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Original Article

Synergistic activity of linifanib and irinotecan increases the survival of mice bearing orthotopically implanted human anaplastic thyroid cancer

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Abstract: Anaplastic thyroid cancer (ATC) is the most aggressive form of thyroid cancer, and novel combined therapies are urgently needed to prolong patient survival. No data are currently available on the preclinical activity of the combination of linifanib, a CSF-1R inhibitor, and irinotecan in ATC. The aim of the study was to evaluate the in vitro and in vivo activity of linifanib plus irinotecan. Proliferation and apoptosis assays were performed on 8305C and 8505C human ATC cell lines exposed to SN-38, the active metabolite of irinotecan, linifanib alone, and their concomitant combination. Synergism was evaluated by the combination index method. Quantification of pospho-CSF-1R levels was performed by ELISA. In vivo ATC orthotopic xenografts were treated with the single drugs, or their combination, to evaluate their impact on survival. Histology and immunohistochemistry were performed on ATC tissue samples. Both SN-38 and linifanib inhibited in vitro the proliferation of 8305C and 8505C cells in a concentration-dependent manner, whereas their concomitant treatment revealed a strong synergism in the ATC cells. A significant pro-apoptotic activity was found in both ATC cell lines treated with linifanib alone and in combination with SN-38. Moreover, linifanib significantly decreased the levels of phospho-CSF-1R after 24 h and 72 h in both 8505C and 8305C cells, and this was also observed with the concomitant administration of SN-38. In vivo, the combination of linifanib and irinotecan produced a greater survival result than either monotherapy, and resulted in a significant higher median survival. In some of the mice the combination produced a complete response with a macroscopic disappearance of the disease, as confirmed by histology. In conclusion, the synergistic ATC antitumor activity of linifanib/irinotecan combination significantly increased the survival of ATC affected mice and induced some complete responses, suggesting a potential role of this schedule in ATC patient's treatment.

Keywords: Anaplastic thyroid cancer, linifanib, SN-38, irinotecan, synergism, orthotopic xenograft, CSF-1R, histology

Introduction

Among the malignancies of the thyroid, anaplastic thyroid cancer (ATC) is the most aggressive type, and novel combination therapies are urgently needed to prolong patient survival and improve clinical outcomes [1]. Currently, surgery, radiotherapy and chemotherapy are not effective in improving overall survival and quality of life of ATC patients [2, 3]. The molecular signalling perturbations discovered to be linked to the development of ATC have led to the development of new preclinical treatment options, such as the combination of some targeted therapies (e.g. the BRAF inhibitor dabrafenib plus the MEK inhibitor trametinib) [4] or the combination of chemotherapy with tyrosine kinase inhibitors (TKIs) [5-7] or inhibitors of histone methyltransferases [8].

Linifanib (ABT-869) is a multi-targeting TKI [9] that showed potent inhibition of colony stimulating factor-1 receptor (CSF-1R) in both enzyme and in cell-based assays [10]. CSF-1R RNA was found to be overexpressed in ATC [11], and its endogenous ligand CSF-1 [12] is fundamen-

tal for the differentiation and the survival of tumor associated macrophages, which largely infiltrate ATC and are associated with a low survival [13]. Recently, we reported that a decrease in the mRNA and protein expression of CSF-1 might contribute to the antitumor activity of drugs such as campthotecins and microtubule inhibitors in combination with TKIs in ATC [6, 7, 14]. A preclinical study noted a potent anti-proliferative and pro-apoptotic effect of linifanib against cancer cells whose proliferation is sustained by mutated and constitutionally active angiogenic TK receptors, like FLT-3 [15]. Furthermore, combinations of linifanib with cytotoxic agents (e.g. platinum derivates and fluoropyrimidines) have shown promising preclinical anti-tumor activity *in vitro* as well as in solid tumor models *in vivo* [16].

To our knowledge, no data are currently available on the preclinical activity of linifanib in ATC and on its combination with irinotecan in different type of tumors, including ATC. The aim of this study was 1) to evaluate the activity of linifanib on ATC, 2) to test the combined schedule of linifanib plus irinotecan, 3) to investigate the *in vivo* effects of the combination on the survival of mice affected by an orthotopic human ATC.

Materials and methods

In vitro experiments

The human ATC cell line 8305C was obtained from DSMZ (Braunschweig, Germany), whereas the human ATC cell line 8505C was from ECACC-94090184. Both cell lines were maintained in RPMI 1640 medium (Sigma Aldrich srl, Milan, Italy) supplemented with 15% FBS and L-glutamine (2 mM; Sigma Aldrich). Irinotecan (a topoisomerase I inhibitor used *in vivo*) [17], SN-38 (the active metabolite of irinotecan used *in vitro*), and linifanib were purchased from Selleckchem (DBA Italia, Milan, Italy), and dissolved in a stock solution of 10 mM in 100% dimethylsulfoxide.

In vitro chemosensitivity was tested on 8305C and 8505C cell lines, as previously described [7]. ATC cells (10^4 cells/well) were treated for 72 h with SN-38 (0.001-100,000 nM) and linifanib (0.01-100 μM) or with their vehicle as control. The concentrations of drug that reduced cell proliferation by 50% (IC_{50}) *vs.* controls were calculated by a nonlinear regression fit of the mean values. The combination of SN-38 with linifanib was explored on the ATC cell lines at the fixed molar concentration ratio of 1:100 (8305C) and 1:10 (8505C). Synergism was calculated on the multiple drug-effect equation and quantified by the combination index (CI) and dose reduction index (DRI) method [7]. The CI and DRI indexes were calculated with the CalcuSyn v.2.0 software (Biosoft, Cambridge, UK).

To quantify the extent of apoptosis, 3×10^5 ATC cells were treated for 72 h with SN-38, linifanib and their concomitant combination at the experimental IC_{50} or with vehicle alone. At the end of the experiment, samples were analyzed using the Cell Death Detection ELISA Plus Kit (Roche, Basel, Switzerland) as per the manufacturer's instructions. The optical density was measured using a Multiskan Spectrum microplate reader (Thermo Labsystems, Milan, Italy) set to a wavelength of 405 nm (with a wavelength of 490-nm correction). All experiments were repeated three times with at least three replicates per sample.

To investigate the phosphorylation level of CSF-1R, ATC cells were treated with SN-38 and linifanib alone and in simultaneous combination at a concentration corresponding to their experimental IC_{50}s or with vehicle alone for 24 h. Tumor cell lysates were assayed as per the manufacturer's instruction with the human phospho-M-CSFR ELISA (DuoSet IC, R&D Systems, Minneapolis, MN, USA). The optical density was determined using the microplate reader set to 450 nm. The phospho-CSF-1R results were expressed as percentage of phospho-CSF-1R of controls because each well was loaded with the same amount of total protein.

In vivo experiments

Six-week-old CD *nu/nu* male mice (Envigo, Milan, Italy) were first anesthetized with i.p. injection of sodium pentobarbital (50 mg/kg). To perform the orthotopic ATC xenograft a previously published procedure was followed with minor changes [18]. Briefly, the skin of the neck was cut, reflecting the salivary glands superiorly; the left thyroid lobe was exposed to inject 5×10^5 8505C cells in 10 μl of serum-free RPMI medium with a Hamilton syringe (Fisher Scientific, USA) attached to a 27 gauge needle. Once the surgery was terminated, the

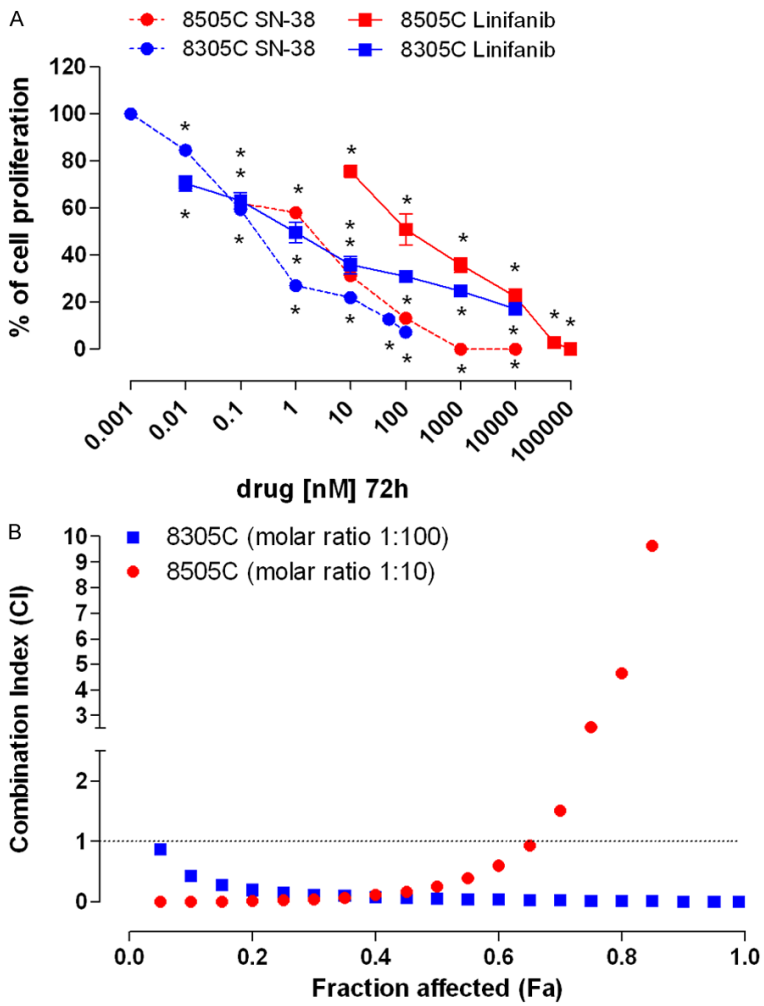

Figure 1. *In vitro* 72 h antiproliferative effects. (A) of SN-38 (*round symbols*) and linifanib (*square symbols*) on proliferation of 8305C (*blue symbols*) and 8505C (*red symbols*) ATC cell lines. *Symbols and bars*, mean values ± S.E., respectively. **P*<0.05 *vs.* vehicle-treated controls. Combination index (CI)-fraction affected (Fa) plot (B) of 72 h-concomitant combination of SN-38 and linifanib in 8305C (*square blue symbols*) and 8505C (*round red symbols*) ATC cells. CI<1, CI=1 (*dotted line*) and CI>1 indicate synergism, additive effect and antagonism, respectively.

cut was sutured with three interrupted 3-0 nylon stitches. Therapy (n=5 mice/group) started 13 days after cell injection. The presence of the tumors was confirmed by ultrasound (Vevo LAZR2100, Fujifilm VisualSonics Inc., Toronto, ON, Canada). Mice were randomly assigned to the different groups just before the beginning of the treatment. Control mice were treated with vehicle alone. Irinotecan, linifanib or their concomitant combination were administered as follows: 1) linifanib 10 mg/kg p.o. daily for 33 days; 2) irinotecan 100 mg/kg i.p. weekly for 4 weeks; 3) combination of linifanib and irinotecan. Mice were euthanized using an anesthetic overdose when the weight loss exceeded 20% normal body mass or the experimentally-induced disease determined a life-threatening condition. The study was approved by the Academic Organization Responsible for Animal Welfare of the University of Pisa, in accordance with the Italian law D.lgs. 26/2014, and by the Italian Ministry of Health (Authorization No. 264/2016-PR).

To perform histology and immunohistochemistry (IHC), the experimental procedures previously described by Di Desidero et al. [19] were followed. Briefly, tumor tissue samples from all the different treatment groups were fixed in 10% neutral-buffered formalin for 12-24 h and embedded in paraffin. Sections of the tumor (5 μm thick) were stained with hematoxylin and eosin. Sections were evaluated for the mitotic index (MI) by counting mitotic figures under microscopy. Immunostainings were performed by a Benchmark immunostainer (Ventana, Tucson, AZ, USA) using the avidin-biotin-peroxidase complex (ABC) method and counterstained with hematoxylin. Negative controls were carried out by omitting the primary antibodies. The microvascular count was determined using anti-CD31 polyclonal antibody (clone JC70; Ventana Medical System); Ki-67 expression was detected using a rabbit monoclonal primary antibody (clone 30-9; Ventana Medical System); the active caspase-3 detection was carried out using a rabbit polyclonal antibody (ab2302; Abcam, Cambridge, UK).

Statistical analysis

The results (mean ± SEM) of all the experiments were subjected to analysis of variance between groups (ANOVA), followed by the Student-Newman-Keuls test. The level of sig-

Table 1. Dose Reduction Index (DRI) values for the drugs combination at 30%, 50%, 70% and 90% levels of inhibition of 8305C and 8505C cell proliferation after 72 h of SN-38 plus linifanib (LIN) concomitant combination treatment

Drug combination	DRI values							
	30%		50%		70%		90%	
	SN-38	LIN	SN-38	LIN	SN-38	LIN	SN-38	LIN
8305C cell								
SN-38+LIN (1:100)	371.6	8.6	554.4	19.3	827.1	43.5	1564.3	157.7
8505C cell								
SN-38+LIN (1:10)	24.9	273.7	4.2	54.1	0.7	10.7	0.04	0.81

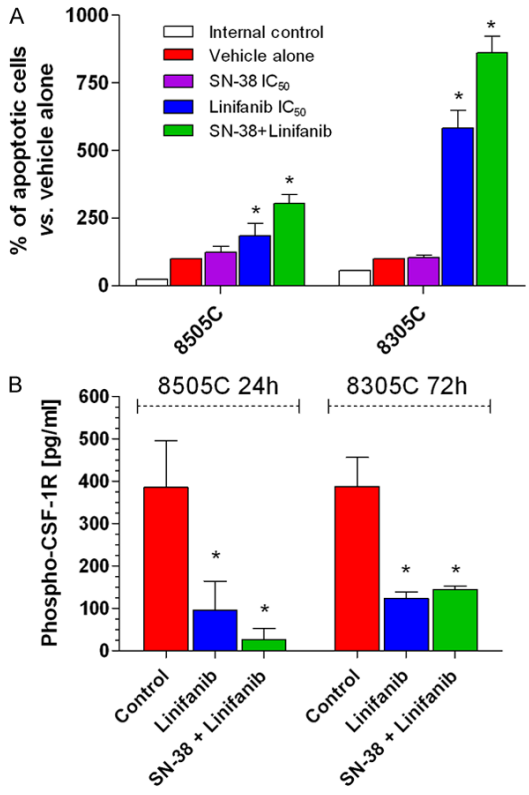

Figure 2. Pro-apoptotic activity. (A) in 8305C and 8505C cell lines treated with SN-38 alone, linifanib alone and in combination with SN-38 at the experimental IC_{50} when compared to the control cells. *Columns and bars*, mean values ± S.E., respectively. *P<0.05 *vs.* vehicle-treated controls. CSF-1R phosphorylation levels (B) in 8505C and in 8305C ATC cells exposed to linifanib, and to the combination of linifanib and SN-38 or with vehicle alone for 24 h and 72 h. Data are expressed as percentage of vehicle-treated cells. *Columns and bars*, mean values ± S.E., respectively. *P<0.05 vs. vehicle-treated controls.

nificance was set at P<0.05. Statistical analyses and survival curves were performed using the GraphPad Prism software package version 5.0 (GraphPad Software, Inc, San Diego, CA).

Results

In vitro experiments

Both SN-38 and linifanib inhibited *in vitro* the cell proliferation of 8305C and 8505C cell lines in a concentration-dependent manner (**Figure 1A**) after 72 h of exposure. SN-38 caused a strong antiproliferative effect on 8305C and 8505C cell lines, as demonstrated by the calculated IC_{50}s of 186.3±14 nM and 11.2±5.9 nM, respectively. Linifanib inhibited the 8305C and 8505C cell proliferation with an IC_{50} of 0.7±0.7 nM and 123.7±44.9 nM, respectively.

Concomitant exposure of linifanib and SN-38 for 72 h caused a strong synergism for all the fractions of 8305C affected cells with a CI<1 (**Figure 1B**) and DRI>1 (**Table 1**), and it showed a marked synergism corresponding to CI<1 for fractions of affected 8505C cells ranging from 0 to 60%, whereas we observed antagonism for higher percentages (**Figure 1B**).

A significant level of apoptosis was found in both ATC cell lines treated with linifanib alone, and in combination with SN-38 at the experimental IC_{50}s when compared to the vehicle-treated control cells (**Figure 2A**). Linifanib significantly decreased the levels of phospho-CSF1R after 24 h and 72 h in both 8505C and 8305C cells (**Figure 2B**). This inhibitory effect was also observed with the concomitant administration of SN-38.

In vivo experiments

To evaluate the *in vivo* effects of the monotherapies and of the simultaneous combination of irinotecan plus linifanib treatments, we set up an orthotopic model of ATC. The presence of the implanted tumors in the thyroid

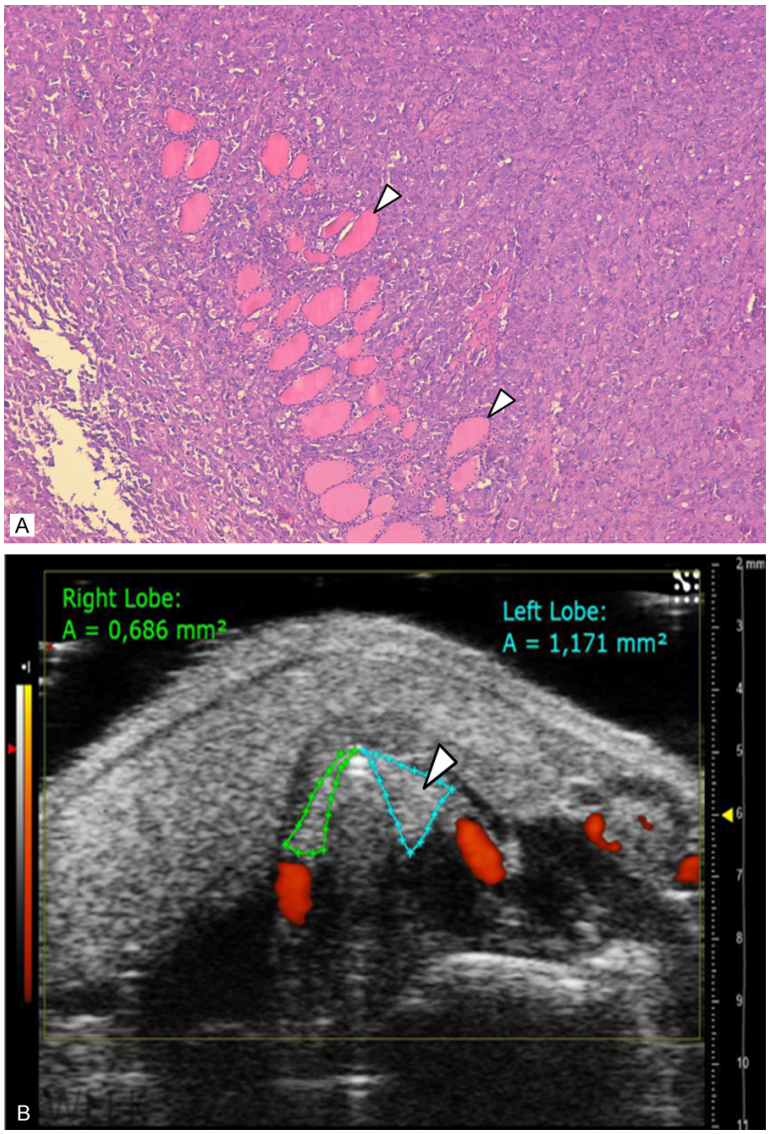

Figure 3. Representative ultrasound image. (A) of the left thyroid lobe with the presence of the tumor (*arrowhead*) after 21 days from the ATC cell injection. Haematoxylin/eosin representative microscopic image (B) of the orthotopic human ATC xenograft with entrapped non-neoplastic thyroid follicles (*arrowheads*) containing colloid.

gland was confirmed by ultrasound imaging during the experiment (**Figure 3A**), and confirmed by histology (**Figure 3B**) at the end of the experimental period. A plot of the resulting survival curve (**Figure 4A**) allowed for a comparison of the overall survival of mice treated with control (vehicle alone), linifanib, or with irinotecan, with that observed with the combination therapy. Interestingly, single-agent linifanib did not show any survival advantage (median survival 52 days) *vs*. control group (55 days). In contrast, the single-agent irinotecan showed a significant increase of overall survival (80 days, $P<0.05$). The combination of linifanib and irinotecan produced a greater survival result than either monotherapy, and resulted in a significant higher median survival of 100 days ($P<0.05$). Moreover, in two (out of five) mice there was a complete response with a macroscopic disappearance of the disease after 120 days of survival (**Figure 4A**), as confirmed by histology. The toxicity profile was minimal for all treatment groups, with a small gain of mouse weight throughout the course of the experiment (**Figure 4B**).

Histological analysis of the tumor tissues, explanted when the mice were euthanized, showed some differences between control treated mice and those that received the combination therapy. Thus, the treatment of irinitecan and linifanib resulted in a lower levels of Ki-67 immunostaining (mean 10±1.3% *vs*. 30±3.4% of control) and a higher the apoptotic index (mean 54±4.2% *vs*. 30±2.3% of control), and a significant decrease in the intratumoral microvessel count (mean 2± 0.3 *vs*. 5±0.8 of control).

Discussion

ATC is an undifferentiated tumor, and is the most aggressive form of thyroid cancer [20, 21]. Although it represents only 1-2% of all thyroid tumors, it accounts for up to 50% of all thyroid cancer-related mortalities. ATC is most common in people over the age of 60, and has a mean survival of only six months after diagnosis [22]. ATC patients present with locally advanced disease or metastasis [23]. In the present study, we report for the first time, the synergistic efficacy of the combined treatment of linifanib and irinotecan on ATC cells

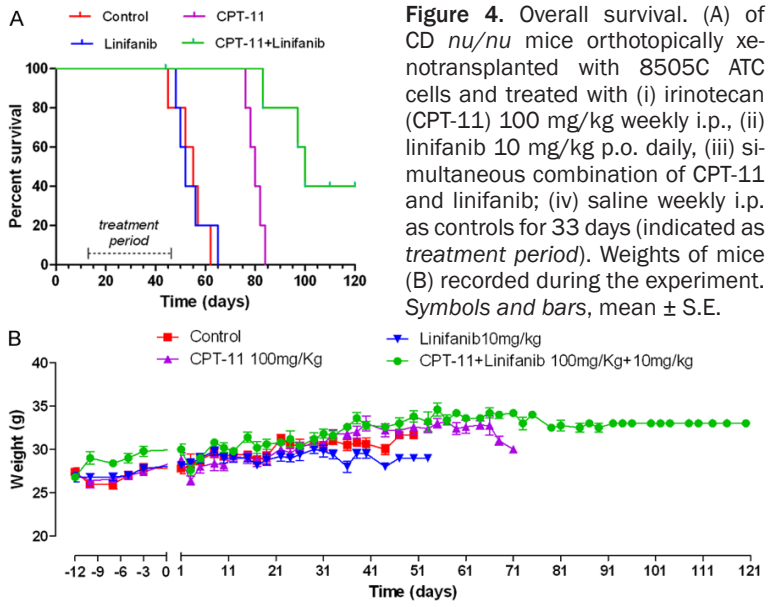

Figure 4. Overall survival. (A) of CD *nu/nu* mice orthotopically xenotransplanted with 8505C ATC cells and treated with (i) irinotecan (CPT-11) 100 mg/kg weekly i.p., (ii) linifanib 10 mg/kg p.o. daily, (iii) simultaneous combination of CPT-11 and linifanib; (iv) saline weekly i.p. as controls for 33 days (indicated as *treatment period*). Weights of mice (B) recorded during the experiment. *Symbols and bars*, mean ± S.E.

and, above all, a significant increase in survival of mice bearing an orthotopically implanted human ATC tumor, with a very favourable toxicity profile. That included a subgroup of mice that showed complete responses to the combination. These findings could open the way to the clinical use of linifanib and irinotecan in the treatment of patients with ATC.

Linifanib has shown a concentration-dependent anti-proliferative and pro-apoptotic activity *in vitro* in our ATC cells expressing CSF-1R, inhibiting its phosphorylation. Although this effect has never been tested before in ATC cells, a similar antiproliferative and pro-apoptotic activity of the drug has been previously described for other cancer cell lines such as epidermoid carcinoma and acute myeloid leukemia cells [9, 24]. However, this direct *in vitro* activity on cancer cells, and the inhibition of the CSF-1-induced angiogenesis by tumor associated macrophages [25] was not sufficiently effective alone to determine an increased survival of ATC affected mice if compared to the vehicle-treated mice of the control group.

A significant increase of activity was demonstrated with the combination of the camptothecin irinotecan. Indeed, irinotecan has already shown an antitumor/therapeutic effect in ATC cell lines and ATC subcutaneous and orthotopic models alone and in combination with vatalanib [26], sunitinib [6] or cetuximab (an anti-EGFR) [27]. The high synergistic *in vitro* activity of linifanib/SN-38 concomitant combination can be explained by different mechanisms previously reported such as: 1) the increased intracellular concentration of the active chemotherapeutic drug (e.g. SN-38) due to the decreased expression of ATP-binding cassette transporters in treated-ATC cells, as shown in other previous combined treatment of camptothecins and TKIs [6, 7], 2) the decreased autocrine effect on CSF-1R due to a lower expression of CSF-1 caused by SN-38 [6], enhanced by the direct inhibition of CSF-1R phosphorylation by linifanib, and 3) the increased apoptotic rate produced by the combination treatment.

In this study we evaluated a combination therapy on an orthotopic ATC model, to the best of our knowledge an in vivo model that better recapitulates features of human tumors than (for example) more simplistic subcutaneous xenograft models. We report an increase in survival in ATC bearing mice treated with the irinotecan/linifanib combination schedule, an anti-tumor effect that may be ascribed at least three mechanisms: 1) the synergic and proapoptotic direct activity on cancer cells, as also suggested by the increased active caspase 3 and the decreased Ki-67 immunostaining; 2) the decreased levels of CSFR-1 phosphorylation in cancer cells; 3) the inhibition of the CSF-1 [25] and VEGFRs [28] mediated angiogenic process by linifanib as confirmed by the a reduced microvessel count in the ATC tumor samples we evaluated.

Our data with linifanib argue against a possible role for such a drug as a monotherapy as an effective strategy for the treatment of ATC, consistent with previously reported data for other TKIs such as sorafenib [29]. In contrast, the combination of linifanib with irinotecan shows promising antitumor activity in mice and ought to be considered for evaluation in the clinic for the treatment of ATC, particularly since such thyroid tumors are currently orphan of a gold standard treatment [30].

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Disclosure of conflict of interest

None.

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