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Original Article

Synergistic efficacy of irinotecan and sunitinib combination in preclinical models of anaplastic thyroid cancer

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ABSTRACT

The identification of new therapeutic strategies is urgently needed for the management of patients affected by anaplastic thyroid cancer (ATC) due to their short survival and poor prognosis. Aim of the study was to determine the activity of the combination irinotecan/sunitinib on ATC cell growth *in vitro* and the antitumor effects *in vivo*. Proliferation assays were performed for 72 h on ATC cell lines exposed to the combination of SN-38, the active metabolite of irinotecan, and sunitinib. The simultaneous combination of sunitinib and SN-38, quantified by the combination index, determined a high synergism on ATC cells, increasing the intracellular concentrations of SN-38. Moreover, the synergistic combination greatly decreases the gene expression and the protein levels of vascular endothelial growth factor, colony stimulating factor 1 and ATP-binding cassette transporter G2 in ATC cells. A significant *in vivo* antitumor effect was observed in ATC xenografts with the simultaneous combination of irinotecan and sunitinib if compared to monotherapy. The simultaneous combination of irinotecan and sunitinib, *in vitro* and *in vivo* demonstrated a significant, synergistic ATC antitumor activity, suggesting a possible and rapid translation of this schedule into the clinics.

Introduction

Anaplastic thyroid cancer (ATC) is among the most aggressive malignancies with extremely short survival, poor prognosis and a disease-specific mortality approaching 100% [1]. ATC accounts for approximately 5%–15% of primary malignant thyroid tumors that have been described to be resistant to standard radiotherapy and chemotherapy [2]. At present, surgery, radiotherapy and chemotherapy are not helpful in improving the survival time and the life quality of such patients [1], and the poor prognosis is attributed to its unlimited growth and invasive migration. Therefore, identifying new possible therapeutic strategies is critical for ATC management. In dated scientific literature, cytotoxic drugs have demonstrated limited or no activity in ATC when administered alone [1,3], whereas, the improvement in the recognition and comprehension of genetic and molecular alterations underlying the development of thyroid cancers [3], led to the development of new treatment options such as tyrosine kinase inhibitors (TKIs). While vandetanib, cabozantinib, sorafenib and lenvatinib have reached a phase III clinical trial with favorable results in medullary thyroid carcinoma and differentiated thyroid carcinoma, it is still unclear if ATC patients may benefit from this therapeutic strategy [3]. Despite several preclinical studies on TKIs, such as sunitinib [4] – approved for the treatment of patients with advanced renal cell carcinoma or advanced gastrointestinal stromal tumors after imatinib therapy – have shown some *in vivo* antitumor activity in ATC [5–8], TKIs monotherapy could be not effective *in vitro*, as in the case of pazopanib [9] or imatinib [10]. Indeed, Bible and colleagues reported that although some pazopanib-treated ATC patients in a phase 2 trial incurred transient disease regression, there were no RECIST responses [11]. One example of an effective preclinical drug combination in a murine orthotopic ATC model was represented by the association of irinotecan (a standard therapeutic choice for

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colorectal cancer) plus cetuximab [12]. To our knowledge, no preclinical data are available on the combination between irinotecan and sunitinib in ATC or other cancer types with the only exception of experiments performed in PC12 tumor-bearing mice, showing an enhanced *in vivo* activity of the combined schedule [13].

The aim of the present study is 1) to investigate whether different combined schedules of sunitinib and irinotecan based chemotherapy may synergize and 2) to reveal the underlying mechanism of these effects, challenging the dogma of untreatable ATC with chemotherapeutic drugs and tyrosine kinase inhibitors.

Materials and methods

Materials and drugs

Cell culture media RPMI, M-199 (used to isolate primary ATC cells) and DMEM, supplements and all other chemicals not listed in this section were obtained from Sigma Aldrich SRL (Milan, Italy). Quantitative real-time PCR reagents were from Applied Biosystems (Foster City, CA, USA). SN-38, the active metabolite of irinotecan, and sunitinib, were purchased from Selleckchem (DBA Italia, Milan, Italy), and dissolved in a stock solution of 10 mM in 100% dimethylsulfoxide (DMSO) for *in vitro* studies. DMSO concentration in the control's media was the same used to make up the highest concentration of sunitinib and SN-38 in growth media for the same experiment.

Cell lines

Primary ATC cells were obtained as previously described [14–16], from thyroid biopsy at the moment of first surgery. The diagnosis was established on commonly accepted clinical, laboratory, and histological criteria [17]. Cells were maintained in DMEM supplemented with 10% FCS.

The human ATC cell line 8305C (*BRAF* V600E mutated) – established from undifferentiated thyroid carcinomas of a 67 year-old-female patient – was from DSMZ (Braunschweig, Germany, DSMZ no.: ACC 133) [18], whereas the human ATC cell line FB3 (*HRas Q61R* mutated) was obtained from Prof. Fulvio Basolo of the University of Pisa, Pisa, Italy [6]. Both cell lines were maintained in RPMI 1640 medium supplemented with 15% FBS and L-glutamine (2 mM). The cultures were free of mycoplasma species and cells were used for tests at the fourth passage.

Antiproliferative assay

In vitro chemosensitivity was tested on 8305C, FB3 cell lines, as previously described [6,19]. 8305C and FB3 cells were treated for 72 h (1×10^4 cells/well of cancer cells in 1 ml of medium) with sunitinib (0.01–100 μM) or SN-38 (0.01–100 μM) or with their vehicle.

In vitro assessment of synergism between sunitinib and SN-38 on ATC cells

The combination of sunitinib with SN-38 was explored on 8305C and FB3 cells with three different treatment schedules at a fixed molar concentration ratio (1:1), as follows: (A) *simultaneous exposure*: sunitinib plus SN-38 for 72 h; (B) *sequential exposure*: sunitinib alone for 24 h, sunitinib plus SN-38 for 24–72 h and SN-38 alone for 72–96 h; (C) *reverse exposure*: SN-38 alone for 24 h, SN-38 plus sunitinib for 24–72 h and sunitinib alone for 72–96 h. Therefore, the total exposure of cells to each drug was 72 h. To evaluate the level of interaction (synergistic, additive or antagonist) between SN-38 and sunitinib the Combination Index (CI) and Dose Reduction Index (DRI) method was followed [20], as previously described by our group [21,22].

Apoptosis measurement in primary ATC cells and FB3 cells

Primary ATC cells were incubated for 24 h with sunitinib (5, 10, 25 μM) in a humidified atmosphere (37 °C, 5% CO_2). At the end of the experiment cells were stained with Hoechst 33342 as previously described [23]. The apoptosis index (ratio between apoptotic and total cells) × 100 was calculated. The Annexin V binding assay was used to further confirm the results of the cell apoptosis test as previously described [23]. Moreover, FB3 cells were treated for 72 h with SN-38 and sunitinib at a concentration corresponding to the experimental IC_{50} of cell proliferation, alone and in simultaneous combination. At the end of the experiment, the apoptosis analysis was performed using the Cell Death Detection ELISA Plus kit (Roche Diagnostics, Milan, Italy).

HPLC analysis of SN-38 intracellular concentrations in ATC cells

The quantitative analysis of irinotecan's main metabolite SN-38 in cells was performed as previously described [21,24]. ATC cells, 8305C and FB3, were treated with vehicle alone, SN-38 (1 μM), sunitinib (1 μM), or a combination of the two (SN-38 1 μM + sunitinib 1 μM) for 2 h [25]. The analysis was performed using system LC Module I Plus (Waters, Milan, Italy) [21,24,26].

Modulation of ABCG2, CSF-1 and VEGF gene expression

To evaluate the expression of the genes encoding human ABCG2, CSF-1, and VEGF proteins, 8305C and FB3 cells were grown in their respective media and treated with SN-38 and sunitinib or in combination in three different treatment schedules (*simultaneous, sequential and reverse exposure*) at a concentration corresponding to the experimental IC_{50} of cell proliferation or with vehicle alone for 72 h. Quantitative RT-PCR was performed with the Applied Biosystems 7900HT sequence detection system, as previously described [6,21]. Validated primers were purchased from Applied Biosystems (*ABCG2*, Assay ID Hs01053796_m1, *CSF*-1 Hs00174164_m1, *VEGF* Hs00170236_m1).

Quantification of ABCG2 and CSF-1 protein levels in ATC cells

To investigate the modulation of protein of ABCG2 and CSF-1 by the simultaneous combination of sunitinib and SN-38, 8305C and FB3 cells were treated for 72 h with sunitinib and SN-38 at the above mentioned concentrations or with vehicle alone. Tumor cell lysates were assayed as per the manufacturer's instruction with the human ABCG2 and CSF-1 ELISA kit (Mybiosource, #MBS762174 – Sigma-Aldrich, #RAB0098-1KT). The optical density was determined using a Multiskan Spectrum microplate reader (Milan, Italy) set to 450 nm. The results were expressed as ng of ABCG2 and CSF-1 per mg of total protein.

Animals and treatments

Six-week-old CD *nu/nu* male mice, supplied by Envigo (Milan, Italy), were housed in microisolator cages on vented racks and manipulated using aseptic techniques. Housing and all procedures involving animals were performed according to the protocol approved by the Academic Organization Responsible for Animal Welfare (OPBA, Organismo Preposto per il Benessere Animale) at the University of Pisa, in accordance with the EU Directive 2010/63/EU for animal experiments and the Italian law D.lgs. 26/2014, and by the Italian Ministry of Health (authorization number 613/2015-PR).

On day 0, $2 \times 10^6 \pm 5\%$ viable 8305C cells/mouse were inoculated subcutaneously and tumor volume were measured, as previously described [6,21]. Mice were treated with irinotecan (100 mg/kg/wk) and sunitinib (25 mg/kg/every two days) alone and in combination in three different schedules treatments – simultaneous and sequentials (i.e. irinotecan for two weeks followed by sunitinib for two weeks, or the reverse schedule). At the end of the experiment, mice were sacrificed by an anaesthetic overdose and tumors were excised and measured.

Immunohistochemistry

Briefly, tumor tissue samples from all the different treatment groups were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin for histology and IHC. Sections (5-μM-thick) were incubated with anti-PECAM-1 antibody (M-20) (1:200, #sc1506, Santa Cruz Biotechnology) and anti-cleaved Caspase-3 (Asp175) (1:300, #sc7272, Santa Cruz Biotechnology) at 4 °C overnight. Negative controls were carried out by omitting the primary antibodies. Immunostaining was accomplished using a Benchmark immunostainer (Ventana, Tuscon, AZ). Immunoreaction was displayed using the avidin–biotin–peroxidase complex (ABC) method. Peroxidase activity was visualized with diaminobenzidine. Counterstaining was performed with haematoxylin. Quantification of caspase 3 positive cells and CD31 was performed as previously described [21,27].

Analysis of data

The analysis by ANOVA, followed by the Student-Newman–Keuls test, was used to assess the statistical differences of data *in vitro* and *in vivo*. *P*-values lower than 0.05 were considered significant. Statistical analyses were performed using the GraphPad Prism software package version 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Sunitinib and SN-38 inhibit ATC cells proliferation in vitro

Both sunitinib and SN-38 inhibited *in vitro* the cell proliferation of thyroid cancer 8305C and FB3 cell lines in a concentration-dependent manner. The 72 h sunitinib exposure inhibited the 8305C (Fig. 1A) and FB3 (Fig. 1B) cell proliferation with an IC_{50} of 1.4 μM and 9 μM, respectively. A higher antiproliferative effect of SN-38 on 8305C (Fig. 1C) and FB3 (Fig. 1D) was found as demonstrated by the calculated IC_{50}s (0.1863 μM and 0.252 μM, respectively).