Vandetanib has antineoplastic activity in anaplastic thyroid cancer, *in vitro* and *in vivo*

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Abstract. The antitumor activity of vandetanib [a multiple signal transduction inhibitor including the RET tyrosine kinase, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) receptor (VEGFR), ERK and with antiangiogenic activity], in primary anaplastic thyroid cancer (ATC) cells, in the human cell line 8305C [undifferentiated thyroid cancer (TC)] and in an ATC-cell line (AF), was investigated in the present study. Vandetanib (1 and 100 nM; 1, 10, 25 and 50 μM) was tested by WST-1, apoptosis, migration and invasion assays: in primary ATC cells, in the 8305C continuous cell line, and in AF cells; and in 8305C cells in CD *nu/nu* mice. Vandetanib significantly reduced ATC cell proliferation (P<0.01, ANOVA), induced apoptosis dose-dependently (P<0.001, ANOVA), and inhibited migration (P<0.01) and invasion (P<0.001). Furthermore, vandetanib inhibited EGFR, AKT and ERK1/2 phosphorylation and downregulated cyclin D1 in ATC cells. In 8305C and AF cells, vandetanib significantly inhibited the proliferation, inducing also apoptosis. 8305C cells were injected subcutaneously in CD *nu/nu* mice and tumor masses became detectable after 30 days. Vandetanib (25 mg/kg/day) significantly inhibited tumor growth and VEGF-A expression and microvessel density in 8305C tumor tissues. In conclusion, the antitumor

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and antiangiogenic activity of vandetanib is very auspicious in ATC, opening the way to a future clinical evaluation.

Introduction

Anaplastic thyroid cancer (ATC) represents ~1% of all thyroid cancer (TC) cases, and is one of the most aggressive human tumors, accounting for 15-40% of TC-related deaths (1,2). ATC is classified as stage IV (American Joint Committee on Cancer), regardless of tumor size, or presence of lymph node or distant metastases (present in ~80% of patients at diagnosis) (3-5); median survival is 6 months. The multimodal treatment [including debulking, chemotherapy (doxorubicin, cisplatin, paclitaxel or docetaxel), and hyperfractionated accelerated external beam radiotherapy] is the most effective treatment, with a median survival of 10 months (6,7). Several genetic alterations have been shown in ATC molecular pathways, leading to tumor aggressiveness and progression [p53, BRAF, RAS, RET/PTC, vascular endothelial growth factor (VEGF) receptor (VEGFR)-1, VEGFR-2, epidermal growth factor receptor (EGFR), PDGFRα, PDGFRβ, KIT, MET, PIK3Ca, PIK3Cb and PDK1] (7,8). New drugs targeting these molecular alterations have been recently evaluated in ATC (7), but to date no significant improvement in patient survival has been observed.

Vandetanib (ZD6474, Caprelsa®) is an oral once-daily multi-tyrosine kinase inhibitor (TKI), that inhibits the activation of RET, EGFR, VEGFR-2, VEGFR-3, and slightly VEGFR-1, and has potent antiangiogenic activity (9). Potent antineoplastic action of vandetanib was shown against transplantable medullary thyroid carcinoma (MTC) in nude mice (10). In patients with aggressive MTC, a phase III clinical study showed that vandetanib improved progression-free survival (30.5 vs. 19.3 months in the control group) (11). It was approved by the Food and Drug Administration, and the European Medicines Agency, in 2011, to treat locally advanced or metastatic MTC (12). Vandetanib has also shown promising

results in aggressive differentiated TC patients not responsive to the usual therapies (13,14). In the present study, we aimed to evaluate the antineoplastic activity of vandetanib in ATC continuous cell lines, and in primary ATC cells, *in vitro* and *in vivo*.

Materials and methods

Drug. The effect of vandetanib (ZD6474, Caprelsa®; Aurogene Srl, Rome, Italy; 1 and 100 nM; 1, 10, 25 and 50 μM) was investigated *in vitro* in primary ATC cell cultures, in the 8305C continuous cell line, and AF cells; and *in vivo* in 8305C cells in CD *nu/nu* mice.

Reagents. RPMI-1640 medium was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA); PCR reagents for quantitative real-time were obtained from Applied Biosystems (Thermo Fisher Scientific, Inc.). The other chemicals and supplements not reported in this section were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Patient source for thyroid tissue. We obtained surgical thyroid tissue samples from: i) eight ATC patients at surgery; and ii) six healthy subjects who underwent parathyroidectomy. Recognized clinical, laboratory and histological criteria were used to establish the diagnosis (15-17). The absence of thyroglobulin (Tg), thyroperoxidase (TPO), thyroid-stimulating hormone (TSH) receptor, and sodium/iodide symporter (NIS) expression was shown by immunohistochemistry, that was positive for cytokeratin (Fig. 1). DNA extraction and microdissection, and the detection of *BRAF* mutation were conducted by PCR Single-strand Conformation Polymorphism (by accepted protocols); such as direct DNA sequencing (15-17). All patients and controls agreed to enter the study, which was approved by the local Ethics Committee of the University of Pisa.

Primary ATC cell culture. We prepared ATC cells using protocols published previously (15-17). Cancer tissues were divided in fragments of 1-3 mm, and washed 3-5 times in M-199 media together with streptomycin (500,000 U/l), penicillin (500,000 U/l) and nystatin (1,000,000 U/l). Fragments 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) and then incubated at 37˚C in 5% CO_2 (all were from Sigma-Aldrich; Merck KGaA).

As soon as the primary culture reached confluence, the cells were transferred into primary tissue culture flasks (Becton-Dickinson Labware, Bedford, MA, USA). To evaluate colony-forming efficacy, cells on their 3rd passage were coated in Methocel™ (Dow Chemical Co., Milan, Italy) (18). The biggest colonies were spread in tissue-culture flasks (15-17). At the 4th passage, the cells were tested. The absence of Tg, TSH receptor (19), NIS (20), and TPO (21) expression was shown by immunocytochemistry. Focal positivity for cytokeratin was observed by immunocytochemistry (20). DNA fingerprinting demonstrated a pattern similar to the original cancer tissue (15-17).
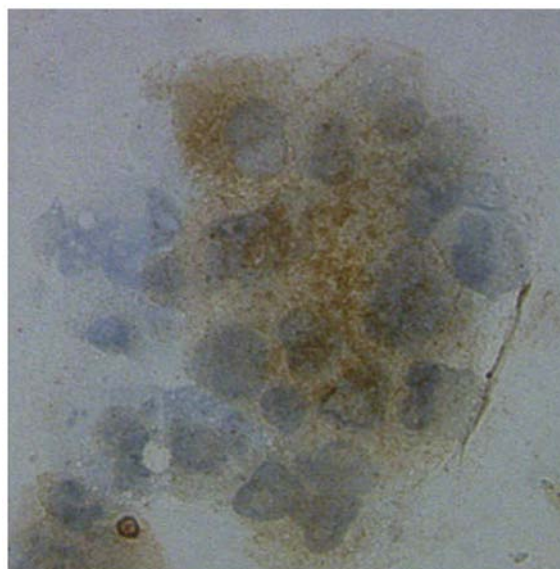

Figure 1. Immunohistochemistry for cytokeratin of anaplastic thyroid cancer (ATC) tissue. The presence of cytokeratin expression is shown in ATC cells (magnification, x400).

Thyroid follicular cell (TFC) culture. TFCs were established as previously reported (22).

AF cells. Among the eight primary ATC cell cultures, one (the AF cell line) grew >50 passages, and was also able to grow in *nu/nu* mice when subcutaneously inoculated.

8305C continuous cell line. 8305C cells (undifferentiated TC cell line, with papillary component; DSMZ, Braunschweig, Germany) were maintained in RPMI-1640 with 15% fetal bovine serum (FBS) with the addition of 2 mM L-glutamine.

Viability and proliferation assay. In order to investigate cell proliferation, we conducted a WST-1 (Roche Diagnostics, Almere, The Netherlands) (16,17,22). We plated TFC, ATC, AF and 8305C cells (at 35,000 cells/ml in 100 μl/well, of 96-well plates), and treated them with vandetanib or with vehicle alone for 24 h. To achieve IC_{50}, the cells were treated with a concentration range of vandetanib (in quadruplicate), and IC_{50} was estimated by linear interpolation. Triplicate experiments were conducted for each cell preparation (16,17,22). The absorbance was estimated at 450 nm after 1 and 2 h from the beginning of tetrazolium reaction.

Cell number counting was used, as well, to estimate the proliferation in all the considered cells, as previously reported (16,17,22).

Apoptosis-Hoechst uptake. Firstly, we plated ATC, 8305C and AF cells (35,000 cells/ml in 100 μl/well; 96-well plates). *Thereafter*, the cells were treated for 48 h with vandetanib (37˚C, 5% CO_2), and dyed with Hoechst 33342 as previously described (22). The apoptotic cells/total cells x100 ratio (apoptosis index) was evaluated.

Annexin V binding assay for apoptosis. The assay was carried out on cells seeded in Lab-Tek II Chamber Slide System (Nalge

Nunc International, Penfield, NY, USA), treated with vandetanib for 48 h, as previously reported (22).

Migration and invasion assays. A 96-well Transwell Permeable Support (Corning Life Sciences, Corning, NY, USA) was used to achieve cell migration and invasion, in agreement with the manufacturer's instructions, applying minor modifications (23,24).

To assess intracellular fluorescence, we used a 96-well plate ELISA reader (excitation at 485 nm and emission at 520 nm). For the migration assay cells were incubated for 12 h; for the invasion assay, 24 h. For the invasion experiments, the inserts were coated with a basement membrane extract solution (Trevigen, Gaithersburg, MD, USA) overnight (37°C, 5% CO_2), before plating cells. For each assay we constructed a standard curve to transform the fluorescence data obtained to the number of invasive or migrated cells. All the data were analyzed by StatView version 5.0 (SAS Institute, Inc., Cary, NC, USA).

ELISA tests in ATC cells
Phospho-EGFR inhibition cell-based assay. ATC cells ($5x10^4$ cells/well) were plated in 1% FBS medium for 24 h, and then treated for 72 h with vandetanib at a concentration similar to the experimental IC_{50} of cell proliferation (25 μM for ATC), and with a higher (50 μM), or with a lower (1 μM) concentration of vandetanib, or with vehicle. We collected cell lysates as previously reported (25) and we assayed them with PathScan phospo-EGFR (Tyr1173) and total EGFR sandwich ELISA kits (Cell Signaling Technology, Inc., Danvers, MA, USA). Optical density (OD) was estimated at 450 nm.

AKT (pThr308), or ERK1/2 (pTpY185/187). ATC cells ($5x10^4$ cells/well) were exposed for 72 h to vandetanib and subsequently lysed (25), and tested for human AKT or ERK1/2 phosphorylation by the PhosphoDetect AKT (pThr308) or by PhosphoDetect ERK1/2 (pThr185/pTyr187) ELISA kits (Calbiochem; EMD Millipore, Billerica, MA, USA). Data were normalized by total protein AKT, or ERK1/2 concentrations evaluated by AKT, or ERK1/2 ELISA kits, respectively. OD was estimated at 450 nm.

Cyclin D1 protein expression. The ATC cells were exposed for 72 h to vandetanib at the above considered concentrations or to vehicle, in order to evaluate the vandetanib-modulated expression of the protein cyclin D1. Cyclin D1 protein amount was measured by lysing cells with (ice-cold 1X) lysis buffer (0.5 ml), as previously reported (25). Lysates were gathered and then sonicated on ice (for 10 sec). Subsequently, the samples were microcentrifuged at 4°C for 10 min and the supernatant was gathered. Cyclin D1 was measured in cancer cell lysates by the human ELISA kit (Uscn Life Sciences, Inc., Wuhan, China). OD was measured 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 weighing 20-25 g, supplied by Envigo (Milan, Italy), were housed in microisolator cages on vented racks and manipulated using aseptic techniques and were allowed unrestricted

access to sterile food and water. We proceeded 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, in agreement with the Italian law D.lgs. 26/2014, and by the Italian Ministry of Health (authorization no. 613/2015-PR). In order to obtain statistically meaningful results, each experiment was conducted with the minimum necessary number of mice. In each mouse we inoculated, subcutaneously, $2x10^6\pm5\%$ viable 8305C cells, on day 0. Animal weights were monitored, and tumor volume (mm^3) was defined as: [(w1 x w1 x w2) x (π/6)], where w1 refers to the smallest tumor diameter (mm), whereas w2 to the largest one. We started the treatment (n=5 mice/group) after 30 days from cell inoculation, when the mean volume was ~100 mm^3. All mice were randomized just before initiation of treatment. Control mice received vehicle alone, or 25 or 12.5 mg/kg/day of vandetanib, injected intraperitoneally (i.p.) for 29 days. An anesthetic overdose of urethan was used to sacrifice mice, and the tumors were then excised and measured.

Microvessel density in the cancer tissue, and immunohistochemistry. Cancer tissues from the three treatment groups were weighed, and then fixed in formalin and subsequently embedded in paraffin. The sections (5 μm) were stained with hematoxylin and eosin, and immunostaining was conducted as previously described (23). An anti-VEGF rabbit polyclonal antibody (diluted at 1:50; cat. no. sc-152; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used to estimate VEGF expression, as the percentage of positive cells in ~1,000 tumor cells. Anti-FVIII polyclonal antibody (cat. no. 760-2642; Ventana Medical Systems, Inc.:Roche Group, Tucson, AZ, USA) was used to determine microvascular count (MVC), as previously described (23).

Statistical analysis. For normally distributed variables, the values are expressed as mean ($\pm$SD), or as median (and interquartile range). Experiments were conducted thrice with ATC from each subject, and here we report the mean in the eight samples obtained by different donors (for TFC and ATC). One-way ANOVA, or Mann-Whitney U or Kruskal-Wallis test, were used to compare mean group values for normally distributed variables. χ^2 test was applied to compare proportions. Post hoc comparisons on normally distributed variables were conducted by Bonferroni-Dunn test. Apoptosis results were analyzed by one-way ANOVA with Newman-Keuls multiple comparisons test. All the data were analyzed by StatView version 5.0 (SAS Institute, Inc.).

Results

In vitro studies in primary cell cultures
Cell proliferation. Vandetanib significantly reduced ATC cell proliferation vs. the control, at 1 and 2 h (P<0.01, by ANOVA, for both) (Fig. 2A). Cell counting confirmed these results: after 1 h, the cell number was 12,120±680/100 μl/well in the ATC control group; 11,998±710 (99%) with vandetanib 1 nM; 11,635±780 (96%) with vandetanib 100 nM; 10,787±690 (89%) with vandetanib 1 μM; 9,332±710 (77%) with vandetanib 10 μM; 8,610±580 (71%) with vandetanib 25 μM; and 3,878±490 (32%) with vandetanib 50 μM (P<0.01,
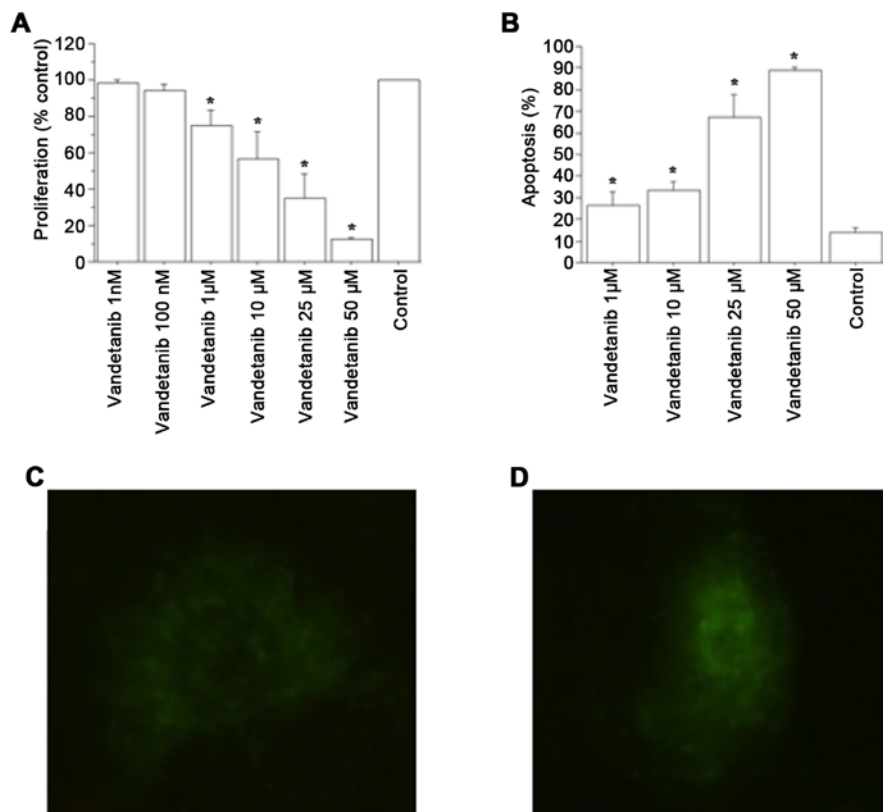
Figure 2. Proliferation (WST-1), and apoptosis assays in primary human anaplastic thyroid cancer (ATC) cells. (A) WST-1 in ATC cells treated with vandetanib for 24 h. Vandetanib significantly reduced ATC cell proliferation vs. control. Bars represent mean (±SD). *P<0.05 vs. control with Bonferroni-Dunn test. (B) ATC cell apoptosis after treatment with vandetanib for 48 h. Apoptosis index was determined by Hoechst staining. Vandetanib dose-dependently induced the percentage of apoptotic cells. Data are presented as mean (±SD) (n=8), and were analyzed by one-way ANOVA (with Newman-Keuls multiple comparisons test, and with a linear trend test) (*P<0.001 vs. control). (C and D) Representative images of the Annexin V binding assay in control and treated ATC cells (with vandetanib 50 μM), respectively.

ANOVA); after 2 h, cell number was 18,950±910/100 μl/well; 18,382±920 (97%) with vandetanib 1 nM; 17,623±990 (93%) with vandetanib 100 nM; 14,592±1,010 (77%) with vandetanib 1 μM; 10,423±1,180 (55%) with vandetanib 10 μM; 6,443±1,190 (34%) with vandetanib 25 μM; and 2,274±750 (12%) with vandetanib 50 μM; (P<0.01, ANOVA). The IC$_{50}$ value, obtained with linear interpolation, was 13±2.9 μM for vandetanib.

The results of WST-1 assay in TFC cells with vandetanib showed a slight but significant reduction in proliferation with respect to the control both at 1 h (P<0.01, ANOVA) with vandetanib 10 μM (94% vs. control), 25 μM (87% vs. control), and 50 μM (81% vs. control), and at 2 h (P<0.01, for both, ANOVA) with vandetanib 10 μM (87% vs. control), 25 μM (81% vs. control), and 50 μM (76% vs. control). Cell counting supported the previously reported results: after 1 h, the cell number was 11,290±730/100 μl/well in the TFC control; 10,612±1,080 (94%) with vandetanib 10 μM; 9,822±940 (87%) with vandetanib 25 μM; and 9,145±880 (81%) with vandetanib 50 μM; (P<0.01, ANOVA); after 2 h, the cell number was 17,950±910/100 μl/well; 15,615±1,090 (87%) with vandetanib 10 μM; 14,540±950 (81%) with vandetanib 25 μM; and 13,640±890 (76%) with vandetanib 50 μM (P<0.01, ANOVA).

Proliferation and BRAF. Three considered ATCs had V600E*BRAF* mutation, while RET/PTC1 and RET/PTC3, H-RAS or N-RAS mutations were not reported in primary ATC cells by real-time PCR. Proliferation was inhibited similarly in ATC from cancers with or without V600E*BRAF* mutation (data not shown).

Apoptosis. Vandetanib increased the number of apoptotic ATC cells dose-dependently (P<0.001; by ANOVA; Fig. 2B). The Annexin V assay corroborated these results (Fig. 2C and D).

Migration and invasion. The effect of vandetanib on migration and invasion was evaluated in ATC cells showing a reduction in both migration (Fig. 3A) and invasion (Fig. 3B).

EGFR inhibition in ATC cells by vandetanib. Vandetanib significantly reduced the phosphorylated (p)EGFR/total EGFR ratio in the ATC cell lysates in a dose-dependent manner (Fig. 4A).

Inhibition of AKT or ERK1/2 phosphorylation in ATC cells by vandetanib. Vandetanib significantly reduced the pAKT/total AKT and pERK1/2/total ERK1/2 protein ratios in the ATC cells (Fig. 4B and C).

Vandetanib decreases cyclin D1 protein levels in ATC cells. Lower cyclin D1 concentrations were detected in ATC cells treated with vandetanib vs. those treated with vehicle, and vandetanib inhibited *cyclin D1* gene expression in a dose-dependent manner (P<0.05; Fig. 4D).

A

	Control	Vandetanib 10 μM	Vandetanib 25 μM	Vandetanib 50 μM
Proliferation at 12 h [% control (mean)]	100%	49%	41%	25%
Migration at 12 h [% control (mean)]	100%	38%	32%	28%

B

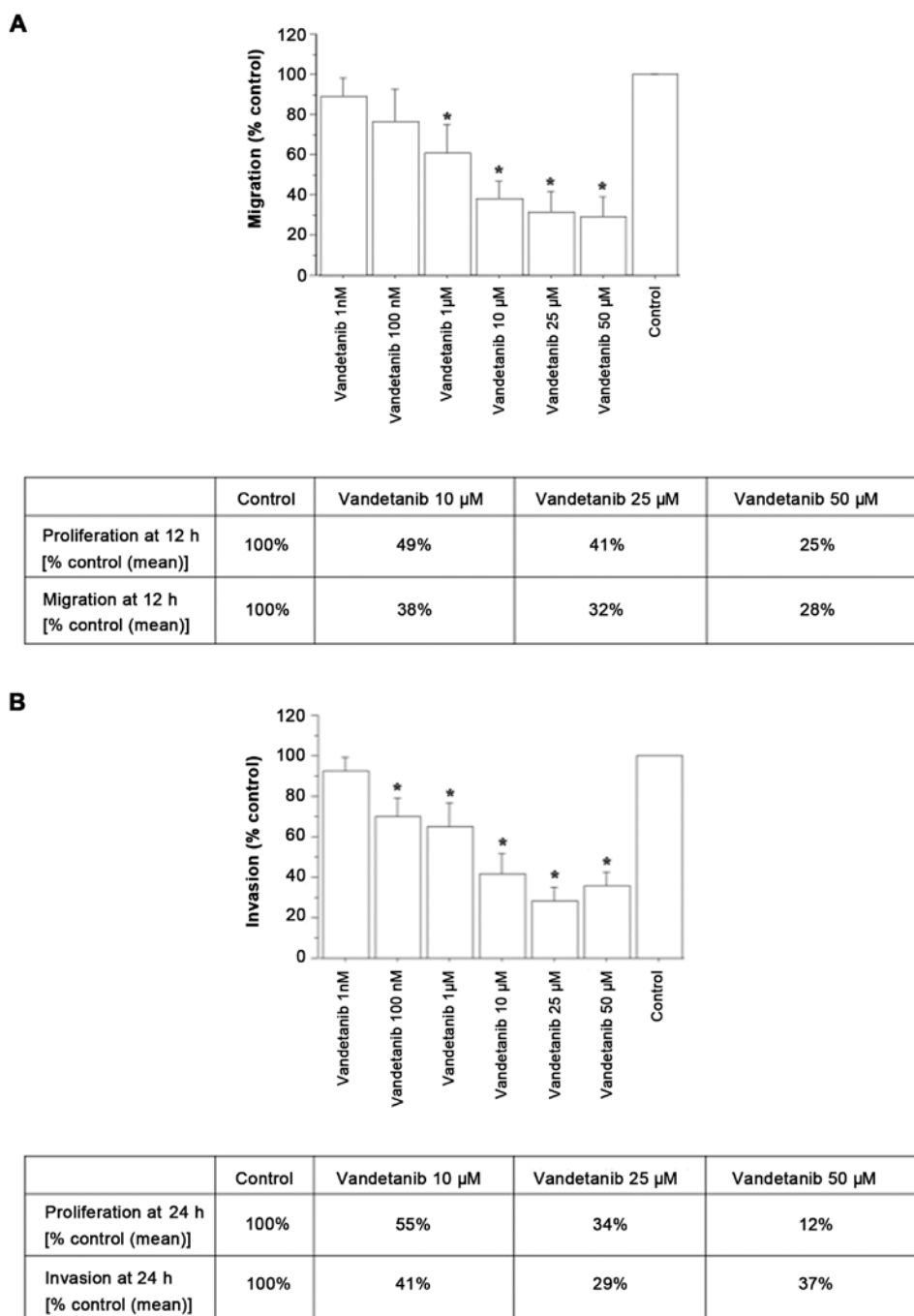|	Control	Vandetanib 10 μM	Vandetanib 25 μM	Vandetanib 50 μM
Proliferation at 24 h [% control (mean)]	100%	55%	34%	12%
Invasion at 24 h [% control (mean)]	100%	41%	29%	37%

Figure 3. Migration and invasion tests in anaplastic thyroid cancer (ATC) cells. ATC cells were incubated for (A) 12 h for migration and (B) 24 h for invasion. For comparison, the inhibition of proliferation (at 12 h) (% with respect to control) and the inhibition of migration are reported in the Table in (A), and the inhibition of proliferation (at 24 h) (% with respect to control) and the inhibition of invasion are shown in (B). Bars represent the mean (±SD). *P<0.05 vs. control (control = medium + FCS 10%) by Newman-Keuls test.

Studies in vitro in AF and 8305C cells. Vandetanib demonstrated a dose-dependent antiproliferative activity in the 8305C cell line (IC_{50} of 9.6±3.4 μM) (Fig. 5A), and in AF cells (IC_{50} of 4.7±1.8 μM) (Fig. 5B). Vandetanib dose-dependently induced the apoptosis of the 8305C cells: apoptotic cells were 16.8% with vandetanib 10 μM, and 20 and 33.2% with vandetanib 25 and 50 μM, respectively (P<0.001; by ANOVA; Fig. 5C). In AF cells, vandetanib dose-dependently increased apoptosis: apoptotic cells were 19.9% with vandetanib 10 μM, and 22.7 and 27.8% with vandetanib 25 and 50 μM, respectively (P<0.001; by ANOVA; Fig. 5D).

In vivo studies

Vandetanib inhibits 8305C tumor growth in mice with no body weight loss. Thirty days after subcutaneous xenotransplantation of 8305C cells in CD *nu/nu* mice, tumor masses reached the average volume of 100 mm^3 and treatment with vandetanib was initiated. In order to establish the optimal antitumor dose, two different doses were administered. At the lower dose of 12.5 mg/kg a slight, but not significant, antitumor activity was recorded (overall from days 16 to 22), and no statistical differences were obtained when tumor volumes in the treatment group were compared to the control group (Fig. 6A).
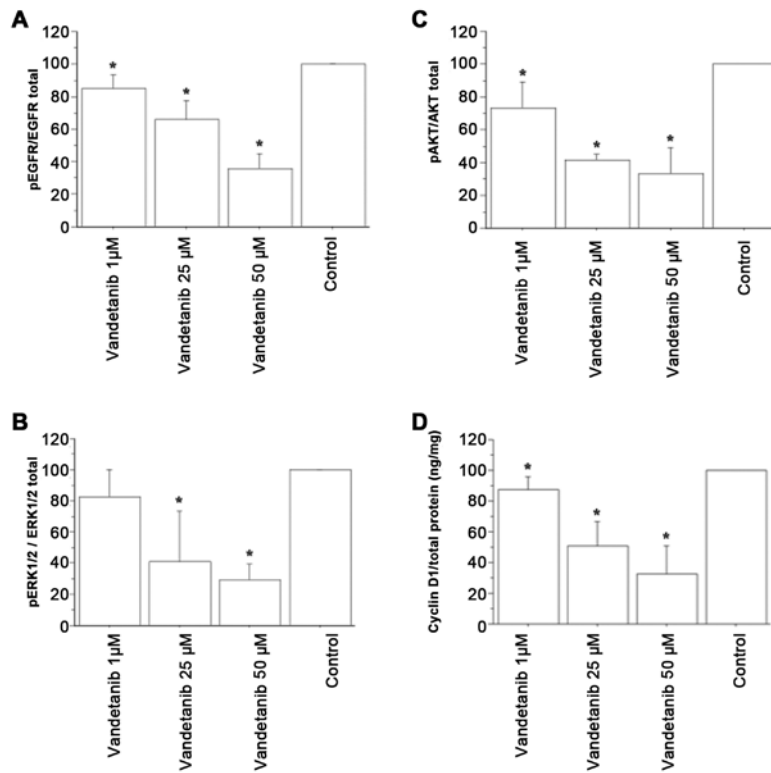
Figure 4. Inhibition of epidermal growth factor receptor (EGFR), AKT, ERK1/2 phosphorylation, or cyclin D1 protein expression in anaplastic thyroid cancer (ATC) cells. (A) Inhibition of EGFR phosphorylation by vandetanib in ATC cells after 72 h of treatment. Experiments were repeated thrice, with eight samples (for each dose). Mean (±SE); *P<0.05 vs. control. (B and C) Inhibition of ERK1/2 (pThr185/pTyr187) and AKT (pThr308) phosphorylation by vandetanib in ATC cells after 72 h of treatment. Experiments were repeated, independently, thrice with eight samples (for each dose). Mean (±SE); *P<0.05 vs. control. (D) Cyclin D1 concentrations in ATC cells exposed to vandetanib or vehicle for 72 h. Cyclin D1 results are expressed as ng/mg of total protein. Experiments were repeated 6 times with eight samples (for each dose). Mean (±SE); *P<0.05 vs. control.
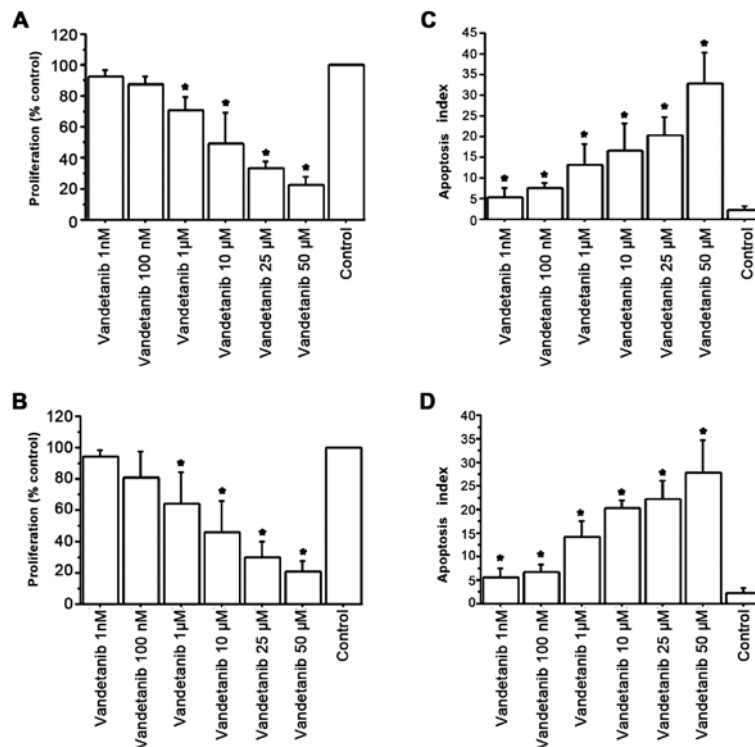

Figure 5. WST-1 and apoptosis assays in 8305C or AF cells. WST-1 assay results in (A) 8305C and (B) AF cells treated with vandetanib for 24 h. A significant inhibition of proliferation vs. control is shown with vandetanib. Bars are mean (± SD). *P<0.05 vs. control with Bonferroni-Dunn test. Apoptosis in (C) 8305C or (D) AF cells exposed to vandetanib for 48 h [mean (±SD) of the samples]. Apoptosis index (ratio between apoptotic and total cells) x100 was determined by Hoechst staining. One-way ANOVA was used to analyze the data (with Newman-Keuls multiple comparisons test, and with a test for linear trend) (*P<0.001 vs. control). IC$_{50}$ vs. controls were estimated by non-linear regression fit (see Results); IC$_{50}$ was 9.6±3.4 μM for 8305C cells, and 4.7±1.8 μM in AF cells.
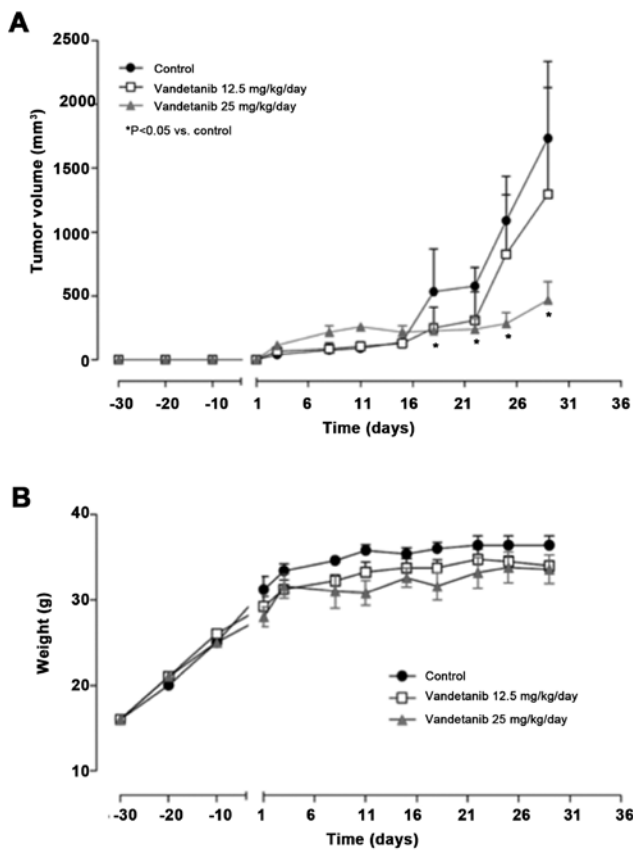
Figure 6. *In vivo* experiments. (A) Dose-dependent antitumor *in vivo* effect of vandetanib at the dose of 12.5 and 25 mg/kg/day intraperitoneally (i.p.), on 8305C tumors xenotransplanted in CD *nu/nu* mice. (B) Weights of mice monitored during the treatment with the two doses of the drug and vehicle alone. Symbols and bars, mean (±SE); *P<0.05 vs. vehicle-treated controls.

In contrast, at the highest dose of 25 mg/kg/day i.p., vandetanib significantly inhibited tumor growth (e.g., at day 25, 282.1 vs. 1,086.9 mm³ of controls P<0.05; Fig. 6A). Notably, no loss of mouse body weight throughout the course of the experiment was observed at both the administered doses, suggesting that the vandetanib treatment was well tolerated even at its optimal antitumor dose (Fig. 6B).

Vandetanib decreases VEGF-A expression and microvessel density in 8305C tumor tissues. 8305C cells led to the formation of a tumor histologically similar to ATC. Vandetanib significantly reduced VEGF-A and FVIII immunostaining. A localized immunoreactivity for VEGF-A was present in cells of the control cancer mass, and vandetanib reduced it (51±9 vs. 37±7; P<0.05), with a concurrent reduction in microvessel density (15±4 vs. controls 26±7; P<0.05).

Discussion

Vandetanib belongs to the TKIs, that are under evaluation for ATC treatment (26).

With the present study, we contributed to the understanding of the anticancer activity of vandetanib, demonstrating that: i) it inhibited primary ATC cell proliferation *in vitro*, through increased apoptosis, and suppressed the migration and invasion abilities as well; and ii) it blocked 8305C cell proliferation

in vitro, increasing apoptosis and reducing 8305C tumor growth in CD *nu/nu* mice as well, with no toxicity.

Our results are in line with those of another study that reported how vandetanib is able to inhibit 8305C cell growth *in vivo*, and to block angiogenesis, decreasing vascular permeability (27). We also observed the important antiangiogenic activity of vandetanib in 8305C xenotransplants.

The antiproliferative action of vandetanib was observed in all the used primary ATC cells, independently from the absence/presence of [V600E]*BRAF* mutation.

Our data support the concept that vandetanib could be used for a multiple signal inhibition (involving RET, VEGFR, EGFR, ERK, AKT, and others), and it also exhibits antiangiogenic activity (28).

It is suggested that AKT plays a crucial role in ATC oncogenesis, and it has been demonstrated that pharmacological and molecular inhibition of PI3K or AKT isoforms are able to reduce the *in vitro* growth and motility of human TC cell lines (29,30). Moreover, both RAS/RAF/MAPK, ERK and PI3K pathways are implicated in the carcinogenesis of TCs, and mutations in such genes have been reported in ATC (31). Since AKT and ERK proteins are activated, once phosphorylated, in ATC, these proteins have been suggested as potential targets of therapy. In the present study in ATC cells, vandetanib significantly inhibited ERK1/2 and AKT phosphorylation.

Furthermore, EGFR phosphorylation in ATC cells was significantly reduced by vandetanib treatment, according to the results obtained by Di Desidero *et al* (32) and our previous results (33), reporting that TKI suppressed EGFR phosphorylation in ATC.

Cyclin D1 regulates cell cycle progression (34), and its expression was observed at different levels in ~67% of ATC cells by Lee *et al* (35). In addition, overexpression of cyclin D1 was reported in 77% of ATC by Wiseman *et al* (36). Vandetanib, a TKI of both VEGFR-2, and EGFR, inhibited cell growth downregulating cyclin E and D1 expression (37). Notably, we showed that vandetanib downregulated the cyclin D1 protein in ATC cells.

We found a significant 8305C cell-derived tumor growth inhibition in CD *nu/nu* mice by vandetanib, without body weight loss, suggesting a minimal toxicity profile, whereas other compounds are known to provoke different adverse effects in humans and animals (38). Nevertheless, we did not collect data concerning the kidney, liver, or other biochemical tests, that will be provided in future studies.

Antineoplastic activity of vandetanib in ATC is the result of different effects on tumoral cells, that include: i) antiproliferative activity; ii) increased apoptosis; iii) inhibition of both migration, and invasion; and iv) inhibition of cancer neovascularization.

New therapeutic attempts for the treatment of ATC are ongoing, even if various limitations are still present for the selective use of new molecules. Even if neoplastic tissue has a potential target (as BRAF), the tumor response is present in only a few patients. As we achieve target inhibition, any response may occur owing to the increased activity of other compensatory pathways, that rescue cancer cell growth. The efficacy of treatment could be increased by assessing the sensitivity of primary ATC cells from each subject to different TKIs. In fact, *in vitro* chemosensitivity tests are able to predict

in vivo effectiveness in 60% of cases (39), while a negative chemosensitivity test *in vitro* is associated with a 90% of ineffectiveness of the treatment *in vivo* (39,40), thus avoiding the administration of inactive chemotherapeutics to these patients (15,16,24,26).

In the present study, we first showed an antitumoral activity of vandetanib (a multi-targeted kinase inhibitor, with antiangiogenic effect) in human primary ATC cell cultures established directly from patients, paving the way for personalized TKI and for future clinical trials.

Acknowledgements

Not applicable.

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 the acquisition of data; TDD, IR, GE, FR, AF, PO, SRP, AP, SP, CLM, SU, EB and GM analysed 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 manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study are appropriately investigated and resolved. 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 section).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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