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Pharmacological effects of vinorelbine in combination with lenvatinib in anaplastic thyroid cancer

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ARTICLE INFO

Chemical compounds studied in this article:
Vinorelbine - IUPAC namemethyl (1*R*, 9*R*, 10*S*, 11*R*, 12*R*, 19*R*)-11-acetyloxy-12-ethyl-4-[(12*S*, 14*R*,)-16-ethyl-12-methoxycarbonyl-1, 10-diazatetracyclo[12.3.1.0, ^{3}, ^{11}.0, ^{4}, ^{9},] octadeca-3(11), 4, 6, 8, 15-pentaen-12-yl]-10-hydroxy-5-methoxy-8-methyl-8, 16-diazapentacyclo[10.6.1.0, ^{1}, ^{9}.0, ^{2}, ^{7}.0, ^{16}, ^{19},] nonadeca-2, 4, 6, 13-tetraene-10-carboxylate- **PubChem CID** 5311497
Lenvatinib - IUPAC name: 4-[3-chloro-4-(cyclopropylcarbamoylamino)phenoxy]-7-methoxyquinoline-6-carboxamide - **PubChem CID:** 9823820

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ABSTRACT

Anaplastic thyroid cancer (ATC) is a rare neoplasia with a poor prognosis. Proliferation and apoptosis assays were performed on ATC cell lines (8305C, 8505C) exposed to vinorelbine, lenvatinib, as well as to concomitant combinations. *ABCB1*, *ABCG2* and *CSF-1* mRNA expression was evaluated by real time PCR. The relative levels of pospho Akt were investigated as part of a human phospho-kinase array analysis, and CSF-1 and VEGFR-2 protein levels were measured by ELISA. The intracellular concentration of lenvatinib in ATC cells was measured by combined reversed-phase liquid chromatography-tandem mass spectrometry. An ATC subcutaneous xenograft tumor model in nude mice was treated with vinorelbine, lenvatinib, or vinorelbine plus lenvatinib.

After treatment with vinorelbine, lenvatinib, a significant antiproliferative effect in ATC cell lines was observed. The concomitant treatment of vinorelbine and lenvatinib revealed synergism for all the fractions of affected cells. A decrease in *ABCB1* expression was reported in both ATC cell lines treated with the lenvatinib plus vinorelbine combination, as was an increase in the intracellular concentration of lenvatinib. The combination caused a decrease in Akt, GSK3α/β, PRAS40 and Src phosphorylation, and in both *CSF-1* mRNA and protein levels. In the subcutaneous tumor model, the combination reduced the tumor volume during the treatment period.

Our results establish the synergistic ATC antitumor activity of a vinorelbine and lenvatinib combination.

1. Introduction

Anaplastic thyroid cancer (ATC) is a rare neoplasia with an incidence of 1–2 % among all thyroid cancers [1]. Lymph node involvement or distant metastases are present in 80 % of patients at the time of diagnosis, and the median overall survival is around 6 months [1]. Multiple therapeutic approaches, including surgery, radiotherapy and chemotherapy, are the current treatment options [2,3]. These therapeutic procedures are of minimal benefit in terms of overall survival and life quality of ATC patients because of the growth and invasion of surrounding tissues of this cancer [4,5]. Consequently, it is important to identify novel therapeutic strategies for the treatment of ATC.

The study of molecular pathways involved in the evolution of ATC led to therapeutic strategies such as tyrosine kinase inhibitors (TKIs).

Abbreviations: ABCB1, ATP-binding cassette sub-family B member 1; ABCG2, ATP-binding cassette super-family G member 2; AI, apoptotic index; Akt, protein kinase B; AMPKα, 5′ adenosine monophosphate-activated protein kinase α1; ATC, anaplastic thyroid cancer; CI, combination index; CSF-1colony, stimulating factor 1; DRI, dose reduction index; GSK, glycogen synthase kinase; HSP60, heat shock protein 60; IHC, immunohistochemistry; Lenv, lenvatinib; MI, mitotic index; p53, tumor protein p53; PRAS40, proline-rich Akt substrate of 40 kDa; SORAF, sorafenib; Src, proto-oncogene tyrosine-protein kinase Src; TAM, tumor associated macrophage; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VNR, vinorelbine

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Lenvatinib is an oral TKI which targets vascular endothelial growth factor receptors -1, -2 and -3 (VEGFR-1, VEGFR-2, and VEGFR-3), fibroblast growth factor receptors 1–4, and platelet-derived growth factor receptors. The antitumor activity of lenvatinib is due to its antiangiogenic properties as well as to direct antitumor effects [6]. Preclinically, lenvatinib has been shown to inhibit ATC cell proliferation, invasion, migration and to increase tumor cell apoptosis. Lenvatinib also significantly reduced tumour growth of ATC subcutaneous xenografts [7]. Recent clinical observations, including a phase II clinical trial, have established the anticancer efficacy of lenvatinib in ATC [8–10]. Sorafenib, a multikinase inhibitor, inhibits tumour angiogenesis and tumor cell growth [11]. Previous preclinical studies on the activity of sorafenib alone in ATC are conflicting [12–14] and clinically it showed minimal activity in ATC patients [15]. Preclinically, the combination of sorafenib with N-hydroxy-7-(2-naphthylthio) heptanomide showed synergistic antitumor activity in a xenograft model of human ATC [16].

Vinorelbine is a microtubule-targeting agent that kills tumor cells by inducing apoptosis [17]. The drug has also been shown to block angiogenesis [18]. I*n vitro* experiments have shown that the combination vinorelbine plus gemcitabine has activity in ATC models [19].

Our aims in this study were to: 1) test for possible synergism in the combined schedule of vinorelbine with lenvatinib (or sorafenib), 2) investigate the antitumor efficacy of the vinorelbine/lenvatinib combination *in vivo* in a model of ATC.

2. Materials and methods

2.1. Materials and drugs

RPMI media and the related supplements for cell cultures were purchased from Sigma Aldrich SRL (Milan, Italy). Reagents for quantitative real-time PCR were purchased from Applied Biosystems (Foster City, CA, USA). Vinorelbine, sorafenib and lenvatinib were acquired from Selleckchem (DBA Italia, Milan, Italy). *In vitro* studies were performed using drugs diluted from a 10 mM stock solution (in 100 % dimethylsulfoxide). In the *in vitro* experiments negative controls had the same concentration of dimethylsulfoxide in the media as did cells that were treated with the highest concentration of vinorelbine, sorafenib and lenvatinib.

2.2. Cell lines

The human ATC cell line 8305C, harbouring the BRAF V600E mutation, was purchased from DSMZ (Braunschweig, Germany, DSMZ no.: ACC 133), and the human ATC cell line 8505C was obtained from ECACC – 94090184. These cell lines were cultured in RPMI media completed with 2 mM L-glutamine and 15 % FBS. Cells were used for experiments at the fourth passage.

2.3. In vitro experiments

2.3.1. Antiproliferative assay

The assay was performed with 8305C and 8505C cells, as previously described [20]. 8505C and 8305C cells were exposed for 72 h (1×10^4 cells/well) to vinorelbine (0.001–10,000 pM), sorafenib (0.01–1,000 μM), and lenvatinib (0.01–1,000 μM), or with vehicle as control. The drug concentrations that decreased cell proliferation by 50 % (IC_{50}) *versus* vehicle-treated cells were determined by a nonlinear regression fit of the mean values obtained in triplicate experiments (at least 9 wells for each concentration).

2.3.2. In vitro determination of synergism between vinorelbine plus lenvatinib or sorafenib on ATC cells

The determination of synergism between vinorelbine and lenvatinib or sorafenib on ATC cells *in vitro* was performed with the objective to: i) investigate the type of pharmacological interaction between the used drugs and ii) suggest a possible dose reduction of each drug used in the combination schedules.

The combination of vinorelbine (0.001 − 10000 pM) with sorafenib (0.01–1,000 μM) and lenvatinib (0.01–1,000 μM) was investigated on 8305C and 8505C cells in a fixed molar concentration ratio (1:10). Synergism was measured on the principle of the multiple drug-effect equation and estimated by the combination index (CI) method [21], where $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additive effect, and antagonism, respectively. To evaluate the level of interaction (synergistic, additive or antagonistic) between vinorelbine and sorafenib or lenvatinib, the CI was calculated with the classic isobologram for mutually exclusive effects, the CI value was assessed as follows:

$$CI = [(D)_1/(DX)_1] + [(D)_2/(DX)_2]$$

where the $(Dx)_1$ and $(Dx)_2$ are the concentrations of vinorelbine and lenvatinib, respectively, that cause a certain percentage of inhibition of cell proliferation and $(D)_1$ and $(D)_2$ are the concentrations of vinorelbine and lenvatinib in combination that block cell proliferation by the same percentage.

The dose reduction index (DRI) represents the theoretical magnitude of concentration decrease that could be obtained for each drug in the combination compared to the concentration of each drug alone that would yield the same effect:

$$(DRI)_1 = (DX)_1/(D)_1 \text{ and } (DRI)_2 = (DX)_2/(D)_2$$

The CI and DRI indexes were calculated with the CalcuSyn v.2.0 software (Biosoft, Cambridge, UK).

2.3.3. Intracellular accumulation of lenvatinib in ATC cells

To explore the synergistic effects observed, a variation of the intracellular lenvatinib level was considered. The intracellular levels of lenvatinib in treated cells were assessed by a quantitative analysis. 8305C and 8505C cells (1×10^6) were incubated with 1 μM lenvatinib in RPMI for 16 h at 37 °C. After incubation, the cells were rinsed twice with PBS pH 5 at 4 °C and detached with a cell scraper. The ATC cells were then lysed by ultrasound (three 10 s pulses). Cell lysates (200 μL) were mixed with 400 μL methanol and centrifuged (5 min; 7500 *g*; 4 °C) to measure the intracellular lenvatinib concentration. The protein concentration in the lysates was assessed by a colorimetric assay (Bio-Rad Laboratories). The lenvatinib levels in the samples were evaluated using a previously described method of combining reversed-phase liquid chromatography-tandem mass spectrometry with positive ion electrospray ionization [22].

2.3.4. ABCB1, ABCG2 and CSF-1 gene expression in ATC cells

To evaluate the expression of the genes encoding human *ATP-binding cassette sub-family B member 1 (ABCB1), ATP-binding cassette super-family G member 2 (ABCG2)* and *colony stimulating factor 1 (CSF-1)*, the 8305C and 8505C ATC cell lines were treated for 72 h with vinorelbine and lenvatinib alone, or in the concurrent combination at a concentration corresponding to their experimental IC_{50} of cell proliferation. Controls were treated with vehicle alone. An Applied Biosystems 7900HT (Applied Biosystems, Carlsbad, CA, USA) sequence detection system was used to carry out the quantitative RT-PCR as previously described [23]. *ABCB1*, *ABCG2* and *CSF-1* (ABCB1, Assay ID Hs01067802_m1, ABCG2, Assay ID Hs01053796_m1, CSF-1, Assay ID Hs00174164_m1) validated primers were used. The manufacturer's instructions were followed to set the polymerase chain reaction thermal cycling conditions. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) amplifications were used for normalization, and the ΔΔCt was calculated; the amount of target was defined as $2^{-\Delta\Delta Ct}$. All experiments were repeated, independently, three times with at least nine samples for each concentration.

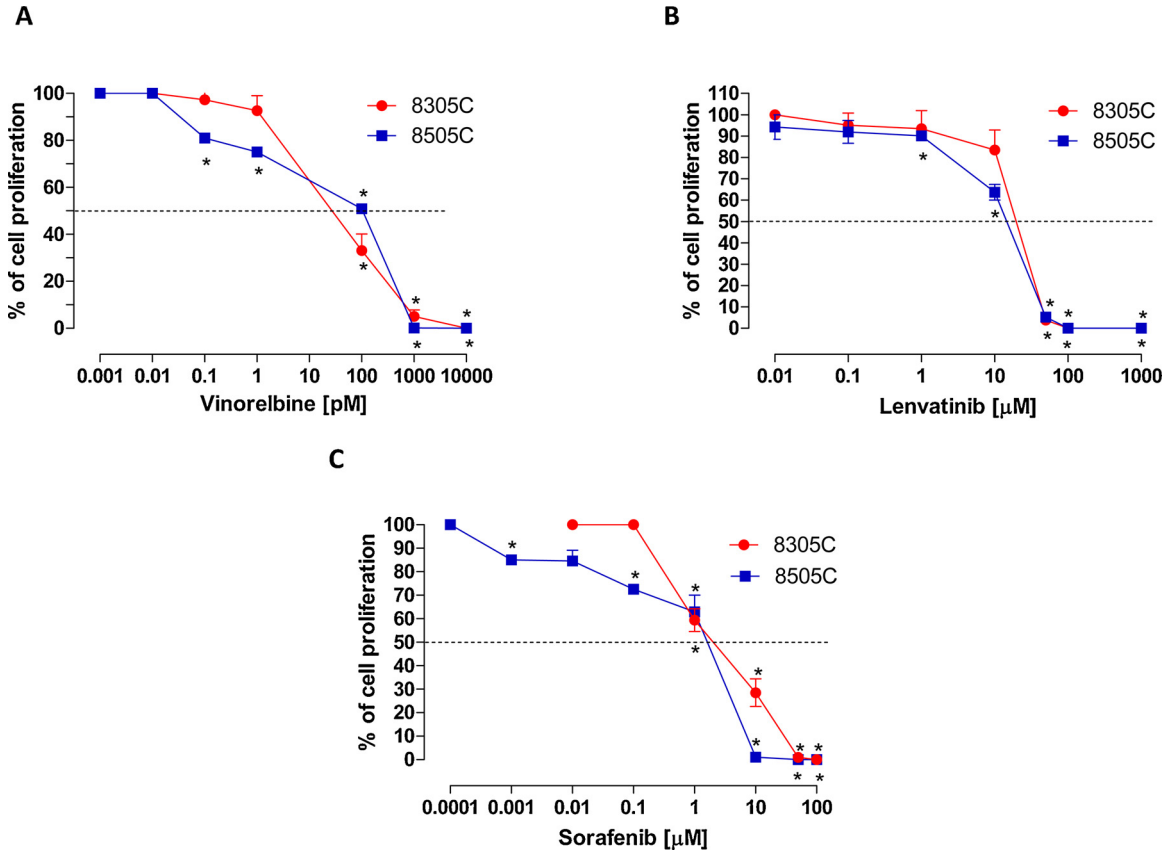


Fig. 1. *In vitro* effect of vinorelbine (**A**), lenvatinib (**B**) and sorafenib (**C**) on proliferation of 8305C and 8505C ATC cell lines. The antiproliferative effects of the drugs were studied using a 72 h exposure. *Symbols and bars*, mean values ± S.E., respectively. *$P < 0.05$ *vs.* vehicle-treated controls.

2.3.5. Quantification of phospho-Akt, CSF-1 and VEGFR-2 protein levels

To assess the levels of phospho-Akt (pAkt), CSF-1, and VEGFR-2, 8305C and 8505C cells were exposed for 72 h to vinorelbine and lenvatinib alone and in concurrent combination at a concentration equal to their IC_{50} of cell proliferation. Cell lysates were analyzed with the human pAkt (PhosphoDetect Akt *pThr*^{308}, Calbiochem, Darmstadt, Germany), CSF-1 (Sigma-Aldrich, #RAB0098−1KT), and VEGFR-2 (Cell Signaling Technology) ELISA kits. The optical density was measured with a Multiskan Spectrum microplate reader (Milan, Italy) set to 450 nm. The protein levels were recorded as ng of CSF-1, or pg of VEGFR-2, per mg of total protein. The pAkt results were scored as percentage of pAkt levels in controls. All experiments were repeated, independently, three times with at least nine samples for each concentration.

2.3.6. Phospho-kinase array

To evaluate the relative levels of phosphorylation of 43 kinase phosphorylation sites and 2 total proteins, 8505C cells were exposed for 72 h to vinorelbine and lenvatinib alone and in concomitant combination at a concentration equal to their IC_{50} of cell proliferation. Cell lysates were analyzed using a Proteome Profiler™ Array (R&D Systems, Inc.), the Human Phospho-Kinase Array (Catalog Number ARY003B), according to the manufacturer's instructions. The results were scored as mean pixel density.

2.4. In vivo experiments

2.4.1. Subcutaneous model of human ATC

Six-week-old Athymic Nude-Foxn1^{nu} male mice (20−25 g) were purchased from Envigo (Milan, Italy) and then housed in microisolator cages on vented racks. Aseptic techniques were followed to manipulate the animals that had access to sterile food and water in an unrestricted manner. All animals procedures were done in agreement with a protocol authorized by the Academic Organization Responsible for Animal Welfare of the University of Pisa, following the Italian law D.lgs. 26/2014, and by the Italian Ministry of Health (authorization No. 264/2016-PR). On day 0 of the experiment, 2×10^6 viable 8505C cells/mouse were injected subcutaneously. Before injection of tumor cell suspension, mice were first anesthetized with i.p. injection of sodium pentobarbital (50 mg/kg). Weights of the mice were measured and tumour dimensions were calculated with callipers; tumour volume (mm^3) was measured in the following manner: $[(w^1 \times w^1 \times w^2) \times (\pi/6)]$, where w^1 and w^2 were the smallest and the largest tumour diameter (mm), respectively. Therapy started when the tumour attained 100 mm^3, 25 days after cell injection. The mice were randomly assigned to four groups (n = 5/group) which were treated as follows, 1) saline once a week i.p. as controls, 2) vinorelbine 10 mg/kg once a week i.p. [24], 3) lenvatinib 25 mg/kg every day orally by gavage, as previously published by our group [7] 4) concomitant combination of vinorelbine + lenvatinib. At the end of the experiment, mice were euthanized using an anesthetic overdose.

2.4.2. Immunohistochemistry (IHC)

IHC was performed as previously described [25]. Briefly, tumour tissue samples were fixed in 10 % neutral-buffered formalin for 12–24 h and embedded in paraffin. Sections of the tumour (5 μm thick) were stained with haematoxylin and eosin. Immunostaining was carried out with a Benchmark immunostainer (Ventana, Tucson, AZ, USA) and using the avidin–biotin-peroxidase complex (ABC) method and counterstained with haematoxylin. Negative controls were performed by omitting the primary antibodies. IHC was evaluated independently by two pathologists (G.A. and G.Fo.). Sections stained with haematoxylin-eosin were evaluated for the mitotic index (MI) by counting mitotic figures under a microscope. The microvessel number was established

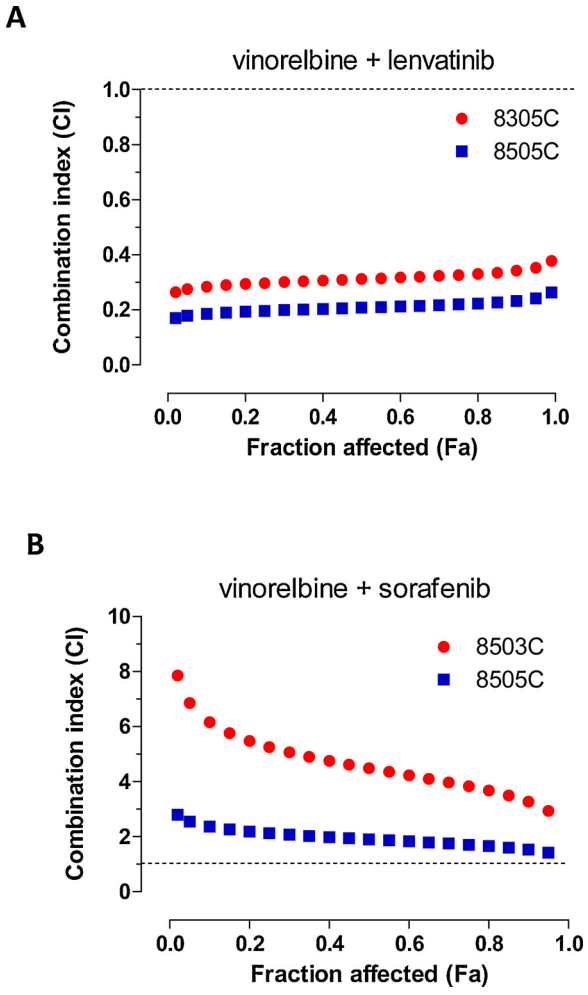


Fig. 2. Combination index (CI)-fraction affected (Fa) plot of 72 h vinorelbine (VNR) and lenvatinib (Lenv) combination in 8505C and 8305C (**A**) ATC cells. Combination index (CI)-fraction affected (Fa) plot of 72 h vinorelbine and sorafenib combination in 8505C and 8305C (**B**) ATC cells. CI < 1, CI = 1 (dashed line) and CI > 1 indicate synergism, additive effect and antagonism, respectively.

Table 1
Dose reduction index (DRI) values of the vinorelbine (VNR) and lenvatinib (Lenv) combination.

Affected Cell Fraction (%)	DRI values	
8505C	**VNR + Lenv**	
	VNR	**Lenv**
30 %	5.0	> 1000
50 %	4.8	> 1000
70%	4.6	> 1000
90%	4.3	> 1000
8305C	**VNR + Lenv**	
	VNR	**Lenv**
30 %	3.3	> 1000
50 %	3.2	> 1000
70%	3.1	> 1000
90%	2.9	> 1000

using anti-CD31 polyclonal antibody (clone JC70; cat. 760–4378, Ventana Medical System). Ki-67 staining was done using the rabbit monoclonal primary antibody (clone 30 − 9; cat. 790–4286; Ventana Medical System). Ki-67 staining was measured as the percentage of cancer cells with positively stained nuclei. The identification of apoptosis was performed using a rabbit polyclonal antibody that recognizes

Table 2
Dose Reduction Index (DRI) values of the vinorelbine (VNR) and sorafenib (SORAF) combination.

Affected Cell Fraction (%)	DRI values	
8505C	**VNR + SORAF**	
	VNR	**SORAF**
30 %	0.485	120.7
50 %	0.528	126.7
70%	0.574	133.1
90%	0.657	143.9
8305C	**VNR + SORAF**	
	VNR	**SORAF**
30 %	0.197	> 1000
50 %	0.223	> 1000
70%	0.252	> 1000
90%	0.306	> 1000

active caspase-3 (diluted 1:2000; cat. ab2302, Abcam, Cambridge, UK). The apoptotic index was determined as a percentage of the apoptotic cells out of the total number of the examined cells.

2.5. Statistical analysis

The investigators responsible for data analysis were blinded to which samples/animals represented treatments and controls.

The results (mean ± SEM) of all the experiments were analyzed with ANOVA, followed by the Student–Newman–Keuls test. The statistical significance was fixed at $P < 0.05$. Statistical analyses were carried out by the GraphPad Prism software package version 5.0 (GraphPad Software, Inc, San Diego, CA).

Power analysis of *in vivo* experiment. In the *in vivo* experiments, 5 animals (n = 5) were included in each of the 4 groups in order to give 80 % power to oneway ANOVA analysis, against a difference in tumor volumes equal or greater than 0.50*sd, with a nominal alpha error rate = 0.05. The power calculation was performed with the G*Power 3 software [26].

3. Results

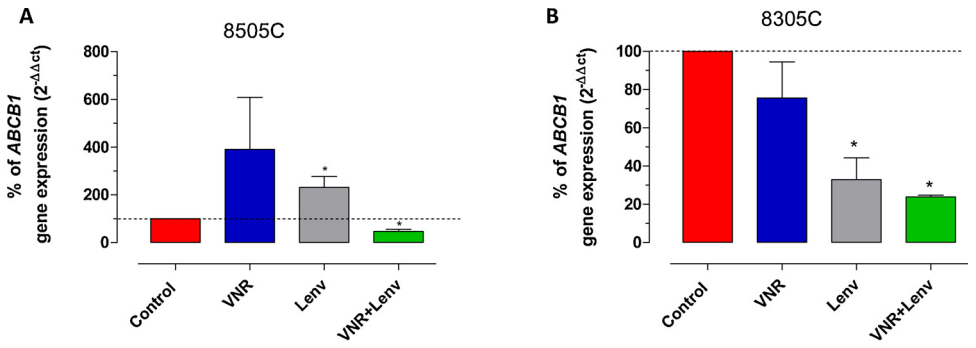
3.1. In vitro experiments

3.1.1. Vinorelbine, lenvatinib and sorafenib inhibited ATC cells proliferation in vitro

An antiproliferative effect was observed in 8505C and 8305C cell lines following vinorelbine treatment, as shown by the calculated IC_{50s} of 130.8 ± 14.78 pM and 8.2 ± 8.15 pM (Fig. 1A), respectively. The 72 h lenvatinib treatment inhibited the 8305C and 8505C cell proliferation with IC_{50} of 18.23 ± 2.82 μM and 14.94 ± 1.14 μM (Fig. 1B), respectively. Following treatment with sorafenib, growth inhibition was observed in the 8305C cell line with an IC_{50} of 2.611 ± 4.39 μM (Fig. 1C), whereas in the 8505C cell line the IC_{50} was 2.11 ± 2.27 μM (Fig. 1C).

3.1.2. Vinorelbine is synergistic with lenvatinib, but not with sorafenib, in vitro

The simultaneous treatment of 8505C and 8305C cells with diverse concentrations of lenvatinib and vinorelbine for 72 h resulted in synergism for all the fraction of affected cells with a CI < 1 (Fig. 2A) and DRI > 1 as shown in Table 1. Synergism always produced a favorable DRI > 1 for both drugs in 8305C and 8505C cells. Conversely, the simultaneous treatments of sorafenib and vinorelbine did not shown any synergism for the fractions of affected cells (Fa) (CI > 1) in either 8505C or in 8305C cells (Fig. 2**B**; Table 2).



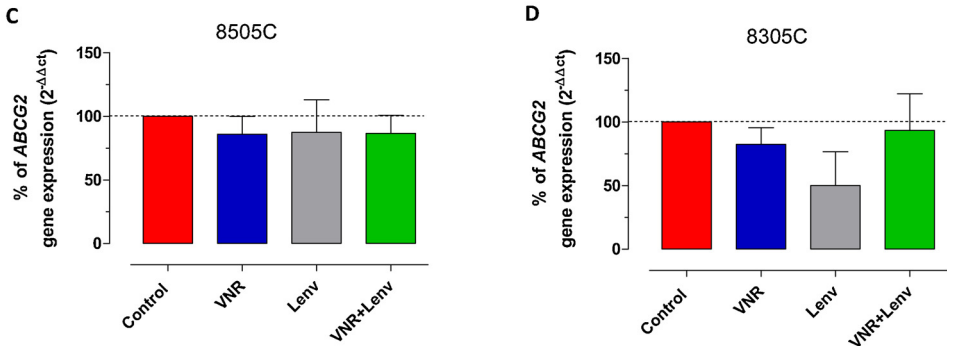


Fig. 3. *ABCB1* gene expression ($2^{-\Delta\Delta ct}$) levels in 8505C (**A**) and 8305C (**B**) ATC cells exposed to vinorelbine (VNR), lenvatinib (Lenv), and their combination or with vehicle alone for 72 h. *ABCG2* gene expression ($2^{-\Delta\Delta ct}$) in 8505C (**C**) and 8305C (**D**) ATC cells exposed to vinorelbine, lenvatinib, and their combination or with vehicle alone (control) for 72 h. Data are expressed as percentage of vehicle-treated cells (dashed line). *Columns and bars*, mean values ± S.D., respectively. *$P < 0.05$ *vs.* vehicle-treated controls.

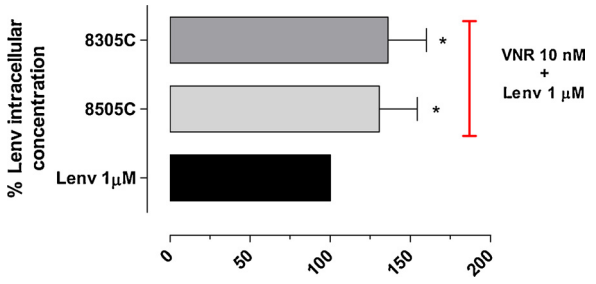


Fig. 4. Intracellular accumulation of lenvatinib (Lenv) in 8505C and 8305C ATC cell lines after exposure to 1 μM lenvatinib alone and in combination with vinorelbine (VNR). *Columns and bars* indicate the mean percentage values (± S.D.) *vs.* treated cells with lenvatinib alone. *$P < 0.05$ with respect to lenvatinib alone.

3.1.3. Gene expression changes induced by lenvatinib and vinorelbine combination

The modulation of *ABCB1* and *ABCG2* gene expressions was evaluated. The *ABCB1* transporter mRNA expression was assessed in 8505C and 8305C cell lines treated with vinorelbine, lenvatinib, and their concomitant combination. In 8505C cells, the *ABCB1* gene expression was decreased (Fig. 3A) for the lenvatinib plus vinorelbine treatment. Similarly, in 8305C cells *ABCB1* mRNA expression decreased following treatment in the concomitant combination of lenvatinib with vinorelbine, and also with lenvatinib alone (Fig. 3B). The *ABCG2* mRNA expression was not altered by the concomitant combination of lenvatinib and vinorelbine (Fig. 3**C** and **3D**, respectively).

3.1.4. Concomitant combination increases intracellular lenvatinib concentrations in ATC lines

Lenvatinib levels in 8305C and 8505C cells were analyzed, and elevated levatinib intracellular concentrations were found in both ATC cell lines (Fig. 4) after treatment with the combination of lenvatinib plus vinorelbine compared to cells treated with lenvatinib alone.

3.1.5. Lenvatinib and vinorelbine combination reduces phospho Akt, GSK-3α/β, PRAS40 and Src in ATC cells

Phosphorylated Akt ($pThr^{308}$) was evaluated in vinorelbine alone and vinorelbine plus lenvatinib-treated cells. We noted a significant pAkt reduction in both 8505C cells (Fig. 5A) and 8305C cells (Fig. 5B) in the concomitant vinorelbine plus lenvatinib combination.

The phospho-kinase array analysis of 8505C cells confirmed the

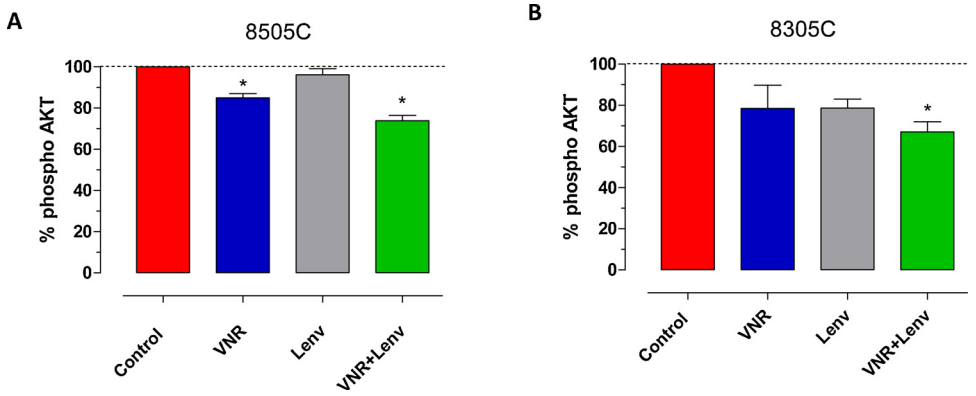


Fig. 5. Akt phosphorylation levels in 8505C (**A**) and 8305C (**B**) ATC cells exposed to vinorelbine (VNR), lenvatinib (Lenv), and their combination or with vehicle alone (control) for 72 h. Data are expressed as percentage of vehicle-treated cells (dashed line). *Columns and bars*, mean values ± S.D., respectively. *$P < 0.05$ *vs.* vehicle-treated controls.

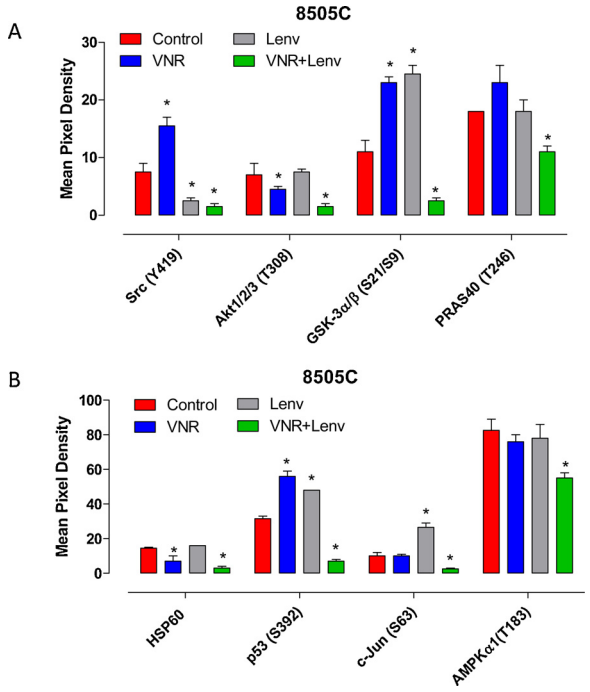


Fig. 6. Proto-oncogene tyrosine-protein kinase Src (Src), protein kinase B (Akt), glycogen synthase kinase-3α/β (GSK-3α/β), proline-rich Akt substrate of 40 kDa (PRAS40) (**A**) and heat shock protein 60 (HSP60), tumor protein p53 (P53), c-Jun, 5′ adenosine monophosphate-activated protein kinase α1 (AMPKα1) (**B**) phosphorylation levels in 8505C ATC cells exposed to vinorelbine (VNR), lenvatinib (Lenv), and their combination or with vehicle alone (control) for 72 h. Data are expressed as men pixel density. X-axis legend: targets (phosphorylation sites). *Columns and bars*, mean values ± S.D., respectively. $^{*}P < 0.05$ *vs.* vehicle-treated controls.

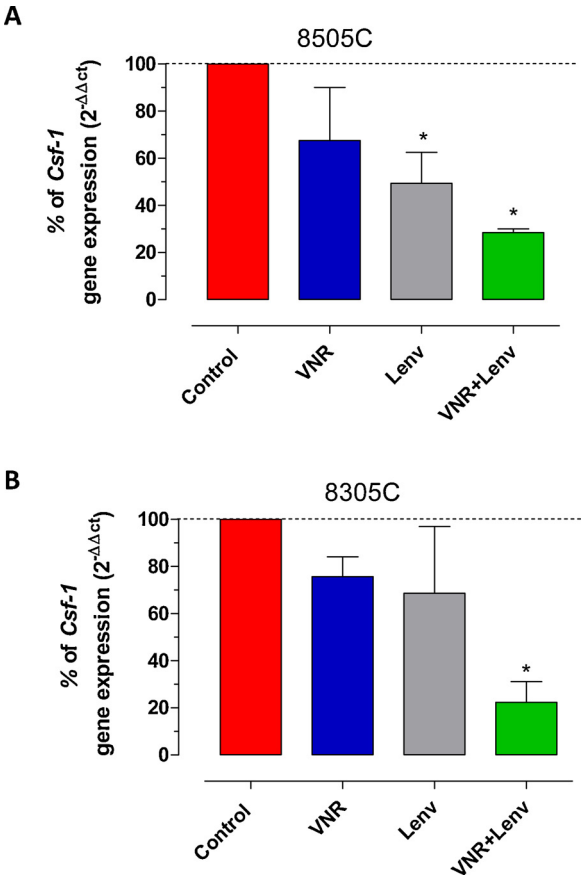


Fig. 7. *CSF-1* gene expression ($2^{-\Delta\Delta ct}$) levels in 8505C (**A**) and 8305C (**B**) ATC cells exposed to vinorelbine (VNR), lenvatinib (Lenv), and their combination or with vehicle alone (control) for 72 h. Data are expressed as percentage of vehicle-treated cells (dashed line). *Columns and bars*, mean values ± S.D., respectively. $^{*}P < 0.05$ *vs.* vehicle-treated controls.

significant decrease of phospho Akt (T308) by the combined treatment (Fig. 6A), and showed the significant inhibition of the phosphorylated forms of multiple proteins downstream of Akt, including GSK-3α/β (S21/S9) and PRAS40 (T246), as well as the significant reduction in the phosphorylation state of Src (Y419) (Fig. 6A). Fig. 6B shows the other targets and phosphorylation sites that were impacted by the concomitant treatment of vinorelbine and lenvatinib, such as p53 (S392), c-Jun (S63) and AMPKα1 (T183).

3.1.6. Lenvatinib and vinorelbine combination reduces CSF-1 expression in ATC cells

In 8505C cells, *CSF-1* mRNA expression was reduced in the lenvatinib alone treatment, and in a higher extent also in the concomitant combination with vinorelbine (Fig. 7A). In 8305C cells *CSF-1* mRNA expression was significantly reduced after the administration of the concomitant lenvatinib and vinorelbine (Fig. 7B). Moreover, CSF-1 levels were significantly reduced after treatment with lenvatinib alone, and with vinorelbine plus lenvatinib - in both 8505C and in 8305C cells (Fig. 8**A** and **B**, respectively). VEGFR-2 protein was not detectable in lysates of either cell line (data not shown).

3.2. In vivo experiments

3.2.1. Subcutaneous ATC xenograft model

8505C cells were injected subcutaneously in CD *nu/nu* mice and the resulting tumours grew rapidly. Both lenvatinib (25 mg/kg/day) and vinorelbine (10 mg/kg/weekly) monotherapies significantly inhibited tumor growth compared to controls (Fig. 9A). The concomitant combination of lenvatinib and vinorelbine inhibited tumor growth, and this was evident 4 days after the start of therapy (Fig. 9A). During the 25 days of treatment, the combination significantly inhibited tumor growth without producing evident toxicity (Fig. 9B).

3.2.2. Immunohistochemical analysis

As summarized in Table 3 we observed differences between ATC subcutaneous control tumours and vinorelbine plus lenvatinib-treated tumours in terms of mitotic index, activated caspase 3 staining (representative microscopic images are shown in Fig. 10**A** and **B**, respectively), CD-31 (Fig. 10**C** and **D**, respectively) and ki-67 immunoreactivity (Fig. 10**E** and **F**, respectively).

4. Discussion

Anaplastic thyroid cancer represents only a small percentage of all thyroid cancers, but it is responsible for the majority of deaths from thyroid cancer because of its aggressive character [27]. ATC is insensitive to any known systemic treatment, such as radiation or chemotherapy [4,28]. The identification of new therapies is therefore a main goal of ATC research. Prospective and retrospective clinical trials have not identified efficacious agents or schedules, in some cases due to an insufficient recruitment of patients [29]. Chemotherapeutic drugs administered alone can be a therapeutic option for ATC patients with inoperable or disseminated disease, but overall cytotoxic agents are not efficacious [4,30]. ATC have shown minimal responses to chemotherapy agents such as anthracyclines, taxanes, platinum derivatives, and methotrexate [29,31]. Occasional complete responses (and numerous adverse drug reactions) are observed with doxorubicin, a topoisomerase II inhibitor, which remains the chemotherapeutic drug of choice for the therapy of advanced disease [29]. Nonetheless, in

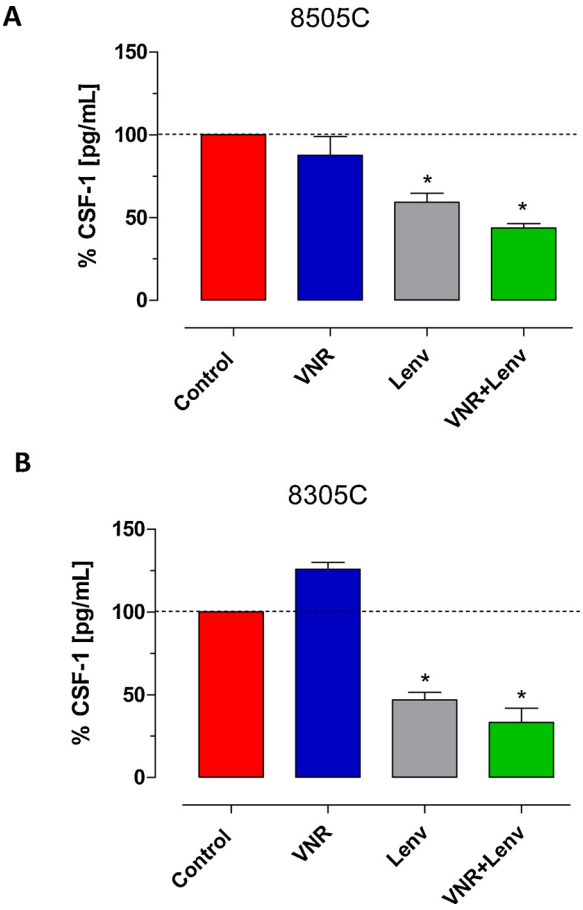


Fig. 8. CSF-1 concentrations in 8505C (**A**) and 8305C (**B**) ATC cells exposed to vinorelbine (VNR), lenvatinib (Lenv), and their combinations or with vehicle alone (control) for 72 h. Data are expressed as percentage of vehicle-treated cells (dashed line). *Columns and bars*, mean values ± S.D., respectively. *$P < 0.05$ *vs.* vehicle-treated controls.

preclinical studies efficacious drug combinations were demonstrated in ATC, such as irinotecan plus sunitinib [23], topotecan plus pazopanib [32], or paclitaxel plus lenvatinib [33]. In the present study, we explored the preclinical activity of the microtubule-targeting drug vinorelbine alone and its combination with the TKIs lenvatinib or sorafenib. Recent data from preclinical models and clinical trials support the idea that combination treatments that involve drugs from different antineoplastic classes have synergistic antitumour activity in ATC as highlighted by two recent reviews of literature on this issue [34,35]. Preclinical studies using drugs already registered by Food and Drug Administration or European Medicines Agency for the treatment of other cancers, such as vinorelbine (*e.g.*, approved for non-small cell lung cancer) or lenvatinib (*e.g.*, approved for differentiated thyroid carcinoma refractory to radioiodine therapy), are fundamental to build rational bases for the compassionate use of these combinations in single patients with advanced ATC. They can also serve as a rationale for new, although presently infrequent, multicenter clinical trials. Our study is therefore an important effort to address the need to evaluate combinations of approved drugs on ATC models; so that the most promising ones may rapidly translate to clinical evaluation.

Our findings show that vinorelbine monotherapy was more effective than lenvatinib monotherapy in ATC cells *in vitro* and in tumors *in vivo*. Above all, the concomitant combination of these drugs was highly synergistic in ATC cells and in the subcutaneous model of ATC. In contrast, although in our hands sorafenib alone showed a higher antiproliferative activity *in vitro* if compared to lenvatinib, the simultaneous treatment of vinorelbine and sorafenib did not show such an activity, but a clear antagonistic effect. For this reason, we did not test further the combination in our experimental settings and in our animal model, focusing our efforts toward the synergistic schedule of lenvatinib plus vinorelbine.

A similar antiproliferative effect was found by Jing and colleagues [33] using another microtubule-targeting drug such as paclitaxel in association with lenvatinib. In that article, paclitaxel was found to be more effective than lenvatinib but their combination blocked colony formation showing synergism and inhibited tumour growth in nude mice compared to lenvatinib (or paclitaxel) monotherapy, as well as inducing apoptosis [33]. Indeed, it has been reported that vinorelbine, but also other chemotherapeutic drugs such as paclitaxel, induces cell accumulation in G2/M phase. Cells accumulated in G2/M phase die *via* apoptosis, exhibiting mitochondrial membrane potential decrease and caspase activation and phosphatidylserine externalization [36]. Furthermore, Jing and colleagues [33] described that lenvatinib potentiated the cell cycle arrest and the apoptotic effects of paclitaxel in the same ATC cell lines used in our study. Interestingly, both lenvatinib and paclitaxel monotherapies increased the proportion of 8305C cells in the G2/M phase of cell cycle and their combination synergistically increased the percentage of cells in G2/M phase as compared to lenvatinib or paclitaxel alone. Thus, it is conceivable that the association of vinorelbine and lenvatinib may exert the same effects on the cell cycle, increasing the percentage of cells in G2/M phase. Moreover, the combination induced ATC cell to die *via* apoptosis as shown in our *in vivo* experiment with an increase of activated caspase 3 immunostaining. Finally, both our study and the study of Jing and colleagues [33] showed that the combination treatments significantly decreased the Ki67 immunostaining (*i.e.* a proliferative marker), in the *in vivo* ATC tumor model.

The possible mechanisms involved in the synergic activity of the combination of vinorelbine and lenvatinib are likely varied, and our efforts focused on the modulation of genes that can change the intracellular concentrations of the drugs such as the ABC transporters, acting as efflux pumps. The reported synergistic action is probably in part a consequence of the observed increase in lenvatinib intracellular concentrations in ATC cells when co-administered with vinorelbine. The importance of ABC transporters in ATC is highlighted by the recent systematic review by Abbasifarid and colleagues [37]. Of note, *ABCG2* had the highest expression rates among the analyzed transporters in ATC, and *ABCB1* was the second most highly expressed. Thus, it was logical to investigate the modulation of the expression of these genes in our ATC model, as that could be a mechanism contributing to the increased intracellular concentration of lenvatinib. In our hands, the combination of vinorelbine and lenvatinib significantly decreased *ABCB1* expression. As an efflux pump, the ABC transporter can extrude various molecules, including tyrosine kinase inhibitors, which can contribute to increased resistance to such drugs. For example, cell lines that overexpress *ABCB1* show increased efflux of STI571/imatinib and are relatively resistant to the drug [38]. Indeed, lenvatinib has been described to be a substrate of ATP-binding cassette transporters such as ABCB1 [39], and the decreased *ABCB1* expression in ATC cells is consistent with the observed increased intracellular level of the drug and thus to a synergic effect on cell proliferation. Conversely, we did not find any significant change in *ABCG2* expression, a transporter reported to alter the intracellular concentration of substrate chemotherapeutic drugs in ATC cells, as previously shown also by our group [32]. In fact, we previously proposed that the association of diverse TKIs such as sunitinib, pazopanib and axitinib with topotecan or with the active metabolite of irinotecan (*i.e.* SN-38) could alter the intracellular concentrations of drugs because of the *ABCG2* downregulation mediated by treatment with TKIs [32,40,41].

Thyroid neoplasms are greatly permeated with macrophages, and 95 % of ATC patients display a large number of infiltrated tumour associated macrophages (TAMs), which is associated with a low survival [42]. Recently, Weinberger and co-workers discovered a group of

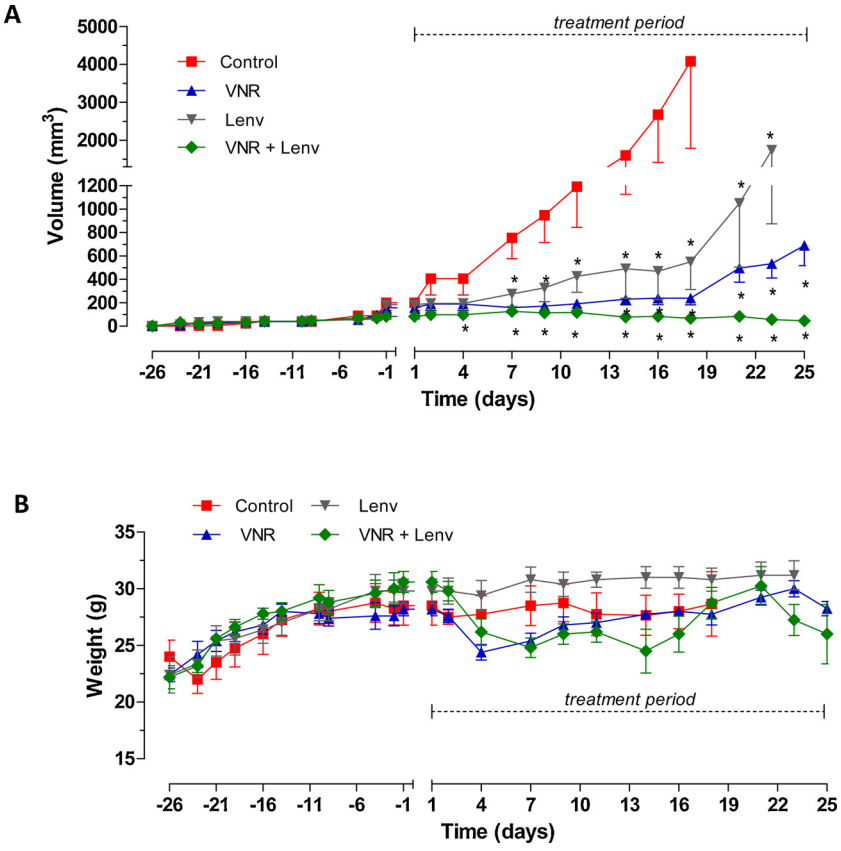


Fig. 9. Antitumour effect (**A**) of (i) vinorelbine (VNR) 10 mg/kg weekly i.p., (ii) lenvatinib (Lenv) 25 mg/kg p.o. daily, (iii) concomitant combination of vinorelbine and lenvatinib; (iv) saline (control) weekly i.p. as controls for 25 days (indicated as treatment period), on 8505C tumours subcutaneously xenotransplanted in CD *nu/nu* mice. Weights of mice (**B**) recorded during the experiment. *$P < 0.05$ with respect to controls. *Symbols and bars*, mean ± S.D.

Table 3
Immunohistochemistry of human ATC subcutaneous tumor after 25 days of treatment. VNR (vinorelbine), Lenv (lenvatinib). Values are shown as mean ± SE; *$P < 0.05$ *vs.* vehicle-treated controls.

	Mitotic index	Ki-67 (% of stained cells)	Microvascular count (CD31+)	Apoptotic index (% of active caspase 3 stained cells)
Control	12.0 ± 2.8	36.0 ± 0.7 %	7.0 ± 0.8	36 ± 0.14 %
Lenv	9.2 ± 3.4	30.0 ± 0.1 %	2.8 ± 1.3*	62 ± 0.1 %*
VNR	14.4 ± 4.8	26.0 ± 0.2 %	5.8 ± 2.9	43 ± 0.3 %
VNR + Lenv	3.3 ± 2.3*	13.0 ± 0.3 %*	2.0 ± 1.0*	77 ± 0.2 %*

constantly impaired biological mechanisms and pathways in ATC [27]. They found some genes related to TAMs, lymphocytic penetration into the tumour mass, and phagocytosis by macrophages. For example, the *CSF1R* gene was greatly upregulated in ATC tumours. CSF-1 is fundamental for the differentiation and the survival of macrophages, which may affect the overall gene expression profile in ATC [27]. Clinical studies using CSF-1/CSF-1R inhibitors have been reported on diverse type of cancers. Imatinib [43], dasatinib [44], and sunitinib [45] have been suggested to control the immunosuppressive, and the tumorigenic, capacities of TAMs. We detected a significant decline of the levels CSF-1 protein and of *CSF-1* mRNA expression attributable to the concomitant treatment with lenvatinib and vinorelbine in ATC cells, suggesting that the decrease of *CSF-1* gene mRNA and protein may diminish macrophages stimulation determined by CSF-1 *in vivo*. Interestingly, CSF-1 was previously shown to induce VEGF production [46], one of the main angiogenic factors for ATC [47]. Indeed, the decrease of *CSF-1* expression caused by the combination of vinorelbine and lenvatinib may reduce the stimulus to the angiogenic process and thus the microvessel density, as seen in our *in vivo* experiments. Furthermore, lenvatinib is also a VEGFR-2 tyrosine kinase inhibitor and by such activity may contribute to the inhibition of angiogenesis and to the anti-tumor effects seen in our *in vivo* experiments in nude mice by the combination treatments.

We also studies signalling pathways that have been described to regulate the expression of ABC transporters. In particular, activated Akt regulates downstream effectors, leading to an increased expression of the target genes, including ABC transporter genes [48]. While VEGFR-2 expression was not detectable in our ATC cells, the combination lenvatinib and vinorelbine induced a decrease in the phosphorylation of Akt, glycogen synthase kinase (GSK)-3α/β, proline-rich Akt substrate of 40 kDa (PRAS40) and Src. Zhong and colleagues [49] have described the importance of Akt/GSK3β pathway activation in ATC and its association to increased invasive and migratory abilities of cancer cells. Small molecules such as apatinib induced apoptosis and blocking cell cycle progression in CAL-62 and BHT-101 ATC cells through the decrease of the level of p-Akt and p-GSK3β [50]. Another example of dose-dependent reductions in the phosphorylation state of multiple proteins downstream of Akt, including GSK3β and PRAS40, was obtained with a pan-Akt kinase inhibitor GSK690693, that significantly inhibited proliferation and induced apoptosis in various types of tumor cells [51]. The PRAS40 is a substrate of Akt and a component of the mammalian target of rapamycin complex 1 (mTORC1). PRAS40 phosphorylation is associated with tumor progression by deregulating cellular proliferation and apoptosis [52]. Recently, the combination of erlotinib and crizotinib showed a strong synergism in non-small cell lung cancer cells, associated to G2/M phase arrest, increased apoptosis, with a decrease of the phosphorylation of downstream targets of PI3K/Akt/mTOR pathways, with the largest decrement observed for PRAS40 Thr246 [53]. It

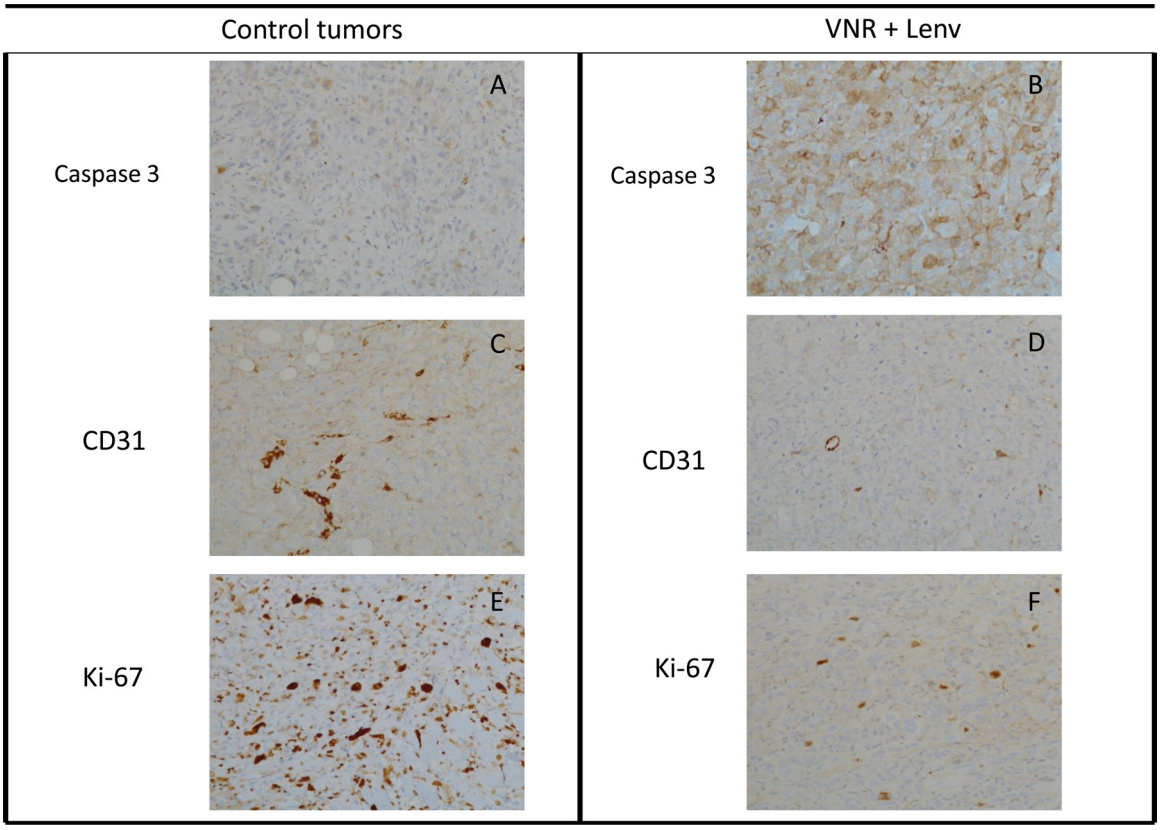


Fig. 10. Representative microscopic images of immunohistochemical staining of subcutaneous tumor samples treated with saline (control), vinorelbine (VNR) and levatinib (Lenv) at the end of a 25-day treatment, including active caspase 3 (**A** and **B**, respectively), CD31 (**C** and **D**, respectively), and Ki-67 (**E** and **F**, respectively). Magnification, X20.

has been also reported that Src is overexpressed in thyroid cancer. Chan and co-workers [54] reported that a Src inhibitor (*i.e.* dasatinib) decreased the growth of ATC cells, and induced the cell-cycle arrest and apoptosis of thyroid cancer cells. These pharmacological effects could be enhanced by the combination with drugs inhibiting the MAPK pathway [55] and with BRAFV600E inhibitors such as PLX4720 [56].

The synergistic antitumor effect of the concomitant combination schedule of lenvatinib and vinorelbine was demonstrated also in the 8505C subcutaneous ATC model. All therapies were well tolerated in the *in vivo* ATC model. Interestingly, during the treatment period, the concomitant combination led to a significant reduction in tumor volume and induced a significant decrease of Ki-67 immunostaining and mitotic index of tumour cells as well as the vascularization of the tumour masses. The decrease of the microvascular count in ATC tissues has been previously described after the treatment with multitarget small molecules such as CLM3 [57], sunitinib [23] and apatinib [50], suggesting the important role in the inhibition of angiogenesis of these drugs. In our experiments, lenvatinib confirmed this *in vivo* effect that was also maintained and increased by the combination with vinorelbine. Furthermore, an increase of active caspase-3, a crucial proapoptotic factor, was also observed in tumors that were treated with the combination, as previously seen also in the combination treatment of lenvatinib and paclitaxel [33].

In conclusion, in this preclinical investigation we report, for the first time, the activity of vinorelbine alone and the synergistic efficacy of the concomitant combination of vinorelbine and lenvatinib in ATC cells *in vitro* and *in vivo*. The observed anti-tumor effect results *via* multiple mechanisms: the modulation of ABCB1 transporter, reduced CSF-1 expression, increased intracellular levels of lenvatinib, as well as the inhibition of the Akt/GSK3β/PRAS40 pathway and the reduction in the phosphorylation state of Src. These results suggest it would be of interest to adapt this combination schedule into future clinical trials or for the compassionate use in single patients with advanced ATC, since both drugs are already registered and in use for other tumour types.

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Authors' contributions

GB and TDD designed the study. TDD, PO, GJC and DG conducted the experiments. GFo and GA performed and analyzed the immunohistochemistry experiments. PA, CK, and LM performed analysis in mice. TDD, PO, DG, GFr and GB analyzed the data. GB, PO, GFr and DG wrote the manuscript. All authors read and approve the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests

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Appendix A. Supplementary data

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