Original Article

Evaluation of *in vitro* and *in vivo* activity of a multityrosine kinase inhibitor, AL3810, against human thyroid cancer

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Abstract

Thyroid cancer is the most common type of endocrine neoplasia. Despite recent breakthroughs in treatment of the disease, the treatment of advanced, progressive thyroid cancers remains challenging with limited therapeutic options available. In this study, we evaluated a novel and orally bioavailable small-molecule multiple tyrosine kinases inhibitor, AL3810, in preclinical models of thyroid cancer *in vitro* and *in vivo*. AL3810 (2–5 μmol/L) dose-dependently inhibited the proliferation of human thyroid cancer cell lines TT, SW579 and TPC-1 *in vitro* with IC_{50} values ranging from 0.59 to 7.03 μmol/L. Specifically, this agent dose-dependently arrested the thyroid cancer cells in the G_1 phase and induced apoptosis. Furthermore, AL3810 dose-dependently inhibited the migration and invasion of SW579 and TPC-1 cells *in vitro*. In SW579 and TT xenograft models, oral administration of AL3810 (5–20 mg·kg^{-1}·d^{-1}) for 21 d potently inhibited the tumor growth; immunohistochemical staining revealed that the antitumor activity of AL3810 was closely correlated with its anti-angiogenesis effect, as evidenced by a dose-dependent reduction of microvessels in tumor tissues. To assess the therapeutic potential of AL3810 in treating thyroid cancer involving RET gene fusion, we showed that AL3810 (1–10 μmol/L) dose-dependently inhibited the proliferation of RET-driven Baf3 cell line Baf3-CCDC6-RET, and the auto-phosphorylation of RET in these cells. Our data suggest that AL3810 is a promising agent for the treatment of thyroid cancer.

Keywords: thyroid cancer; AL3810; multi-tyrosine kinases inhibitor; angiogenesis; RET

Introduction

Thyroid cancer is the most prevalent endocrine malignancy, accounting for 1%-1.5% of all deaths due to cancers[1]. This percentage has increased by 2.4% annually from 1980 to 1997 and by 6.6% annually from 1997 to 2009[2]. Thyroid cancer can be divided into 3 primary histologic types, differentiated, medullary, and anaplastic-thyroid cancer, and the characteristics and prognoses of these histologic subtypes differ[3]. Currently, the treatment of thyroid cancer may include surgical resection, radioactive iodine (RAI) after surgery, and thyroid stimulating hormone (TSH) inhibition[4, 5]. However, many patients are refractory to RAI, and alternative options for treatment remain limited[6].

In the last decade, the scientific community has made significant strides toward a better understanding of the biology of thyroid cancer, and this research has facilitated significant progress in the development of novel and more effective treatment modalities for advanced thyroid cancer[7, 8]. Like most endocrine tumors, thyroid tumors are richly vascularized, and initial clinical studies exploited the important role of increased angiogenesis in thyroid cancer[9]. Since increased VEGF, VEGFR and fibroblast growth factor receptor expression are significantly associated with advanced-staged thyroid cancer[10-12], the use of inhibitors against these angiogenesis signaling pathways may represent a viable approach to control malignant thyroid cancer[9, 13, 14]. Furthermore, current clinical trials examining these small inhibitors have shown promising results, although the efficacy of these agents varies widely[15, 16]. In addition to the angiogenesis pathway, RET mutations also reportedly participate in the development and aggressive phenotype of thyroid cancers[17]. Specifically, somatic mutations in the RET gene are present in 20%–80% of sporadic medullary thyroid cancer (MTC) cases[18, 19]. Moreover, several rearranged forms of RET have been identified in up to 80% of papillary thyroid cancer patients[20, 21]. In all cases, RET kinase is activated independently

of ligand binding and induces the malignant transformation of cells[22, 23], and uncontrolled RET activity is both sufficient and necessary to cause neoplastic phenotype[24]. Therefore, RET represents a potential target for thyroid cancer therapy[25].

AL3810, also named E-3810, was developed as a novel, orally bioavailable small-molecule inhibitor that targets multiple angiogenic kinases, including VEGFR1, VEGFR2, FGFR1 and PDGFR[26, 27]. At present, this agent is undergoing advanced clinical trials in China, Europe and the USA. Our previous studies showed that AL3810 is a potent anti-angiogenic agent and significantly inhibits tumor growth in established human renal, liver and pancreatic cancer xenograft models[28]. We also found that AL3810 moderately inhibits RET kinase at the molecular level[28]. Based on these studies, angiogenesis plays a very important role in the development and metastasis of thyroid cancer, and RET is another important oncogene related to medullary and papillary thyroid cancer. Therefore, we exploited the therapeutic effect of AL3810 against thyroid cancer.

Materials and methods
Compounds
AL3810 was originally designed by US Advenchen labs, and its purity was 99.1%. Sorafenib, vandetanib (ZD6474) and cabozantinib (XL184) were all purchased from Selleck (Houston, TX, USA). For *in vitro* studies, these compounds were dissolved in DMSO as a 10 mmol/L stock solution and stored at -20°C. For *in vivo* studies, AL3810 was dissolved with 0.5% carboxymethyl cellulose sodium.

Cell cultures
The human thyroid cancer cell lines SW579, TT, and TPC-1 and mouse cell line Baf3 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). SW579 cells were grown in L-15 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). TT cells were grown in F12K (ATCC) supplemented with 15% FBS and nonessential amino acids (NEAA; Sigma, St Louis, MO, USA). TPC-1 cells were grown in DMEM (Gibco) supplemented with 10% FBS. Baf3 cells were grown in RPMI-1640 (Gibco) supplemented with 10% NBSC (Gibco) and IL3. Baf3 cells transfected with RET were grown in RPMI-1640 (Gibco) supplemented with 10% FBS and 2 μg/mL puromycin.

Proliferation assays
Thyroid cancer cells were seeded in 96-well plates at an appropriate density of 3000-10 000 cells per well and cultured for 24 h before exposure to increasing doses of AL3810. After being cultured for 3 or 5 d, TT, TPC-1 and SW579 cells were fixed with 10% trichloroacetic acid (TCA) and stained with SRB (Sigma) in 1% acetic acid (*v/v*). SRB in the cells was dissolved with 150 μL of 10 mmol/L Tris-HCl and measured at 560 nm with a spectra MAX190 (Molecular Devices, Sunnyvale, CA, USA). Treated Baf3 cells were incubated with 10% 1.5 mg/mL resazurin (Sigma) and for another 1–3 h. The fluorescence intensity was then detected at an excitation wavelength range

of 540±35 nm and an emission wavelength range of 590±35 nm using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Burlington, VT, USA)[29]. The dosages corresponding to the half-maximal inhibition (IC_{50}) were calculated using a SoftMax pro-based nonlinear 4-parameter regression analysis. IC_{50} values (mean±SD) are presented as histograms.

Western blot assays
The cells were grown to half confluence in six-well plates and then exposed to various concentrations of compounds for 2 h to detect the phosphorylation of tyrosine kinase receptors and downstream signal transduction pathways. Equal amounts of protein were probed with primary antibodies against phospho-STAT3 (p-STAT3) (Tyr705), STAT3, phospho-ERK1/2 (p-ERK1/2), ERK1/2, phospho-AKT (p-AKT) (ser473), AKT, phospho-RET (p-RET) (Tyr905) and RET (Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight.

Annexin V/PI apoptosis assays
An Annexin V-FITC/PI double-staining apoptosis detection kit (KeyGEN Biotech, Nanjing, China) was used to quantitatively measure the percentage of apoptotic cells. Briefly, cells were seeded in six-well plates at the appropriate density and then cultured in medium alone or with various concentrations of AL3810 for 48 h or 72 h. The probed cells were collected, stained, and examined using a FACS Calibur Instrument (BD Biosciences, Franklin Lake, NJ, USA). The resultant apoptosis data were analyzed with the FlowJo software.

Cell cycle assays
The cells were cultured in medium alone or with various concentrations of AL3810 for 24 h or 48 h. The probed cells were collected and stained with RNA enzyme and PI and then examined using a FACS Calibur Instrument (BD Biosciences). The resultant data were analyzed with the BD Cell Quest Pro software.

Colony formation assays
TPC-1 and SW579 cells were cultured for 10–15 d in 6-well plates at the appropriate density until colonies were visible. The colonies were then fixed in 10% formaldehyde and 10% acetic acid at room temperature for 10 min and then stained with 0.1% crystal violet for 15 min. The remaining crystal violet was removed by washing the cells with water before drying them at room temperature. Colony formation was then quantitatively measured by dissolving crystal violet in 33.3% (*v/v*) acetic acid and measuring the absorbance at 600 nm with a spectra MAX190 (Molecular Devices).

Monolayer wound-healing assays
TPC-1 and SW579 cells were seeded into 96-well plates overnight to form a monolayer with 90% confluence and subsequently cultured in medium alone or with various concentrations of AL3810 for 24 h. The monolayer was then wounded by creating a scratch with a p200 pipette tip (Axygen Scientific, Union City, CA, USA). After washing the monolayer twice

with PBS to remove the detached cells, the adherent cells were cultured in medium containing indicated concentration of AL3810 for 0, 6, 12 and 24 h, and microphotographs were then captured at ×10 magnification using an Olympus IX51 Research Microscope with a DP-70 camera (Olympus, Tokyo, Japan).

Transwell assays

TPC-1 and SW579 cells were cultured in medium alone or with various concentrations of AL3810 for 24 h. The cells were then digested and added to the top chambers of twenty-four-well Transwell plates (8 µm; Corning Costar Corp, Corning, NY, USA) containing 100 µL serum-free medium or medium containing various concentrations of AL3810; the bottom chambers were filled with 600 µL of complete medium containing 10% FBS. Cultures were maintained for 3, 6 and 10 h. The migrated cells were fixed in 95% ethanol and stained with 0.1% crystal violet, and images were then captured with an inverted fluorescence microscope (Olympus B-51, Tokyo, Japan). The number of cells that had penetrated the membrane was measured by dissolving crystal violet in 33.3% (v/v) acetic acid and measuring the absorbance at 600 nm with a spectra MAX190 (Molecular Devices).

Immunohistochemistry assays

Xenograft tumor tissues were fixed in formalin, embedded and deparaffinized before being incubated with CD34 antibody (1:50, Abcam, UK) and TUNEL *in situ* cell death detection Kit-POD reagent (Roche, Basel, Switzerland) at 4 °C overnight. After being washed with PBS, the slides were incubated with DAKO secondary antibody (EnVision™ Detection System, DAKO), and peroxidase activity was detected with diaminobenzidine (DAB). Three random areas from three individually stained tumors were captured with a Leica Upright Metallurgical Microscope (Leica Microsystems, Wetzlar, Germany).

Tumor xenograft assays

Athymic BALB/c nude mice aged 4–6 weeks were housed and maintained under specific pathogen-free conditions with a 12 h light/dark cycle at 25±1 °C and received food and water *ad libitum*. All experiments were carried out according to the Institutional Ethical Guidelines on Animal Care and were approved by the Institute of Animal Care and Use Committee at the Shanghai Institute of Materia Medica. The mice were randomly assigned to the control (12 mice) and treatment groups (six mice per treatment group). The control group was administered only vehicle, whereas treatment groups received AL3810, XL184 or Sorafenib every day by oral gavage for the duration indicated in each treatment. The sizes of the tumors were measured twice per week using microcalipers. The tumor volumes (V) were calculated as follows: $V=1/2(\text{length}\times\text{width}^2)$. The individual relative tumor volumes (RTV) were calculated as follows: $RTV=V_t/V_0$, where V_t is the volume on each day, and V_0 is the volume at the beginning of the treatment. The therapeutic effect of the compounds was described as the volume ratio of treatment to control T/C (%)=100%×(mean RTV of the treated group/mean RTV of the

control group).

Statistical analysis

All experiments were performed at least three independent times. Student's *t* test was applied for statistical comparisons using GraphPad Prism 6. Unless otherwise indicated, the results are expressed as the mean±SD from at least three independent experiments. Differences were considered to be significant at *P*<0.05 and *P*<0.01.

Results

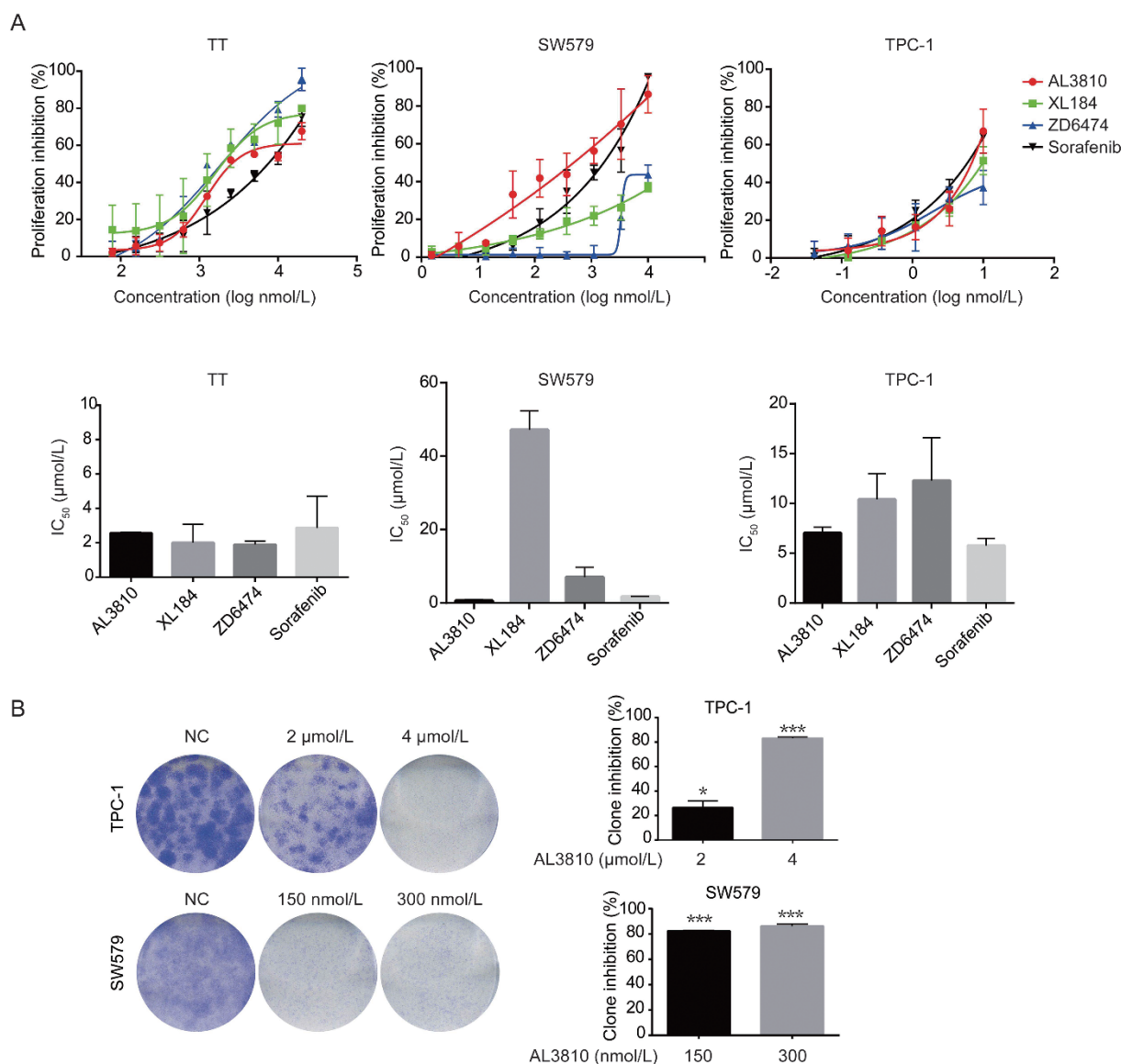### AL3810 inhibits the proliferation of thyroid cancer cells *in vitro*

The antiproliferative effect of AL3810 on human thyroid cancer cell lines was evaluated based on the IC_{50} values. To this end, three thyroid cancer cell lines were incubated in increasing concentrations of AL3810 in media supplemented with 10% FBS, and proliferation was measured using an SRB assay. After 72 h, AL3810, XL184 and ZD6474 dose-dependently inhibited the proliferation of thyroid cancer cell lines, with mean IC_{50} values ranging from 0.59 to 7.03 µmol/L (Figure 1A), and colony formation assay data supported these results. Thus, AL3810 efficiently reduced colony formation in TPC-1 and SW579 cells (Figure 1B).

AL3810 inhibits growth of thyroid xenografts *in vivo*

To further explore the antitumor efficacy of AL3810 *in vivo*, human thyroid xenograft models were established using TT and SW579 cells, which exhibit differing histology. Briefly, the tumors were allowed to grow to sizes between 100 and 300 mm³ before the initiation of daily oral treatment with AL3810 for 3–4 weeks. Treatment with AL3810 did not affect body weight (Figure 2A). AL3810 showed significant antitumor activity at a concentration of 2 mg/kg, with a T/C value of 33.3% in the SW579 model, and almost completely inhibited tumor growth at 5, 10, and 20 mg/kg, with T/C values of 2.43%, 0.77% and 0.79% on d 21, respectively. AL3810 was significantly more effective in inhibiting tumor growth than 60 mg/kg Sorafenib and 30 mg/kg XL184 (Figure 2A). Moreover, the effect of AL3810 appeared to be sustained, with tumor volumes of 35 mm³ 2 weeks after drug withdrawal in the control group and 22 mm³ in animals treated with 20 mg/kg AL3810 for 3 weeks (Figure 2B). To mimic therapy for advanced thyroid cancer, SW579 xenografts were allowed to grow to 1800 mm³ before the initiation of daily treatment with AL3810, which markedly reduced tumor volume. Specifically, the mean tumor volume decreased to 300 mm³ after treatment with 10 mg/kg AL3810 for 5 weeks (Figure 2C). Similarly, AL3810 substantially inhibited TT tumor growth in a dose-dependent manner at 2 and 10 mg/kg, with a T/C value of 28.92% at 10 mg/kg (Figure 2D). These results demonstrate that AL3810 exhibits the significant antitumor activity in these two thyroid cancer xenograft models, which exhibit differing histology.

AL3810 has potent anti-angiogenesis activity in thyroid cancer xenografts

Because the inhibition of angiogenesis may serve as a novel

Figure 1. AL3810 exhibits potent anti-proliferative activity against thyroid cancer cell lines TT, SW579 and TPC-1 *in vitro*. (A) Inhibition of TT, SW579 and TPC-1 cell proliferation by AL3810 and the IC_{50} values of AL3810, XL184, ZD6474 and Sorafenib. (B) Colony formation was measured with crystal violet staining. $^*P<0.05$ and $^{***}P<0.01$.

thyroid cancer treatment strategy and AL3810 is an angiogenesis inhibitor, the anti-angiogenesis efficacy of AL3810 was evaluated in the same human thyroid cancer xenograft models described above. To this end, tumor microvessels were immunohistochemically stained with an antibody against the endothelial cell marker CD34, which showed that treatment with AL3810 dramatically decreased the number of microvessels in the tumor compared with the control group (Figure 2E). Specifically, the number of microvessels was decreased by 81.88% and 92.83% in SW579 xenograft tumors treated with 5 mg/kg and 10 mg/kg AL3810, respectively, and 59.06%, 81.10% and 63.78% in TT xenograft tumors treated with 0.4 mg/kg, 2 mg/kg and 10 mg/kg AL3810, respectively (Figure 2E). Taken together with the antitumor activity of AL3810

described above, these results suggest that AL3810 inhibits thyroid cancer by inhibiting angiogenesis.

AL3810 arrests thyroid cancer cells in the G_1 phase and induces apoptosis

Flow cytometry was used to assess changes in the cell cycle and apoptosis in thyroid cancer cells treated with various concentrations of AL3810. The results showed that AL3810 arrested TPC-1 and SW579 cells in the G_1 phase and induced apoptosis in a concentration-dependent manner (Figure 3A and 3B). Specifically, treatment with 1 μmol/L AL3810 for 24 h increased the percentage of SW579 cells in the G_1 phase from 48.5% (control) to 64.2%, and treatment of the same duration with 2 μmol/L AL3810 further increased the G_1 population to 64.6% and con-
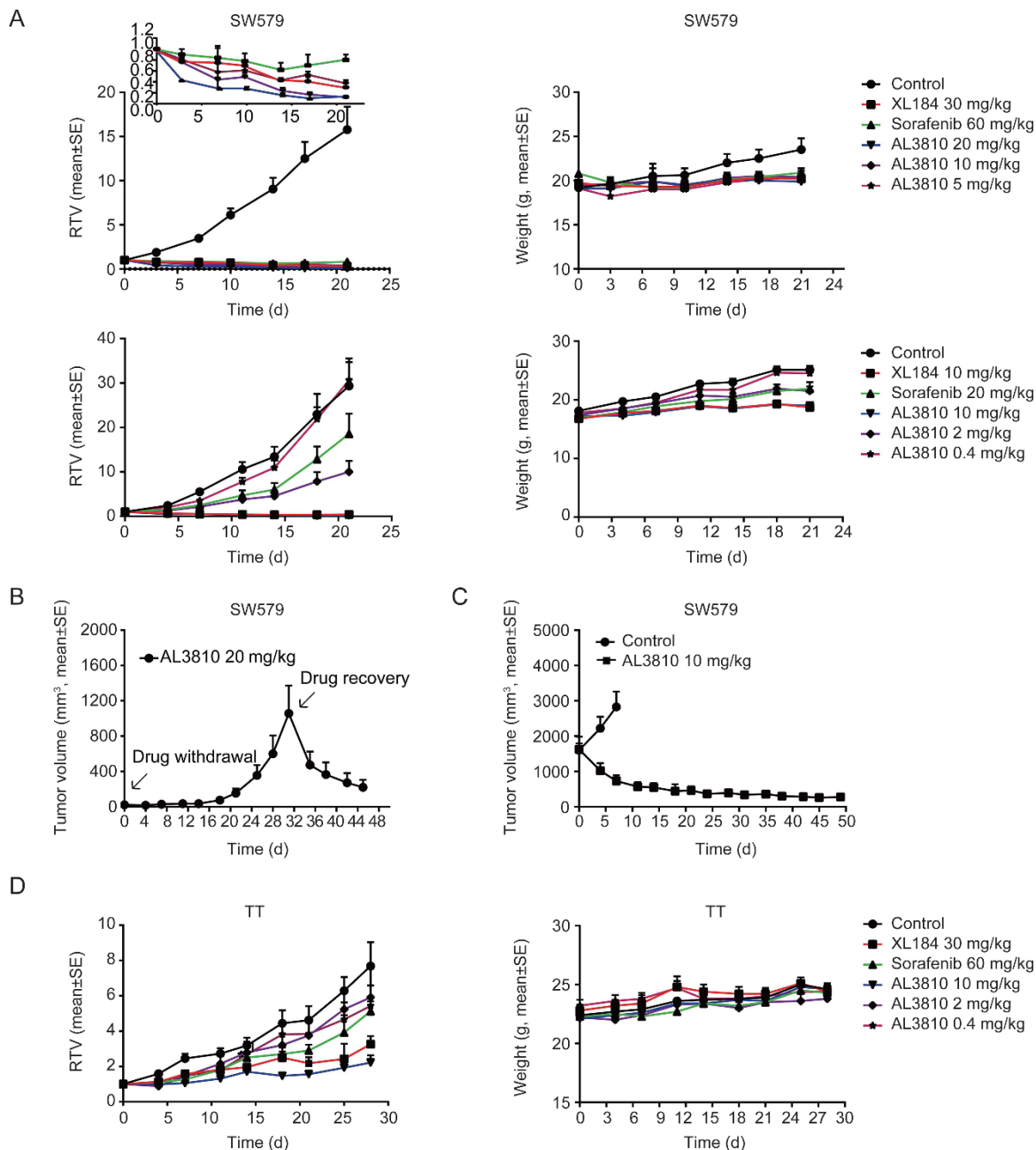
Figure 2A–2D. AL3810 inhibits thyroid tumor growth and decreases tumor microvessel density *in vivo*. (A) The activity of AL3810 against thyroid cancer was tested using SW579 cells (A, B and C) and TT (D) xenograft models. The animals were randomly divided into groups, and AL3810 was orally administered once daily at the indicated dose and duration. Changes in body weight were not observed (right panel of A and D).

comitantly decreased the S and G_2/M populations. We then detected the expression of proteins involved in the G_1 phase after treating cells with AL3810 for 24 h, which showed that AL3810 markedly up-regulated p21 and p27 protein and down-regulated CyclinD1, CDK2 and P-Rb, but it did not impact the cell cycle proteins CyclinE and c-Myc (Figure 3C).

In addition, after treatment with 0.5 μmol/L AL3810 for 48 h, approximately 20% of SW579 cells underwent apoptosis (Figure 3D). Furthermore, to confirm the effect of AL3810 on tumor apoptosis, thyroid cancer TT and SW579 xenografts that had been treated with vehicle or AL3810 were dissected and immunohistochemically stained for the apoptosis marker TUNEL after the last administration. Compared with the control group, TUNEL staining in TT and SW579 xenograft tumors from mice treated with AL3810 was dramatically increased (Figure 3E), which further confirmed that AL3810 induced apoptosis in thyroid cancer cells.

AL3810 suppresses the migration of thyroid cancer cells
Scratch wound-healing and Transwell assays were performed
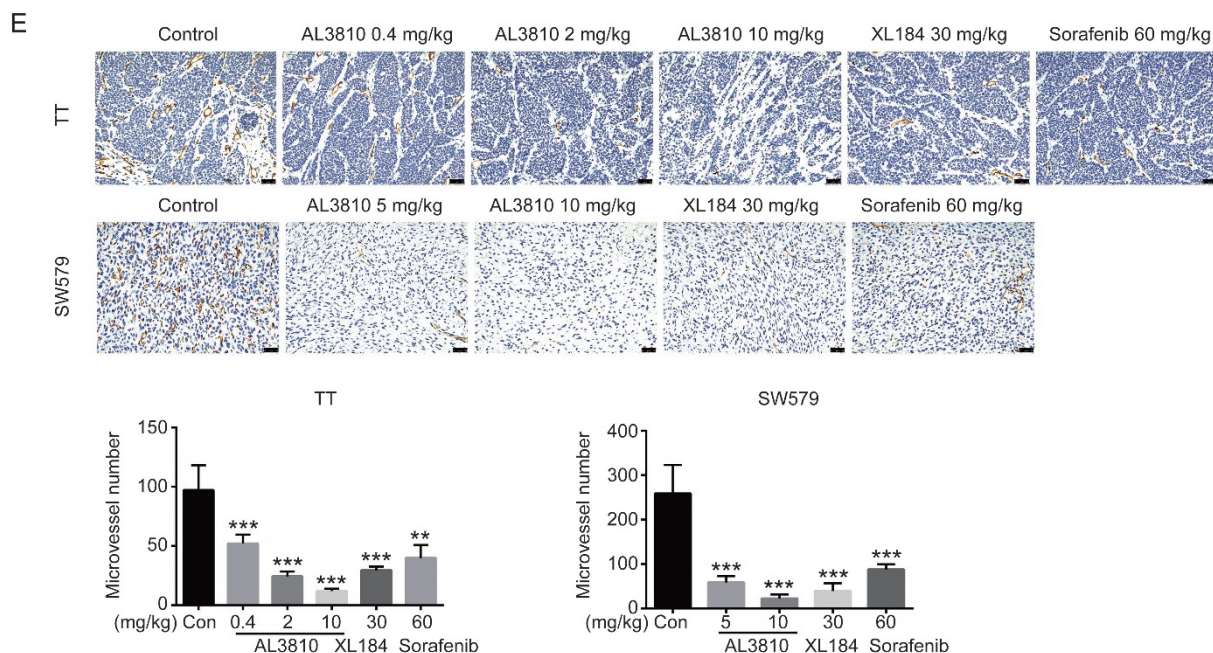
Figure 2E. AL3810 inhibits thyroid tumor growth and decreases tumor microvessel density *in vivo*. (E) Tumors were harvested after treatment and immunohistochemically analyzed for microvessel density (CD34) (E, the scale bar was 50 µm). The microvessel numbers are quantified in a histogram. [**]$P<0.01$ and [***]$P<0.001$.

to explore the effect of AL3810 on the migration of thyroid cancer cells. As shown in Figure 4A, the control SW579 and TPC-1 cells migrated into the denuded area in the wound-healing assay and almost covered the exposed surface. In contrast, a much smaller number of cells migrated into the denuded area after treatment with AL3810. The same results were observed in the Transwell migration assay, as indicated by a marked decrease in the number of cells that had migrated through the filter of the Transwell chamber in the AL3810-treated groups (Figure 4B). Furthermore, the quantitative analysis confirmed that AL3810 significantly decreased the relative migration efficacy of SW579 and TPC-1 cells (lower panel of Figures 4A and 4B). Thus, AL3810 significantly suppressed the migration and invasionof thyroid cancer cellsin a dose-dependent manner.

AL3810 shows RET inhibition *in vitro*

Evidence from cell-free kinase assays suggests that AL3810 inhibits RET kinase (IC$_{50}$=200 nmol/L). Furthermore, RET plays an important role in thyroid cancer progression. Therefore, the antitumor activities of AL3810 were evaluated in RET gene fusion-driven thyroid cancer models to assess the therapeutic potential of AL3810 in thyroid cancer involving RET gene fusions. First, we characterized the ability of AL3810 to inhibit the kinase activity of mutated RET V804M, which was previously shown to render resistance to other RET inhibitors in biochemical assays. Unfortunately, this agent was not active against this mutated RET variant (Figure 5C).

To further evaluate the inhibition of RET gene fusions in cells by AL3810, we generated stable transfectants of Baf3 cells

with plasmids encoding either CCDC6-RET or CCDC6-RET-V804M. As a control, we also generated mock-transformed Baf3 cells. Resazurin assay results indicated that AL3810 significantly inhibited the proliferation of the RET-driven Baf3 cell line Baf3-CCDC6-RET. This inhibition was similar to that of XL184 and ZD6474, with IC$_{50}$ values of 2.77 µmol/L, 2.61 µmol/L and 2.71 µmol/L, respectively (Figure 5A). In contrast, this agent did not affect the growth of Baf3 parent cells at a concentration of 20 µmol/L (Figure 5B). We then examined the change in RET phosphorylation and downstream signaling. AL3810 treatment dose-dependently inhibited RET auto-phosphorylation in Baf3 CCDC6-RET cells (Figure 5D), and 0.625 µmol/L AL3810 reduced this phosphorylation, whereas 2.5 µmol/L AL3810 almost completely abrogated this phosphorylation in Baf3 RET wild-type cells. AL3810 treatment also reduced ERK phosphorylation at similar doses used to reduce RET phosphorylation, indicating that this agent suppressed the oncogenic signaling of RET in these cells.

We further explored the influence of AL3810 against tyrosine kinase RET and downstream signaling in thyroid cancer. To this end, Western blotting was performed to detect changes in intracellular signaling after treating TT and TPC-1 cells with various concentrations of AL3810 for 2 h. The results showed that AL3810 time- and dose-dependently decreased the phosphorylation of RET and downstream ERK1/2 and AKT in these RET fusion cells (Figure 5E).

Discussion

In this study, we evaluated the antitumor and anti-angiogenesis activities of AL3810, an angiogenesis inhibitor that tar-
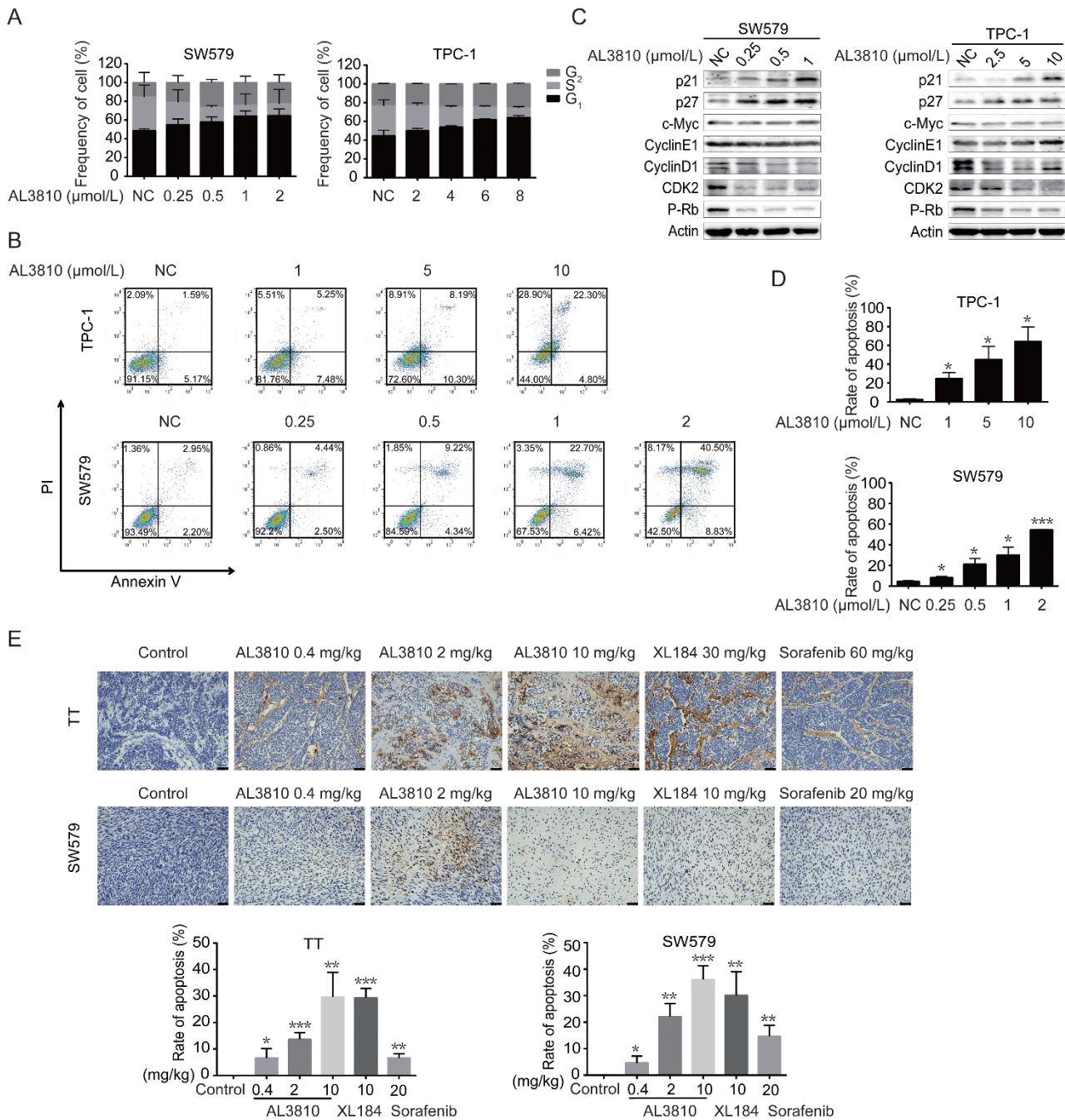
Figure 3. AL3810 arrests thyroid cancer cells in the G_1 phase and induces apoptosis. SW579 and TPC cells were treated with increasing concentrations of AL3810 for 24 h or 48 h *in vitro*, and the cell cycle (A) and apoptosis (B) were assessed by flow cytometry. Cells were collected for Western blotting with the indicated antibodies after treatment with AL3810 (B). (D) Apoptosis was assessed using Annexin V⁺-PI staining, and the quantitative results are shown in a histogram. Each condition was studied at $n \geq 3$, $^{*}P<0.05$ and $^{***}P<0.001$. (E) Representative TUNEL staining images of untreated SW579 and TT xenografts and xenografts treated with AL3810 (the scale bar indicates 50 μm). $^{*}P<0.05$ and $^{**}P<0.01$.

gets multiple RTKs, in human thyroid cancer models. Orally administered AL3810 significantly inhibited tumor angiogenesis in thyroid xenografts, as evidenced by a decrease in CD34 staining and subsequent inhibitions in tumor growth. However, AL3810 did not show potent antiproliferative activity against thyroid cancer cell lines *in vitro*, which also suggests that AL3810 inhibits thyroid cancer models primarily by inhib-

iting angiogenesis. Furthermore, we showed that AL3810 inhibits RET enzymatic function. Encouragingly, AL3810 potently inhibits human thyroid cancer both *in vitro* and *in vivo* compared to XL184, ZD6474 and Sorafenib.

Clinical trials of multikinase inhibitors that target vascular endothelial growth factor (VEGF) receptor signaling have shown efficacy in thyroid cancer[30, 31]. Thyroid cells require
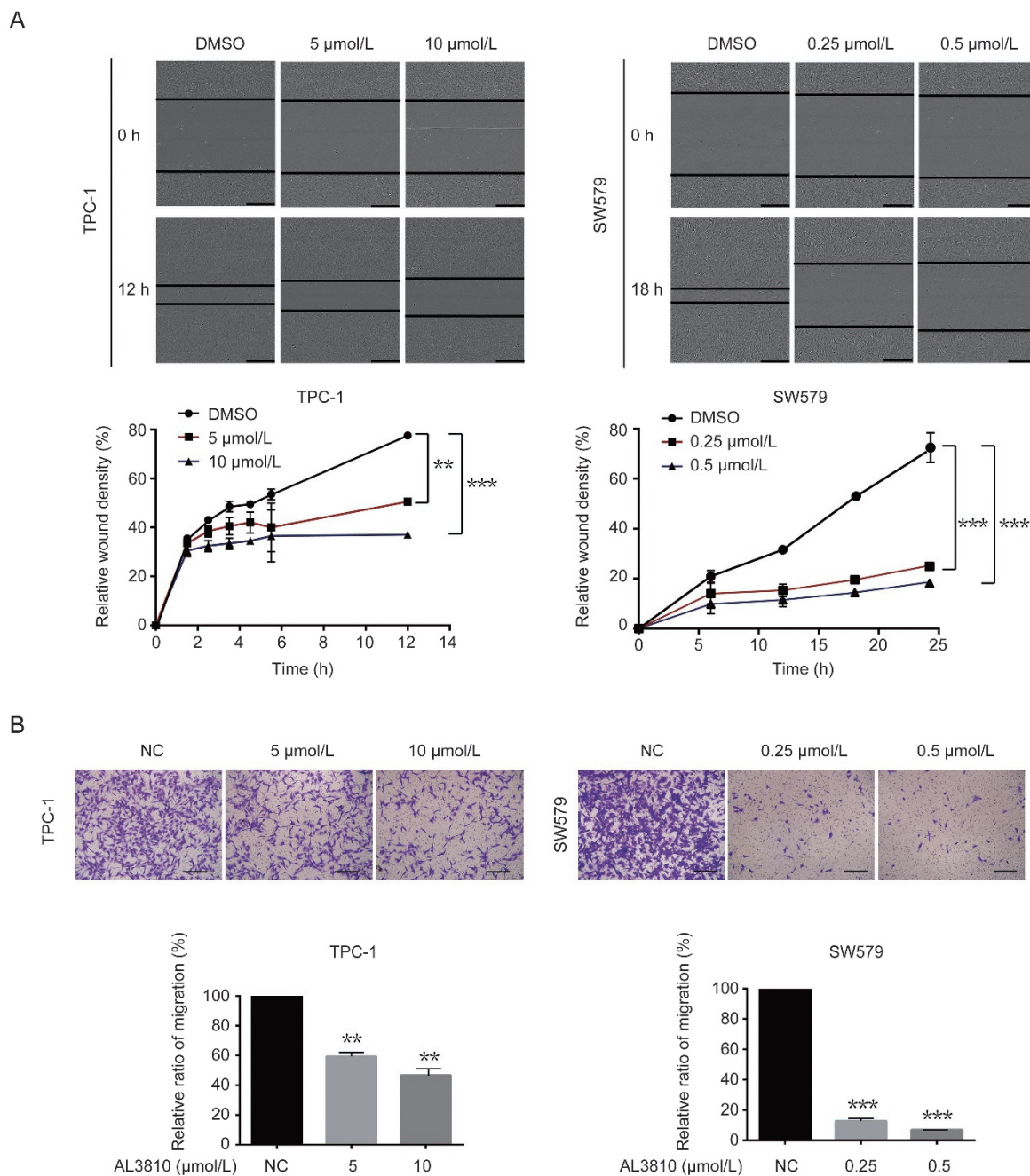
Figure 4. AL3810 suppresses the migration and invasion of thyroid cancer cells. SW579 and TPC-1 cells were treated with increasing doses of AL3810 for the indicated times, and the migration of cells was measured in wound-healing (A, the scale bar indicates 300 μm) and Transwell assays (B, the scale bar indicates 50 μm). $^{**}P<0.01$ and $^{***}P<0.001$.

contact with capillaries to function normally and secrete trophic signals for capillary endothelial cells, primarily VEGF[32]. On transformation and loss of polarity, a disorganized tumor vasculature may result in cancer-cell hypoxia, a loss of immune surveillance, increased VEGF receptor activation, and a dependence on VEGFR signaling that can be leveraged therapeutically[33]. Accordingly, the *in vivo* administration of AL3810 significantly reduced the growth of xenografts and efficiently inhibited the activation of endothelial cell-expressed

VEGFR2 and PDGFR tyrosine kinases, which decreased tumor microvessel density. The magnitude of the antiangiogenic effect of AL3810 suggests that its overall antitumor effects may be due to its antiangiogenic properties.

RET is a well-defined clinical target in thyroid cancer, as recently demonstrated by XL184 and ZD6474, two multikinase inhibitors that are approved for the treatment of advanced MTC[34-37]. Although AL3810 inhibits RET kinase activity at submicromolar concentrations in enzymatic assays, the high
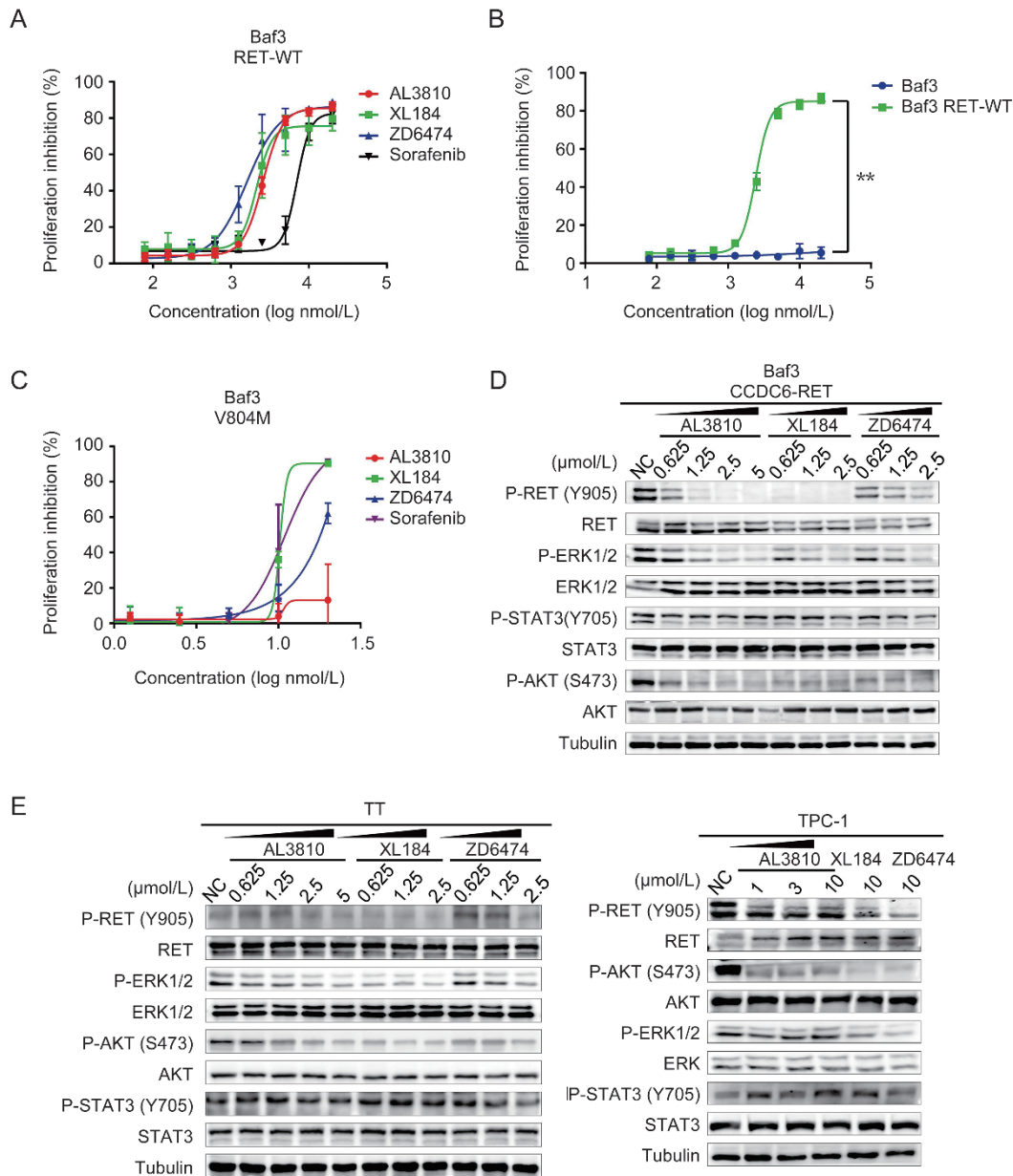
Figure 5. AL3810 inhibits RET. AL3810 inhibited the proliferation of RET-driven Baf3 cells (A) but had no effect on parental Baf3 cells (B) and Baf3 cells transfected with RET V804M (C). Baf3/CCDC6-RET cells (D) and the RET-positive thyroid cancer cell lines TT and TPC-1 (E) were collected and examined by Western blotting with the indicated antibodies after treatment with various concentrations of AL3810 for 2 h. **P<0.01.

potency shown by this compound against RET-dependent Baf3 cells was comparable to its activity against RET-negative cells, with at least 10-fold selectivity versus Baf3 parent cells. These results demonstrate that the inhibition activity in RET may contribute to antitumor activity of AL3810.

In conclusion, AL3810 showed promising antitumor activity against human thyroid cancer, which was due to its inhibition of angiogenesis. In addition, the AL3810-mediated inhibition of RET may also inhibit thyroid tumors harboring RET alterations. These data suggest that AL3810 is an effective therapeutic agent in thyroid cancer that warrants further clinical development.

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