

Lenvatinib exhibits antineoplastic activity in anaplastic thyroid cancer *in vitro* and *in vivo*

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Abstract. Lenvatinib is an oral, multitargeted tyrosine kinase inhibitor (TKI) of VEGFR1-VEGFR3, FGFR1-FGFR4, PDGFR α , RET and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) signaling networks involved in tumor angiogenesis. We have evaluated the antitumor activity of lenvatinib in primary anaplastic thyroid cancer (ATC) cells, in the human cell line 8305C (undifferentiated thyroid cancer) and in an ATC-cell line (AF). The AF cell line was obtained from the primary ATC cultures and was the one that grew over 50 passages. The effect of lenvatinib (1 and 100 nM; and 1, 10, 25 and 50 μ M) was investigated in primary ATC, 8305C and AF cells as well as in AF cells in CD nu/nu mice. Lenvatinib significantly reduced ATC cell proliferation ($P < 0.01$, ANOVA) and increased the percentage of apoptotic ATC cells ($P < 0.001$, ANOVA). Furthermore, lenvatinib inhibited migration ($P < 0.01$) and invasion ($P < 0.001$) in ATC. In addition, lenvatinib inhibited EGFR, AKT and ERK1/2 phosphorylation and downregulated cyclin D1 in the ATC cells. Lenvatinib also significantly inhibited 8305C and AF cell proliferation, increasing apoptosis. AF cells were subcutaneously injected into CD nu/nu mice and tumor masses were observed 20 days later. Tumor growth was significantly inhibited by lenvatinib (25 mg/kg/day), as well as the expression of *VEGF-A* and

microvessel density in the AF tumor tissues. In conclusion, the antitumor and antiangiogenic activities of lenvatinib may be promising for the treatment of anaplastic thyroid cancer, and may consist a basis for future clinical therapeutic applications.

Introduction

Lenvatinib is an oral, multitargeted tyrosine kinase inhibitor (TKI) of vascular endothelial growth factor receptors 1-3 (VEGFR1-VEGFR3), fibroblast growth factor receptors 1-4 (FGFR1-FGFR4), PDGFR α , RET and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) signaling networks involved in tumor angiogenesis (1).

In vitro studies have evaluated lenvatinib in preclinical models. Lenvatinib decreased the auto-phosphorylation of KIF5B-RET, CCDC6-RET and NcoA4-RET, inhibited the proliferation of CCDC6-RET human thyroid and lung cancer cell lines and blocked the tumorigenicity of RET gene fusion-transformed NIH3T3 cells (2). Orally administered lenvatinib exhibited antitumor activity in xenograft models of five differentiated thyroid cancer (DTC) cell lines, five anaplastic thyroid cancer (ATC) cell lines and one medullary thyroid cancer cell line (3). Lenvatinib exhibited antiangiogenic activity in five DTC and five ATC xenografts, while its antiproliferative activity was shown *in vitro* only in 2/11 thyroid cancer cell lines (i.e. RO82-W-1 and TT cells). Lenvatinib was also able to inhibit RET phosphorylation in TT cells with the activating mutation C634W (3).

In vivo phase II (4,5) and phase III (6) studies in patients with aggressive DTC not responsive to radioiodine have demonstrated that the administration of lenvatinib is associated with an improvement in progression-free survival (PFS) compared with placebo (median PFS 18.2 vs. 3.6 months). Following the results of this phase III study, lenvatinib has been approved for the treatment of patients with locally recurrent or metastatic, progressive, radioactive iodine refractory DTC (7).

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ATC is one of the most aggressive human types of tumor. Lymph node or distant metastases are present in ~80% of patients at diagnosis (8-10) and the median survival rate is 6 months (11,12). Multimodal treatment, including debulking, hyperfractionated accelerated external beam radiotherapy and chemotherapy (doxorubicin, paclitaxel, docetaxel and cisplatin) is the most effective treatment strategy, and improves median survival rate to ~10 months (13,14).

Several genetic alterations have been identified in ATC molecular pathways, involving p53, BRAF, RAS, RET/PTC, VEGFR1, VEGFR2, EGFR, PDGFR α , PDGFR β , KIT, MET, PIK3Ca, PIK3Cb and PDK1, that lead to tumor aggressiveness and progression (14,15). New drugs targeting these molecular alterations have been recently evaluated in ATC (14).

Recent anecdotal evidence and a phase II clinical study have reported the antineoplastic activity of lenvatinib in ATC (16-20). In the present study, we aimed to evaluate the antineoplastic activity of lenvatinib in ATC continuous cell lines and in primary ATC cell cultures both *in vitro* and *in vivo*.

Materials and methods

Chemicals and supplements. Lenvatinib (1 and 100 nM; and 1, 10, 25 and 50 μ M) was evaluated in primary ATC cell cultures, in 8305C cells (DSMZ, Braunschweig, Germany) and AF cells, and in AF cells in CD nu/nu mice. Chemicals and supplements were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). RPMI-1640 medium was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). PCR reagents for quantitative PCR were purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.).

Thyroid tissues. Thyroid samples were surgically collected from 9 ATC patients and from 5 healthy subjects undergoing parathyroidectomy. The diagnosis was made on the basis of clinical and histological criteria by a recognized laboratory (21-23). By immunohistochemistry it was demonstrated that TSH receptor, sodium/iodide symporter (NIS), thyroperoxidase (TPO) and thyroglobulin (Tg) were not expressed in thyroid tissues.

DNA extraction and microdissection and detection of BRAF mutation were conducted through PCR single strand conformation polymorphism assays, using accepted protocols such as direct DNA sequencing (21-23). All patients agreed to take part in the study and provided written informed consent. The study was authorized by the local Ethics Committee of the University of Pisa.

Cell cultures

Human primary ATC cell cultures. ATC cell cultures were established as previously described (21-23). Tumor samples were divided into pieces of 1-3 mm with a lancet or clippers. The obtained fragments were washed 3-5 times in M-199 media containing penicillin (500,000 U/l), streptomycin (500,000 U/l) and nystatin (1,000,000 U/l). Then, neoplastic samples were suspended in Dulbecco's modified Eagle's medium (DMEM) with penicillin/streptomycin (50 mg/l), glutamine (1% w/v) and fetal calf serum (FCS) (20% v/v), at 37°C and 5% CO₂.

As primary cultures reached confluence, the cells were separated with a trypsin solution, then moved into flasks for the primary tissue cultures (Becton-Dickinson Labware, Bedford, MA, USA). After reaching the third passage, the cells were coated with methocel (24) for the evaluation of colony-forming efficiency. Subsequently, the biggest colonies were isolated and amplified in flasks for tissue cultures (21-23) and the required tests were performed at the fourth passage.

The absence of expression of the TSH receptor (25), Tg, NIS (26) and TPO (27) was investigated by immunocytochemistry, as was the presence of cytokeratin (26), which exhibited a partial and focal positivity. A pattern similar to that of the original neoplastic tissue was reported by DNA fingerprinting (21-23).

Thyroid follicular cell (TFC) culture. Primary TFC cultures were established as previously described (28). The specimens were minced and digested with collagenase (1 mg/ml; Roche Diagnostics, Almere, The Netherlands) in RPMI-1640 (Whittaker Bioproducts, Inc., Walkersville, MD, USA) for 1 h at 37°C. Semi-digested follicles were removed, sedimented for 2 min, washed and cultured in RPMI-1640 with 10% fetal bovine serum (FBS; Seromed, Biochrom, Germany), 2 mM glutamine and 50 mg/ml penicillin/streptomycin at 37°C and 5% CO₂ in plastic 75-cm² flasks (Sarstedt, Verona, Italy).

AF cell line. Nine primary ATC cell cultures were established and among them the AF cell line grew in nu/nu mice, after being subcutaneously inoculated.

8305C cell line. As the control, 8305C cells, an undifferentiated thyroid cancer continuous cell line (DSMZ), with a papillary component, were seeded in RPMI-1640 with 15% FBS and 2 mM L-glutamine.

Evaluation of cell viability and proliferation. In order to investigate cell proliferation, we conducted an MTT assay, using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (WST-1; Roche Diagnostics) (22,23,28). The 8305C, AF, ATC and TFC cell lines were plated (35,000 cells/ml) at 100 μ l/well and treated for 24 h with lenvatinib at different concentrations or the vehicle alone (four wells for each concentration). The IC₅₀ value was determined with linear interpolation. Triplicate experiments were conducted for each cell preparation (22,23,28). The absorbance at 450 nm was estimated at 1 and 2 h from the beginning of the tetrazolium reaction.

Furthermore, to assess the proliferation rate of the TFC, ATC, AF and 8305C cell lines, cell number counting was also performed (22,23,28).

Apoptosis: Hoechst uptake and Annexin V binding assay. The 8305C, AF and ATC cells were plated in wells (35,000 cells/ml, in 100 μ l/well) and treated for 48 h with lenvatinib in a humidified atmosphere (37°C, 5% CO₂). The cells were then dyed with Hoechst 33342 (28). Subsequently, the apoptosis index (apoptotic cells/total cells x 100) was determined. The apoptosis evaluation was conducted using the Annexin V binding method. The cells were plated in the Lab-Tek II Chamber Slide system (Nalge Nunc International, Penfield, NY, USA)

and treated with lenvatinib for 48 h. The apoptosis index was calculated as previously reported (28).

Migration and invasion tests. Migration and invasion assays were performed using Transwell permeable supports (Corning Life Sciences, Corning, NY, USA) (29,30). Cell cultures were starved for 5 h in serum-free medium at 37°C, 5% CO₂, then collected with a solution of PBS and 5 mM EDTA. Total cell number was calculated. After centrifugation, the cells were seeded at a concentration of 0.5x10⁵ cells/well in serum-free medium.

To produce a gradient, 10% v/v FCS (or serum-free medium as negative control) was added to receiver wells with increasing concentrations of lenvatinib and then, the medium was removed from the lower compartments, and calcein AM (2 µg/ml; Sigma-Aldrich) was added for 1 h. An ELISA reader, with filters set to 485 nm for excitation and 520 nm for emission, was used to assess the intracellular fluorescence.

For the migration assay, cells were incubated for 12 h and for the invasion assay, cells were incubated for 24 h. A basement membrane extract (Trevigen, Gaithersburg, MD, USA) was used overnight (37°C, 5% CO₂) for invasion. To obtain the number of migrated or invasive cells with respect to the fluorescence values, a standard curve with various cell concentrations was generated.

ELISA tests in ATC cells

Phospho-EGFR inhibition cell-based assay. ATC cells were plated (5x10⁴ cells/well) in 1% FBS medium and treated for 72 h (after 24 h of incubation) with lenvatinib at a concentration close to the experimental IC₅₀ of the cell proliferation test (25 µM for ATC), or with a higher (50 µM), or lower (1 µM) concentration or with vehicle. Cell lysates were then harvested (31) and evaluated using PathScan phospho-EGFR (Tyr1173) and total EGFR ELISA kits (Cell Signaling Technology, Inc., Danvers, MA, USA). Optical density (OD) was assessed at 450 nm.

ERK1/2 (pTpY^{185/187}) and Akt (pThr³⁰⁸) ELISA. ATC cells were plated (5x10⁴ cells/well) and treated with lenvatinib for 72 h (31). Then, cell lysates were evaluated for human ERK1/2 and Akt phosphorylation using PhosphoDetect ERK1/2 (pThr¹⁸⁵/pTyr¹⁸⁷) and the PhosphoDetect Akt (pThr³⁰⁸) ELISA kits (Calbiochem; EMD Millipore, Billerica, MA, USA). To normalize the obtained data, total protein ERK1/2 and Akt concentrations were determined with ERK1/2 and Akt ELISA kits, respectively. OD was estimated at 450 nm.

Cyclin D1 protein expression is quantified in lenvatinib-treated ATC cells. To evaluate the effect of lenvatinib on protein cyclin D1 modulation, ATC cells were treated with lenvatinib for 72 h (at the previously indicated concentrations) or with vehicle alone (31). The amount of cyclin D1 was quantified in cell lysates, obtained using lysis buffer (ice-cold 1X; 0.5 ml), with sonication on ice for 10 sec. After microcentrifugation for 10 min at 4°C, supernatants were collected and assessed using a human cyclin D1 ELISA kit (USCN Life Science and Technology Co., Wuhan, China). OD was assessed at 450 nm and the obtained data were reported as cyclin D1 ng/mg of total protein.

In vivo studies

Animals and treatment. Six-week-old CD nu/nu male mice, provided by Envigo (Milan, Italy), were housed in microisolator cages on vented racks and manipulated using aseptic techniques. Housing and procedures involving animals were conducted according to the protocol approved by the Academic Organization Responsible for Animal Welfare [Organismo Preposto per il Benessere Animale (OPBA)] at the University of Pisa, according to the Italian law D.lgs. 26/2014, and with the approval of the Italian ministry of Health (authorization no. 613/2015-PR).

Each experiment employed the minimum number of mice needed to obtain statistically meaningful results. On day 0, 4x10⁶±5% viable AF cells/mouse were subcutaneously inoculated. Animal weights were monitored and tumor volume (mm³) was defined as: [(w₁ x w₁ x w₂) x (π/6)], where w₁ and w₂ were the smallest and the largest tumor diameter (mm), respectively. Treatment (n=6 mice/group) was initiated 20 days after cell inoculation, when the mean volume was ~100 mm³. All mice were randomized shortly before the initiation of treatment. Control mice received vehicle alone. Lenvatinib was administered at 25 mg/kg by gavage daily without interruption for 16 days. Mice were sacrificed using an anesthetic overdose, after which tumors were excised and measured.

Tumor tissue: Immunohistochemistry and microvessel density determination. Neoplastic samples from the two treatment groups were weighed, then fixed in formalin and subsequently embedded in paraffin. Sections of 5-µm thickness were stained by hematoxylin and eosin (H&E), as previously described (29).

VEGF expression was evaluated with an anti-VEGF rabbit polyclonal antibody (cat. no. sc-152; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:50 dilution. Expression was presented as a percentage of positive cells out of at least 1,000 tumor cells. Microvascular count (MVC) was evaluated using anti-FVIII polyclonal antibody (cat. no. 760-2642; Ventana Medical Systems) as previously reported (29).

Statistical analysis. Data are presented as the mean (± SD) for normally distributed variables, or as the median and interquartile range. Experiments were conducted in triplicate from each subject and the mean of the samples was reported for TFC and ATC cells. One-way ANOVA, Mann-Whitney U or Kruskal-Wallis test were used to compare mean group values for normally distributed variables. The χ² test was used to compare group proportions. Post hoc comparisons on normally distributed variables were performed using the Bonferroni-Dunn test. Analysis of apoptosis results was performed using one-way ANOVA with the Newman-Keuls multiple comparison test.

Results

In vitro studies in ATC cells

Evaluation of cell proliferation. Data obtained from the WST-1 test in ATC cells demonstrated a significant reduction in cell proliferation rate vs. the control group with lenvatinib at 1 h from the beginning of the tetrazolium reaction. Cell counting confirmed these results. After 1 h, the cell number was 11,850±620/100 µl/well in the ATC control group;

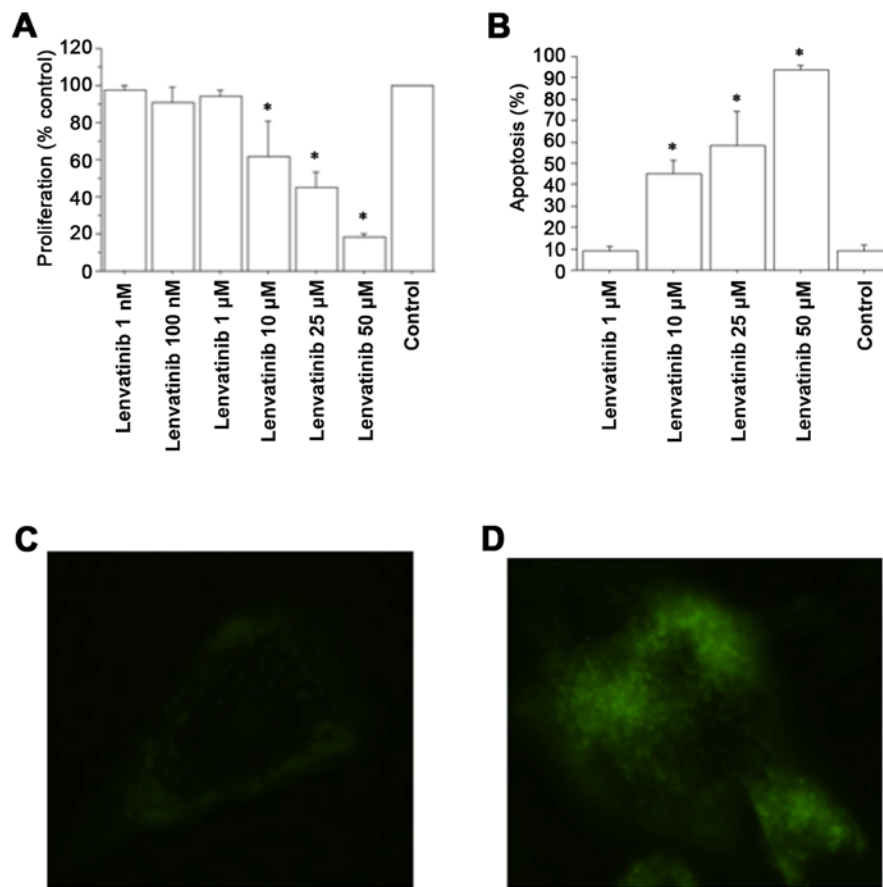


Figure 1. Proliferation (WST-1) and apoptosis assays in primary human ATC. (A) WST-1 assay in primary ATC cell cultures treated with lenvatinib for 24 h. Lenvatinib significantly reduced the proliferation of ATC cells vs. the control. Bars represent the mean (\pm SD). * $P < 0.05$ vs. control with Bonferroni-Dunn test. (B) ATC cell apoptosis after treatment with lenvatinib for 48 h. Apoptosis was strongly and dose-dependently increased by lenvatinib. Apoptosis was evaluated by Hoechst staining. Data are expressed as the mean (\pm SD) ($n=9$), and analyzed by one-way ANOVA with Newman-Keuls multiple comparison test, and with a linear trend test ($P < 0.001$ vs. control). (C and D) Representative example of the Annexin V binding assay in control ATC and treated cells (with lenvatinib 50 μ M), respectively. ATC, anaplastic thyroid cancer.

11,613 \pm 680 (98%) with lenvatinib 1 nM; 11,496 \pm 890 (97%) with lenvatinib 100 nM; 11,376 \pm 790 (96%) with lenvatinib 1 μ M; 9,480 \pm 600 (80%) with lenvatinib 10 μ M; 7,229 \pm 460 (61%) with lenvatinib 25 μ M; and 4,503 \pm 450 (38%) with lenvatinib 50 μ M ($P < 0.01$, ANOVA). The WST-1 assay in ATC cells also demonstrated a significant reduction in the proliferation rate vs. the control group with lenvatinib at 2 h from the beginning of the tetrazolium reaction ($P < 0.01$, for both, ANOVA) (Fig. 1A). Cell counting confirmed these results; after 2 h, the cell number was 18,720 \pm 820/100 μ l/well in the ATC control group; 18,532 \pm 780 (99%) with lenvatinib 1 nM; 17,784 \pm 990 (95%) with lenvatinib 100 nM; 18,158 \pm 810 (97%) with lenvatinib 1 μ M; 11,232 \pm 1,100 (60%) with lenvatinib 10 μ M; 8,425 \pm 960 (45%) with lenvatinib 25 μ M; and 2,995 \pm 750 (16%) with lenvatinib 50 μ M; ($P < 0.01$, ANOVA). The IC_{50} value for lenvatinib, obtained by linear interpolation, was 19 \pm 2.5 μ M.

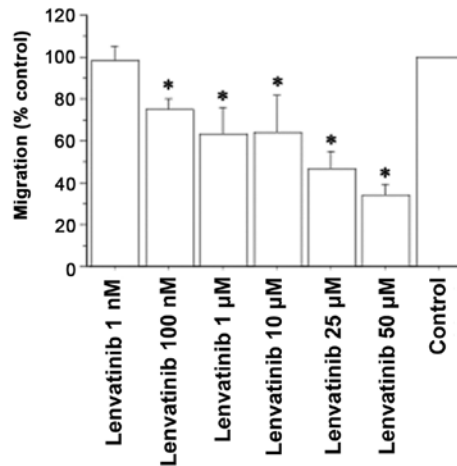
Data obtained from the WST-1 assay in TFC cells following lenvatinib treatment demonstrated a slight but significant reduction in the proliferation rate vs. the control group both at 1 h ($P < 0.01$, ANOVA) with lenvatinib 10 μ M (96% vs. control), 25 μ M (90% vs. control) and 50 μ M (85% vs. control) and at 2 h ($P < 0.01$, ANOVA) with lenvatinib 10 μ M (90% vs. control), 25 μ M (85% vs. control) and 50 μ M (81% vs. control). Cell counting confirmed these results: after 1 h, the

cell number was 10,150 \pm 620/100 μ l/well in the TFC control; 9,642 \pm 1,100 (95%) with lenvatinib 10 μ M; 9,238 \pm 960 (91%) with lenvatinib 25 μ M; and 8,625 \pm 950 (85%) with lenvatinib 50 μ M; ($P < 0.01$, ANOVA); after 2 h, the cell number was 17,500 \pm 820/100 μ l/well; 15,925 \pm 1,120 (91%) with lenvatinib 10 μ M; 14,874 \pm 1,060 (85%) with lenvatinib 25 μ M; and 14,350 \pm 980 (82%) with lenvatinib 50 μ M ($P < 0.01$, ANOVA).

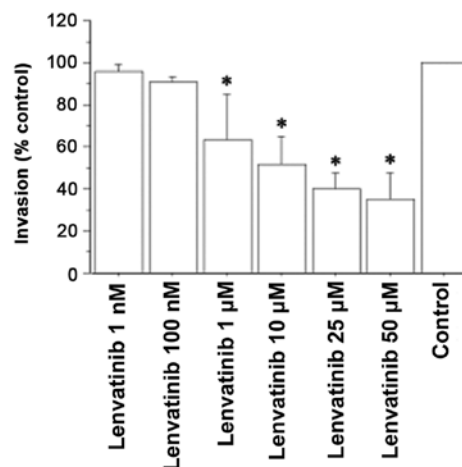
Proliferation and BRAF. The $V600E$ BRAF mutation was detected in three ATC samples. *RET/PTC1* and *RET/PTC3*, *N-RAS* or *H-RAS* mutations evaluated by quantitative PCR were not detected in primary ATC cell cultures. Proliferation was inhibited in a similar manner in ATC from tumors in the presence/absence of $V600E$ BRAF mutation (data not shown).

Apoptosis evaluation. Lenvatinib dose-dependently increased apoptotic ATC cells ($P < 0.001$, ANOVA; Fig. 1B). The Annexin V assay corroborated these results (Fig. 1C and D).

Migration and invasion tests. After reaching subconfluence, primary ATC cell cultures were treated with increasing concentrations of lenvatinib. Lenvatinib inhibited migration (Fig. 2A) and invasion (Fig. 2B), as evaluated by the Transwell chamber (Corning Life Sciences).

A

	Control	Lenvatinib 10 μM	Lenvatinib 25 μM	Lenvatinib 50 μM
Proliferation at 12 h [% control (mean)]	100%	75%	56%	28%
Migration at 12 h [% control (mean)]	100%	64%	48%	35%

B

	Control	Lenvatinib 10 μM	Lenvatinib 25 μM	Lenvatinib 50 μM
Proliferation at 24 h [% control (mean)]	100%	60%	45%	16%
Invasion at 24 h [% control (mean)]	100%	51%	40%	34%

Figure 2. Migration and invasion assays in ATC cells. ATC cells were incubated with lenvatinib for (A) 12 h for the migration assay and for (B) 24 h for the invasion assay. For comparison, the inhibition of proliferation (at 12 h) (% with respect to control) and the inhibition of migration are reported in the table below Fig. 2A, as well as the inhibition of proliferation (at 24 h) (% with respect to control) and the inhibition of invasion in the table below Fig. 2B. Bars represent the mean (\pm SD); * P <0.05 vs. control (control, medium + FCS 10%) by Newman-Keuls test. ATC, anaplastic thyroid cancer.

Inhibition of EGFR. Lenvatinib significantly and dose-dependently decreased the phosphorylated form of EGFR in ATC cell lysates (Fig. 3A).

Inhibition of Akt or ERK1/2 phosphorylation. Phosphorylated/non-phosphorylated Akt or ERK1/2 proteins (evaluated by

ELISA) in lenvatinib-treated samples were significantly reduced in ATC cell cultures (Fig. 3B and C).

Lenvatinib reduces cyclin D1 protein levels. Lenvatinib dose-dependently inhibited cyclin D1 gene expression in ATC cell cultures (Fig. 3D; P <0.05). The intracellular cyclin D1 protein

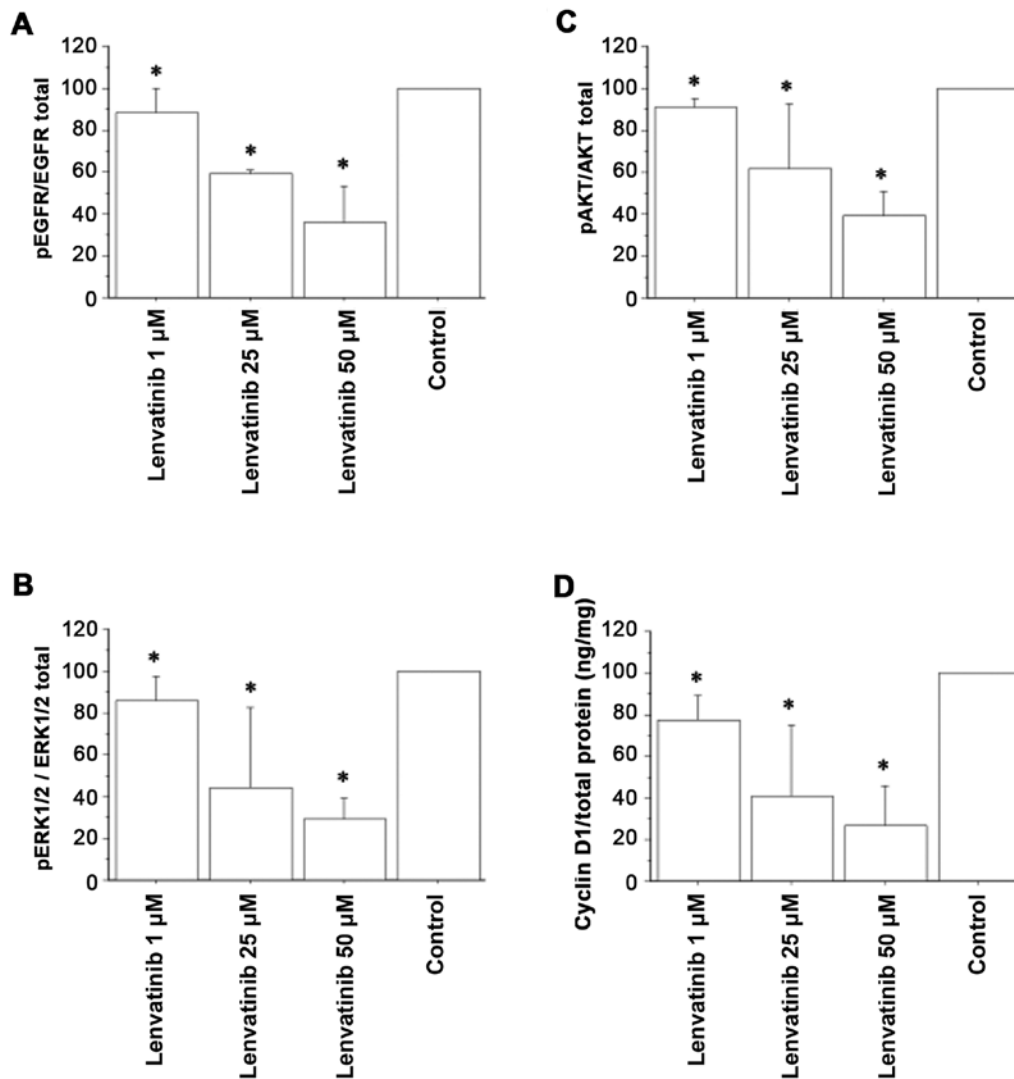


Figure 3. Inhibition of EGFR, Akt and ERK1/2 phosphorylation and cyclin D1 protein expression in ATC cells. (A) Inhibition of EGFR phosphorylation by lenvatinib in ATC cells after 72 h of treatment. Experiments were performed in triplicate, independently, with at least 9 samples for each dose. Data are presented as the mean values (\pm SE); * P <0.05 vs. control (treated with vehicle). (B and C) Inhibition of ERK1/2 (pThr¹⁸⁵/pTyr¹⁸⁷) and Akt (pThr³⁰⁸) phosphorylation by lenvatinib in ATC cells after 72 h of treatment. Experiments were performed in triplicate, independently, with at least 9 samples for each dose. Data are presented as the mean values (\pm SE); * P <0.05 vs. control (treated with vehicle). (D) Cyclin D1 protein concentrations in ATC cells treated with lenvatinib or with vehicle alone for 72 h, quantified by ELISA. Cyclin D1 values were reported as ng/mg of total protein. Experiments were performed, independently, 6 times with at least 9 samples for each dose. Data are presented as the mean values (\pm SE); * P <0.05 vs. control (treated with vehicle).

was evaluated in cells exposed to lenvatinib or to vehicle. Lenvatinib reduced cyclin D1 concentrations compared with vehicle-treated cells.

In vitro studies in 8305C and AF cells. Lenvatinib had a dose-dependent antiproliferative activity in 8305C cells (IC_{50} of $6.3 \pm 2.2 \mu\text{M}$) (Fig. 4A) and in AF cells (IC_{50} of $8.2 \pm 3.1 \mu\text{M}$) (Fig. 4B). Furthermore, lenvatinib increased apoptotic 8305C cells in a dose-dependent manner. Following exposure to lenvatinib $10 \mu\text{M}$, 15% of cells were apoptotic and with lenvatinib 25 or $50 \mu\text{M}$, 23.3 and 29.8% of cells were apoptotic, respectively (Fig. 4C; P <0.001, by ANOVA). Apoptotic AF cells also increased in a dose-dependent manner. Following exposure to lenvatinib $10 \mu\text{M}$, 19.8% of cells were apoptotic and with lenvatinib 25 or $50 \mu\text{M}$, 25 and 30.8% of cells were apoptotic, respectively (Fig. 4D; P <0.001, by ANOVA).

In vivo studies

Lenvatinib reduces AF tumor growth with no weight loss. Twenty days after the subcutaneous xenotransplantation of AF cells in CD nu/nu mice, tumor masses reached an average volume of 100 mm^3 and the treatment started. Lenvatinib (25 mg/kg/day i.p.) significantly reduced tumor growth, from day 7 after treatment started, compared with the controls (Fig. 5A; e.g., at day 7, 107.3 mm^3 vs. 408.1 mm^3 in controls and at day 16, 119.3 mm^3 vs. 1016.1 mm^3 in controls; P <0.05). Notably, no loss of weight was observed throughout the course of the experiment indicating that lenvatinib treatment was well tolerated (Fig. 5B).

Lenvatinib reduces VEGF-A expression and microvessel density in AF tumor tissues. Inoculation of AF cells led to the formation of a tumor that was histologically consistent with ATC. Lenvatinib significantly reduced VEGF-A and FVIII immunostaining. A localized immunoreactivity for VEGF-A

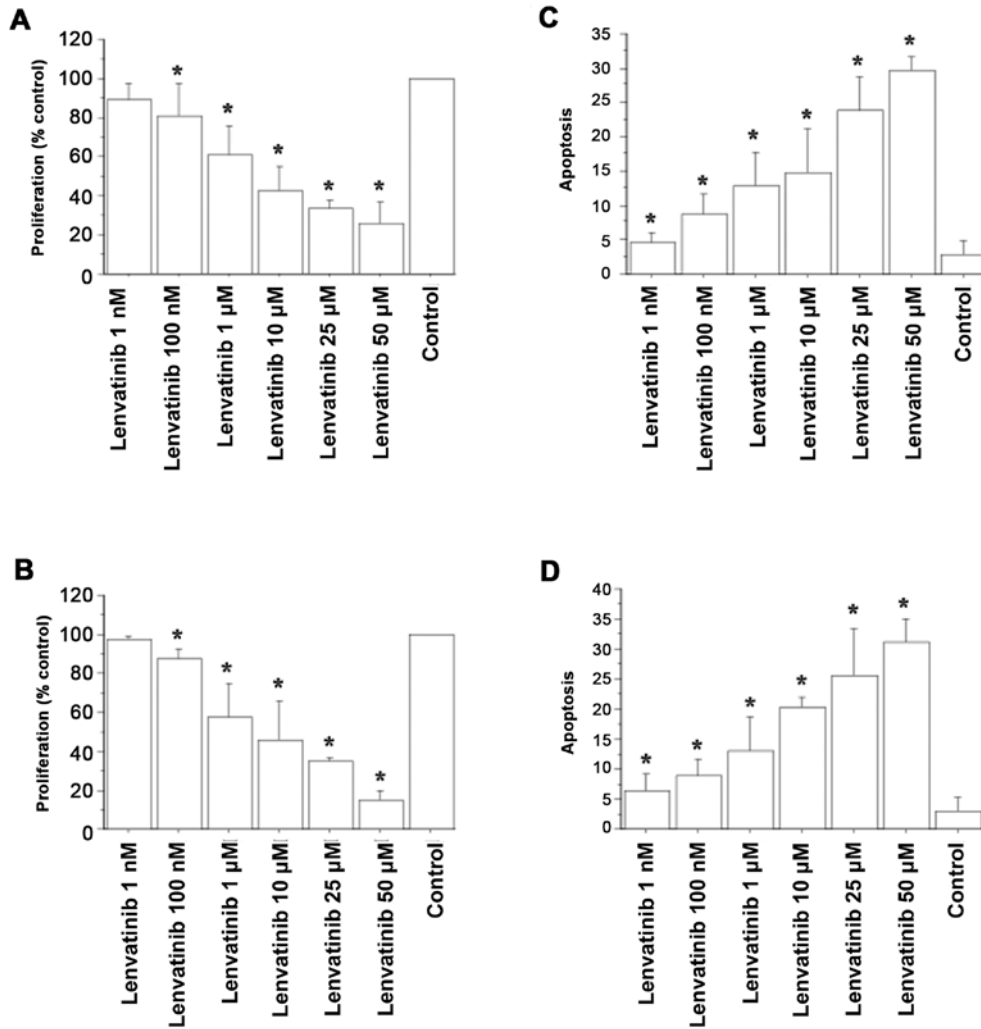


Figure 4. WST-1 and apoptosis assays in 8305C and AF cells. (A) WST-1 in 8305C or (B) AF cells treated with lenvatinib for 24 h. Lenvatinib significantly reduced the proliferation of ATC cells vs. the control. Bars represent the mean (\pm SD); * $P < 0.05$ vs. control by Bonferroni-Dunn test. Apoptosis in (C) 8305C or (D) AF cells treated with lenvatinib for 48 h. Lenvatinib strongly and dose-dependently increased apoptosis (apoptosis was evaluated by Hoechst staining). One-way ANOVA was used to analyze the data (with Newman-Keuls multiple comparison test and with a test for linear trend). Data are presented as the mean values (\pm SE) $P < 0.001$ vs. control. IC_{50} vs. control was evaluated by nonlinear regression (see Results); IC_{50} was $6.3 \pm 2.2 \mu M$ for 8305C cells and $8.2 \pm 3.1 \mu M$ for AF cells. ATC, anaplastic thyroid cancer.

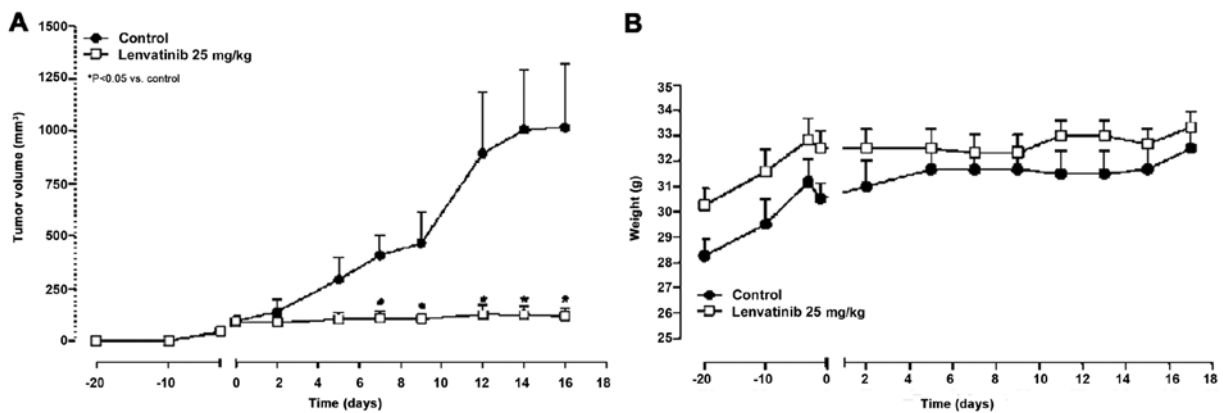


Figure 5. *In vivo* experiments. (A) Antitumor *in vivo* effect of lenvatinib at the dose of 25 mg/kg/day by gavage, on AF tumors xenotransplanted in CD nu/nu mice. (B) Weights of mice monitored during the treatment with lenvatinib or vehicle alone. Symbols and bars, mean (\pm SE); * $P < 0.05$ vs. vehicle-treated controls.

was identified in cells of the control cancer mass which was reduced by lenvatinib (48 ± 8 vs. 35 ± 6 ; $P < 0.05$), with a simul-

aneous reduction of microvessel density (14 ± 5 vs. controls 23 ± 6 ; $P < 0.05$).

Discussion

Research on the effects of TKIs for the treatment of ATC is ongoing (32). In the present study, we demonstrated that lenvatinib inhibited primary ATC cell cultures proliferation *in vitro*, while also increasing apoptosis and inhibiting migration and invasion. In addition, lenvatinib inhibited the proliferation of 8305C and AF cells *in vitro*, while also increasing apoptosis and reduced AF cell tumor growth in CD nu/nu mice with no toxicity. These results were consistent with previous studies that identified an ability of lenvatinib to inhibit tumor growth of ATC cell lines *in vivo* and to disrupt angiogenesis by decreasing vascular permeability. An important antiangiogenic activity of lenvatinib in 8305C xenotransplants has also been reported (2,3). In the present study, the antiproliferative effect of lenvatinib in primary ATC cells was observed in all the samples, independently from the absence or presence of ^{V600E}BRAF mutation. This is probably due to lenvatinib being a multiple signal transduction inhibitor with antiangiogenic effect.

The pharmacological and molecular inhibition of PI3K or AKT isoforms can reduce *in vitro* growth and motility in human TC cell lines (33,34). RAS-RAF-MAPK, ERK and PI3K pathways are implicated in the carcinogenesis of TCs and mutations in these genes are present in ATC (35). In ATC, ERK and AKT proteins were phosphorylated and activated and were thus considered as possible therapeutic targets. In the present study, we demonstrated that lenvatinib inhibited ERK1/2 and AKT phosphorylation in ATC cells. In addition, lenvatinib was demonstrated to reduce EGFR phosphorylation, which is consistent with the data reported by Di Desidero *et al* (36) and with our previous results on EGFR phosphorylation inhibition by CLM3 in ATC cells (37).

A previous study has indicated the important role of cyclin D1 in the regulation of cell cycle progression (38). Cyclin D1 expression was identified by Lee *et al* (39) in 67% of ATCs and by Wiseman *et al* (40) in 77% of ATCs. Lenvatinib is a dual TKI, acting on EGFR and VEGFR-2, and is able to inhibit cell growth by downregulating the expression of cyclin D1 and E (41). In the present study we demonstrated that lenvatinib potentially downregulates cyclin D1 protein in the ATC cells.

Lenvatinib exhibited a low-toxicity profile, since it significantly inhibited AF cell growth in CD nu/nu mice with no weight loss, unlike other compounds that cause various side-effects in humans and animals (42). However, further studies are required in order to elucidate potential side-effects on the function of the kidney, liver and other systems. Nevertheless, it may be hypothesized that the antineoplastic activity of lenvatinib in ATC is the result of multiple effects on tumor cells, namely: i) an antiproliferative activity; ii) increased apoptosis; iii) inhibition of migration and invasion; and iv) inhibition of cancer neovascularization.

Currently, novel therapeutic options for ATC are being developed, although some limitations still exist in the selective use of new molecules. For example, even if there are potential targets in the tumor tissue, such as BRAF, tumor response may only occur in a fraction of patients, and this could be due to the activation of compensatory signal pathways, allowing cancer cell proliferation. The effectiveness

of the treatments could be increased by testing the sensitivity of primary ATC cells from each subject to different TKIs, as *in vitro* chemosensitivity tests can predict *in vivo* effectiveness in 60% of cases (43). In addition, a negative chemosensitivity test *in vitro* is associated with a 90% chance of ineffectiveness *in vivo* (43,44). This is important in order to avoid the administration of inactive chemotherapeutics to patients (21,22,30,32).

In the present study, we revealed for the first time the antitumoral effect of lenvatinib, a multi-targeted kinase inhibitor, in primary human ATC cell cultures obtained from patients. These findings could open the way to the clinical use of lenvatinib in the treatment of patients with ATC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SMF, GB, PM, AA and PF made substantial contributions to conception and design, and to acquisition of data; TDD, GE, IR, FR, PO, SRP, AP, SP, CLM, SU, EB and GM analyzed the data; SMF, GB, TDD, AA and PF have been involved in drafting the manuscript; AA revised it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients and controls agreed to enter the study, which was approved by the local Ethics Committee of the University of Pisa (see Materials and methods).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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