SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

NOME E COGNOME: __Teresa Di Desidero_____

UNIVERSITÀ: ____ Università di Pisa _____

DIPARTIMENTO (in caso di borsa per soggiorno all'estero specificare l'ente presso cui si è svolta la ricerca): _____Medicina Clinica e Sperimentale_____

TUTOR(in caso di borsa per soggiorno all'estero specificare il tutor dell'ente presso cui si è svolta la ricerca): _____Dott. Guido Bocci_____

TIPOLOGIA DI BORSA RICEVUTA: ___MSD-SIF _____

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale):___Finale_____

TITOLO DELLA RELAZIONE: Tyrosine kinase inhibitors as novel candidates for the treatment of thyroid cancer_____

RELAZIONE:

Introduction

Anaplastic thyroid cancer (ATC) is among the most aggressive malignancies with extremely short survival and poor prognosis. ATC accounts for approximately 5% to 15% of primary malignant thyroid tumors that are resistant to surgery, radiotherapy, and chemotherapy [1]. No curative options are available for patients with ATC, and the poor prognosis is attributed to its unlimited growth and invasive migration. Therefore, identifying new therapeutic strategies is critical for ATC management. Classical cytotoxic drugs have demonstrated limited or no activity in ATC when administered alone [2], whereas, the improvement in the recognition and comprehension of genetic and molecular alterations underlying the development of ATC, (e.g. mutational activation of BRAF), led to the development of new treatment options [3] 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 [2]. Despite several preclinical studies on TKIs have shown some *in vivo* antitumor activity in ATC [4-7], monotherapy could be not clinically effective, as in the case of pazopanib [8] or imatinib [9]. Bible KC reported that although some pazopanib-treated ATC patients in a phase 2 trial incurred transient disease regression, there were no RECIST responses [10]. Given the postulate that ATC is resistant to monotherapy with standard chemotherapeutic agents or TKIs, the deep investigation of different combined schedules of targeted therapies and chemotherapy may help to challenge the dogma of untreatable ATC.

Materials and methods

In vitro studies

Materials, drugs and cell lines

Cell culture media RPMI were purchased from Gibco BRL (Paisley, UK), quantitative real-time PCR reagents were from Applied Biosystems (Foster City, CA, USA), supplements and all other chemicals not listed in this section were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SN-38, the active metabolite of irinotecan, and sunitinib were dissolved in a stock solution of 10 mM in 100% dimethylsulfoxide (DMSO) for in vitro studies.

The human undifferentiated thyroid cancer (with papillary component) cell lines 8305C (DSMZ Germany) and FB3 were maintained in 15% FBS RPMI medium suplemented with L-glutamine 2 mM.

Antiproliferative assay

In vitro chemosensitivity was tested on 8305C and FB3 cell lines. Cells were plated in 24-well sterile plastic plates and treated for 72 h ($1x10^4$ 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. At the end of the experiment, cells were harvested with trypsin/EDTA and viable cells counted with a hemocytometer. The data are presented as the percentage of the vehicle-treated cells. The concentration of drug that reduced cell proliferation by 50% (IC50) vs. controls were calculated by nonlinear regression fit of the mean values of the data obtained in triplicate experiments (at least 9 wells for each concentration).

In vitro assessment of synergism between sunitinib and SN-38 on tumor 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, as follows: (A) simultaneous exposure : sunitinib (0.01-100 µM) plus SN-38 (0.01-100 µM) for 72 h; (B) sequential exposure : sunitinib (0.01-100 µM) alone for 24 h, sunitinib (0.01-100 µM) plus SN-38 (0.01-100 µM) for 24-72 h and SN-38 (0.01-100 µM) alone for 72-96 h; (C) reverse exposure : SN-38 (0.01-100 µM) alone for 24 h, SN-38 (0.01-100 µM) plus sunitinib (0.01-100 µM) for 24-72 h and sunitinib (0.01-100 µM) alone for 72-96 h. Therefore, the total exposure of each drug was 72 h. To evaluate the level of interaction (synergistic, additive or antagonist) between SN-38 and sunitinib the Combination Index method was followed [11]. Briefly, synergism or antagonism for sunitinib plus SN-38 was calculated on the basis of a multiple drug-effect equation, and quantitated by the combination index (CI) where CI<1, CI=1, and CI>1 indicates synergism, additive effect, and antagonism, respectively. Based on the classic isobologram for mutually exclusive effects, the CI value was calculated as:

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

As an example, at the 50% inhibition level, $(D_x)_1$ and $(D_x)_2$ are the concentrations of sunitinib and SN-38, respectively that induce a 50% inhibition of cell growth; $(D)_1$ and $(D)_2$ are the concentrations of sunitinib and SN-38 in combination that also inhibits cell growth by 50% (isoeffective as compared with the single drugs alone). The dose-reduction index (DRI) defined the degree of dose reduction that is possible in a combination for a given degree of effect as compared with the concentration of each drug alone:

$(DRI)_1=(Dx)_1/(D)_1$ and $(DRI)_2=(D_x)_2/(D)_2$

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

Modulation of ABCG2, ANG2, C-met, CXCR4, CSF-1, CSF-3, Hif1α and VEGF gene expression

To evaluate the expression of the genes encoding human ABCG2, ANG2, C-met, CXCR4, CSF-1, CSF-3, Hif1α 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 IC50 of cell proliferation or with vehicle alone for 72 h. Briefly, RNA (1 µg) was reverse transcribed using Omniscript RT Kit (Qiagen, Germantown, MD), according to the manufacturer's protocol. The resulting cDNA was diluted (2:3) and then amplified by SYBR green-based real-time RT-PCR (QuantiTect® SYBR Green PCR Kit, QIAGEN) was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with gene-specific primers purchased from Applied Biosystems. The PCR thermal cycling conditions and optimization of primer concentrations were followed as per manufacturer's instructions. Amplifications were normalised to HMBS (Hs_HMBS_1_SG, QuantiTect® Primer Assay Qiagen), and the quantitation of gene expression was performed using the ΔΔCt calculation, where Ct is the threshold cycle; the amount of target, normalised to the endogenous control and relative to the calibrator (vehicle-treated control cells), is given as $2^{-\Delta\Delta Ct}$. All experiments were repeated, independently, three times with at least 9 samples for each concentration.

High-performance liquid chromatography analysis of SN-38 concentrations in ATC cells.

The quantitative analysis of irinotecan's main metabolite SN-38 in cells was performed as previously described [12] with minor modifications. ATC cells 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 hours. At the end of the experiment, the cells were collected and centrifuged. The cells were frozen and thawed three times consecutively. Proteins in the lysate were quantified by colorimetric assay (Bio-Rad Laboratories). Briefly, concentrations of SN-38 were evaluated after extracting with methanol containing 0.1% HCl 10 N. The samples were then centrifuged, and the clear supernatant was evaporated to dryness under nitrogen flow in a thermostated bath at 45°C. The resulting pellet was reconstituted in methanol acidified with 0.1% HCl 10 N and eluted through a µBondapack C18 stationary phase column (300 × 3.9 mm, 10 µm; Waters, Milford, MA) by KH2PO4 0.1 M/acetonitrile (60:40, vol/vol; pH 6.0) containing 3 mM sodium heptanesulfonate. The chromatographic system LC Module I Plus (Waters) was equipped with a Model 474 scanning fluorescence detector with excitation and emission wavelengths set at 375 and 525 nm, respectively [13]. Data analysis was performed using Millennium 2.1 software (Waters). Standard calibration curves were generated by adding SN-38 to 1 ml of PBS, resulting in final concentrations that ranged from 2500 to 0.16 ng/ml. The range of linearity of the high-performance liquid chromatography method was from 2500 to 0.16 ng/ml.

***In vivo* studies**

Animals and treatments

The CD nu/nu male mice, weighing 20 g, were supplied by Charles River (Milan, Italy) and were allowed unrestricted access to sterile food and tap water. Each experiment employed the minimum number of mice needed to obtain statistically meaningful results.
On day 0, $2x10^6$ ± 5% viable 8305C cells/mouse were inoculated subcutaneously. Animal weights were monitored and upon appearance of a subcutaneous mass, tumor dimensions were measured every two days using calipers. Tumor volume (mm3) was defined as follows: [(w1 x w1 x w2) x (π/6)], where w1 and w2 were the smallest and the largest tumor diameter (mm), respectively. The mice were randomized into groups of six animals. Treatment was delivered once the tumor reached ~100mm3. Mice were treated with irinotecan (CPT-11 100 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. CPT-11 for two weeks followed by sunitinib for two weeks, or the reverse schedule), as described above. Mice from the control group were

injected i.p. with saline. The experimental period ended after the last administration of the combination schedules. Mice were sacrificed by an anesthetic overdose, and tumors were excised, measured, and sampled for IHC. Drug efficacy was based on percentage of the average treated tumor volume divided by the average vehicle control tumor volume (% T /C) [28].

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 cancer cell proliferation *in vitro*

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 and FB3 cell proliferation with an IC50 of 1.41±0.69 µM and 9.02±15.6 µM, respectively; (Figure 1 A and B). In contrast, a higher antiproliferative effect of SN-38 on 8305C and FB3 was found as demonstrated by the IC50 (0.1863±0.014 µM and 0.252±0.077 µM, respectively; Figure 1 A and B).

Synergistic effect of sunitinib and SN-38 on cancer cell proliferation

Simultaneous exposure of 8305C and FB3 cells to different concentrations of sunitinib and SN-38 for 72 h showed a strong synergism (CI<1 and DRI>1). Synergism corresponding to CI<1 always yielded a favorable DRI>1 for both drugs. Furthermore, also the sequential and reverse exposure of sunitinib and SN-38 for 72 h were synergistically active on 8305C and FB3 cell proliferation.

Modulation of ABCG2, ANG2, C-met, CXCR4, CSF-1, CSF-3, Hif1α and VEGF gene expression

In order to study the effect of sunitinib and SN-38 in the three different treatment schedules to the variation of the expression of angiogenesis-related genes, the expression of the genes encoding human ABCG2, ANG2, C-met, CXCR4, CSF-1, CSF-3, Hif1α and VEGF proteins was quantified in the 8305C and FB3 cell lines exposed to the IC50 experimental drug concentrations in the three different treatment schedules described above. The simultaneous combination of sunitinib and SN-38 greatly inhibited the gene expression of human ABCG2, ANG2, C-met, CXCR4, CSF-1, CSF-3, Hif1α and VEGF in 8305C and FB3 cells. Of note, the decreased expression of these angiogenesis-related genes was obtained also in the sequential and reverse treatment schedule.

Sunitinib and irinotecan combination exerts its antitumor activity *in vivo*

8305C cells were injected s.c. in CD nu/nu mice. Tumors in control animals showed a exponential enlargement in their dimensions. A significant *in vivo* antitumour effect on 8305C xenografts was observed with the simultaneous combination of irinotecan and sunitinib, resulting in a significant tumour regression after 26 days of treatment. The toxicity profile was favorable for sunitinib and the combination, with no loss of weight throughout the course of the experiment.

Conclusions.

In our study we report, for the first time to our knowledge, the remarkable efficacy of the sunitinib and irinotecan treatment combination in the anaplastic thyroid cancer. The simultaneous combination of irinotecan and sunitinib, *in vitro* and *in vivo* demonstrated a highly significant, synergistic antitumor activity in ATC cells suggesting a possible translation of this schedule into the clinics.

References

[1] Smallridge RC, Marlow LA, Copland JA (2009). Anaplastic thyroid cancer: molecular pathogenesis and emerging therapies Endocr Relat Cancer 16, 17-44. (PMID 18987168);

[2]Alonso-Gordoa T, Diez JJ, Duran M, Grande E (2015). Advances in thyroid cancer treatment: latest evidence and clinical potential Ther Adv Med Oncol 7, 22-38. (PMID 25553081);

[3] Begum S, Rosenbaum E, Henrique R, Cohen Y, Sidransky D, Westra WH (2004). BRAF mutations in anaplastic thyroid carcinoma: implications for tumor origin, diagnosis and treatment Mod Pathol 17, 1359-1363. (PMID 15195111);

[4] Kim S, Yazici YD, Calzada G, Wang ZY, Younes MN, Jasser SA, El-Naggar AK, Myers JN (2007). Sorafenib inhibits the angiogenesis and growth of orthotopic anaplastic thyroid carcinoma xenografts in nude mice Mol Cancer Ther 6, 1785-1792. (PMID 17575107);

[5] Di Desidero T, Fioravanti A, Orlandi P, Canu B, Giannini R, Borrelli N, Man S, Xu P, Fontanini G, Basolo F, et al. (2013). Antiproliferative and proapoptotic activity of sunitinib on endothelial and anaplastic thyroid cancer cells via inhibition of Akt and ERK1/2 phosphorylation and by downregulation of cyclin-D1 J Clin Endocrinol Metab 98, E1465-1473. (PMID 23969186);

[6] Gule MK, Chen Y, Sano D, Frederick MJ, Zhou G, Zhao M, Milas ZL, Galer CE, Henderson YC, Jasser SA, et al. (2011). Targeted therapy of VEGFR2 and EGFR significantly inhibits growth of anaplastic thyroid cancer in an orthotopic murine model Clin Cancer Res 17, 2281-2291. (PMID 21220477);

[7] Zhang L, Gaskins K, Yu Z, Xiong Y, Merino MJ, Kebebew E (2014). An *in vivo* mouse model of metastatic human thyroid cancer Thyroid 24, 695-704. (PMID 24262022);

[8] McLarnon A (2012). Thyroid cancer: Pazopanib alone is not effective against anaplastic thyroid cancer Nat Rev Endocrinol 8, 565. (PMID 22847238);

[9] Dziba JM, Ain KB (2004). Imatinib mesylate (gleevec; STI571) monotherapy is ineffective in suppressing human anaplastic thyroid carcinoma cell growth *in vitro* J Clin Endocrinol Metab 89, 2127-2135. (PMID 15126530);

[10] Bible KC, Suman VJ, Menefee ME, Smallridge RC, Molina JR, Maples WJ, Karlin NJ, Traynor AM, Kumar P, Goh BC, et al. (2012). A multiinstitutional phase 2 trial of pazopanib monotherapy in advanced anaplastic thyroid cancer J Clin Endocrinol Metab 97, 3179-3184. (PMID 22774206);

[11] Chou TC (2010). Drug combination studies and their synergy quantification using the Chou-Talalay method Cancer Res 70, 440-446. (PMID 20068163).

[12] Allegrini G, Falcone A, Fioravanti A, Barletta MT, Orlandi P, Loupakis F, Cerri E,

SOCIETÀ ITALIANA DI FARMACOLOGIA

Masi G, Di Paolo A, Kerbel RS, et al. A pharmacokinetic and pharmacodynamic study on metronomic irinotecan in metastatic colorectal cancer patients. Br J Cancer (2008) 98, 1312–1319.

[13] Canu B, Fioravanti A, Orlandi P, Di Desidero T, Alì G, Fontanini G, Di Paolo A, Del Tacca M, Danesi R, Bocci G. Irinotecan synergistically enhances the antiproliferative and proapoptotic effects of axitinib *in vitro* and improves its anticancer activity *in vivo*. Neoplasia. 2011 Mar;13(3):217-29.

The data of the present study are currently under submission to a scientific journal. This report shows only an extended abstract of the entire study.

Dott. Guido Bocci

Dott.ssa Teresa Di Desidero