistically significant differences are indicated: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  for comparison with control.



**Figure 5**

Inhibition of the growth of C643-derived xenograft tumor by KA. Time course of tumor growth and animal weight was measured in each group at the indicated time points of various treatments. Data are presented as mean  $\pm$  SD (n=5/group). A. Time course of tumor growth was measured in each group at the indicated time points of various treatments. B. Pictures were animal weight at the end time points of different treatments. Bar graphs represents mean tumor weight from mice with the indicated treatments. C. Representative Ki-67 stained sections of xenograft tumors. Bar graphs represent mean  $\pm$  SD of the numbers of Ki-67-positive cells from 5 microscopic fields in each group (right panel). D. Representative H&E stained liver and kidney sections from the indicated mice. Statistically significant differences are indicated: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  for comparison with control.