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Original Article

Galunisertib, a TGF-β receptor (TβR)-I inhibitor, suppresses growth and invasion of an anaplastic thyroid cancer 8505C cell in vitro and in vivo

Xinhui Li[1], Jianhui Zhuang[2], Kejun Zhang[3], Yuan Liu[4], Anbing Dong[4]

Departments of [1]Thoracic Surgery, [3]Thyroid Surgery, The Affiliated Hospital of Qingdao University, Qingdao, China; [2]Department of Surgery, People's Hospital of Rizhao, Shandong, China; [4]Department of Radiotherapy, Linyi Tumour Hospital, Linyi, Shandong, China

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Abstract: Background and objective: The transforming growth factor-beta (TGF-β) signaling pathway is known to play a critical role in promoting tumor growth and metastasis. Blocking this pathway has been found to inhibit tumor growth and metastasis. Previous study has found that siRNA targeting TGF-β1 could inhibit growth and invasion of the anaplastic thyroid cancer (ATC) cells. Galunisertib (LY2157299) is a selective ATP-mimetic inhibitor of TGF-β receptor (TβR)-I activation currently under clinical investigation in hepatocellular carcinoma (HCC) and glioma patients. However, the mechanisms of action of Galunisertib in ATC cells have been unclear. In the present study, we explored the effects and mechanisms of galunisertib on an ATC 8505C cell line *in vitro and in vivo.* Methods: The effects of Galunisertib on cell proliferation, apoptosis, and invasion in vitro was evaluated. Using an orthotopic mouse model, we determined the antitumor and anti-metastasis effects of Galunisertib in vivo. The effect of Galunisertib on TGF-β1/Smad-2, pERK1/2, pAKT, MMP-9, VEGF, NF-κBp65, PUMA signaling was detected by western blot assay in vitro. Ki67 was detected by immunohistochemical attaining. Results: Galunisertib was found to downregulate TβR-I, pSmad2, pERK1/2, MMP-9 and VEGF but upregulate NF-κBp65 and PUMA expression. However, no effect on pAKT was found. Galunisertib inhibited proliferation, invasion, and induced apoptosis of 8505C cells in vitro and in vivo. Conclusions: These data provide the first evidence that Galunisertib could effectively inhibit ATC tumor growth and metastasis, indicating that TGF-β1 inhibitors may have a higher therapeutic efficacy in ATC.

Keywords: Anaplastic thyroid cancer, apoptosis, metastasis, TGF-β1/Smad-2/3, galunisertib

Introduction

Thyroid cancer is the most common malignancy of the endocrine system [1]. The majority of thyroid cancers (95%) are derived from follicular cells and are broadly divided into well-differentiated, poorly differentiated (PDTCs) and undifferentiated thyroid carcinomas (ATCs). The most common thyroid cancers are well differentiated papillary (PTC) and follicular (FTC) thyroid carcinomas, which typically have an excellent prognosis [2]. ATC incidence typically peaks at the 6-7th decade of life (mean age at diagnosis 55-65 years), women representing 55-77% of all patients. ATC represents < 2% of all thyroid tumors. It is a highly aggressive tumor that belongs to the group of killer tumors with median survival time not longer than 6-8 months. Surgery, chemotherapy and radiotherapy are the conventional therapeutic strategies performed in the attempt to improve survival. Unfortunately, very often they do not succeed any clinical benefit but only palliative [3]. Given our poor ability to control ATC progression with conventional modalities, new paradigms are needed for treating this disease. Gene therapy is under investigation.

Transforming growth factor-βs (TGF-β) are multifunctional growth factors that play particularly complex roles in the growth, progression, and metastatic potential of cancers [4]. TGF-β1 is highly expressed in a various cancers such as prostate cancer [5], ovarian carcinoma [6],

hepatocellular carcinoma [7], bladder carcinoma [8], breast cancer [9], and papillary thyroid carcinoma [10, 11], and aberrant TGF-β1 expression is associated with more aggressive tumors and poor prognosis.

TGF-*β* signalling is propagated via cell surface serine/threonine kinases, TGF-*β* type I receptor (T*β*RI) and TGF-*β* type II receptor (T*β*RII). Both receptors are expressed on thyrocytes at equimolar amount [12] and, upon ligand binding by type II receptor, T*β*RI is recruited to an heteromeric complex and phosphorylated by T*β*RII, thus activating its serine/threonine kinase in order to phosphorylate the transcription factors R-Smads (Smad2 and Smad3).

Several small molecule inhibitors targeting the TGF-βRI serine/threonine kinase activity have been developed, including LY2157299 monohydrate (galunisertib) [13], which has been found to inhibit pSMAD2 expression in different tumor models. Galunisertib is now being investigated in a clinical trials and has very recently been shown to elicit anti-tumor effects in patients with glioma and hepatocellular carcinoma [14-16].

Previous study found that targeting the TGF-β1 signaling by siRNA may be more effective to prevent organ metastasis and primary tumor formation in ATC cells. In the present study, we investigate the effect of galunisertib on a ATC 8505C cell for proliferation and invasion in vitro and vivo and investigate its effect on TGF-β signaling.

Materials and methods

Reagents

The following primary antibodies were used from Santa Cruz Biotechnology: phospho-ERK (T202/Y204), phospho-Akt (S473), SMAD2, phospho-Smad2, TGF-β1 and TGF-βRI; ERK1/2, Akt, MMP-9, VEGF and β-actin. PUMA and NF-κBp65 were from Applied Biosystems.

Cell line

The human anaplastic thyroid cancer cell lines 8505C was purchased from American Type Culture Collection (ATCC, Shanghai, China). 8505C cells has high endogenous levels of TGF-β1 and TGF-β receptor (TβR)-I. The cells were maintained in DMEM with 10% FCS, at 37°C in 5% carbon dioxide and 95% air.

Galunisertib treatment

8505C cells were exposure to galunisertib (10 uM) for 24-72 hrs in vitro, and 100 mg/kg/bid oral gavage groups in vivo.

Western blotting

Cells in culture medium were cultured to 60-70% confluency. Inhibitors were added at the given concentrations as shown above, and at the indicated time point, the adherent cells were lysed directly in the dish for 30 minutes on ice with buffer containing 10 mM Tris pH 7.5, 0.5% Triton X-100, 5 mM EDTA, 0.1 mM Phenylmethanesulfonyl fluoride, 10 mM pepstatin A, 10 mM leupeptin, 25 mM aprotinin, 20 mM NaF, 1 mM pyrophosphate, and 1 mM orthovanadate. Separate cytosol and nuclear protein lysates were prepared by using the Active Motif Nuclear Extract Kit (Active Motif Europe, Rixensart, Belgium), according to the manufacturer's protocol. For routine quantitation of proteins, following the manufacturer's protocol (Pierce, Rockford, IL). 40 μg of protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% Tris-glycine gels for detection of all the proteins. After gel electrophoresis and transfer to nitrocellulose, the membranes were stained in 0.5% Ponceau S with 1% acetic acid to confirm the equal loading and transfer efficiency. Membranes were incubated at 4°C overnight in a blocking solution containing 5% bovine skim milk and 0.1% Tween 20 (Fischer Scientific, Pittsburgh, PA) in TBS (10 mM Tris-HCl with 150 mM NaCl, pH 7.6), then probed with specific primary and secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized by chemiluminescence solution and exposure to X-ray film.

For *in vivo* study, the tumor tissues were homogenized for tissue lysate extraction. Both cell lysate and tissue lysate were centrifuged and the supernatants were collected. After protein estimation with Bio-Rad protein assay (Hercules, CA, USA), a calculated volume of lysate was mixed with laemmli sample buffer, whereby the mixture was resolved by 10% SDS/PAGE gel and then electroblotted onto a nitrocellulose membrane. The membrane was probed

with primary antibody to pERK1/2, pAKT, MMP-2, MMP-9, VEGF, TβR-I, p-Smad2, PUMA, NF-κBp65 and β-actin.

Tumor invasion assay

8505C cells were treated with galunisertib (10.0 μM) for 72 hrs. The cells were then plating (1.25×10^5 per well) in the BD Matrigel invasion chambers (BD Biosciences). Medium in the upper chamber was supplemented with 5% FCS. In the lower chamber, FCS concentration was 10%. After 24 h, cells migrated into the lower chamber were stained and counted. Experiments were carried out in triplicate and repeated twice.

Cell cytotoxicity assay

8505C cells were seeded as triplicates in 96-well plates at a density of 5,000 cells per well in 150 ml medium and was exposure to galunisertib (10.0 μM). Cells were incubated for 72 hrs, washed two times with PBS, and 100 ml of a solution containing 100 mg 4-methylumbelliferyl-heptanoate per ml PBS was added. Plates were incubated at 37°C for 1 hour and measured using the MTT assay following the manufacturer's instructions. Data are represented as mean ± SEM absorbance values, based on 3 independent experiments, normalized to control cells.

Flow cytometry assay

8505C cells were treated with galunisertib (10.0 μM) for 72 hrs. To quantify the apoptotic cells, the cells were incubated with FITC-Annexin V and propidium iodide (PI) according to the manufacturer's instructions (BD Pharmingen). The apoptotic cells were monitored by flow cytometry (FACSCanto; BD Biosciences).

Animal studies

Animal studies were approved by the People's hospital animal Care and Use Committee and conducted in accordance with NIH guidelines. 8505C cells suspended in Matrigel (5×10^6 cells/200 mL) were inoculated subcutaneously into the right flank of 4- to 6-week-old female athymic nude (nu/nu) mice. Once palpable, tumor volumes were calculated with calipers using the following formula: length × width × height × 0.5236. After tumors reached approximately 100 mm^3 in average size, animals were sorted into 7 groups. Animals were treated with untreated groups, DMSO vehicle alone, galunisertib 100 mg/kg/bid oral gavage groups. Mice were sacrificed, and tumors were dissected after 6 weeks. For lung metastasis assay (n = 5 for each group), mice were sacrificed, the lungs were fixed, paraffin embedded, cut, and stained with H&E staining after six weeks.

Ki67 immunohistochemistry

The tissue section was deparaffinized before undergoing antigen retrieval step with citrate buffer. One paraffin section was made from the center of one tumor. Paraffin sections were incubated with anti-Ki67 antibody, then subsequently incubated with Alexa Fluor 546 goat anti-rabbit IgG (A-11010; Life Technologies). Sections were counterstained with DAPI and visualized by an confocal microscope. Four fields of one section were observed, and positive cells were counted using a hemocytometer. Data are the average of four independent count in one section.

TUNEL assay

The experimental procedures were carried out according to the manufacturer's protocol. Briefly, the tissue section was deparaffinized before rehydration with decreasing concentrations of ethanol. After washing with 0.85% NaCl and PBS, the tissue section was fixed with 4% formaldehyde for 15 mins. Following washing with PBS, the tissue section was covered with proteinase K solution for 8-10 mins. After another PBS wash, the tissue section was again fixed with 4% formaldehyde for 5 mins. Following PBS wash, the tissue section was covered with equilibrium buffer for 5-10 mins before addition of TdT reaction mixture. After incubation under dark condition for 1 h, the tissue section was incubated with SSC solution for 15 mins, followed by a final PBS wash. After DAPI counterstain, the tissue section was examined and photographed with a fluorescence microscope (Olympus BX51, Shinjuku, Japan). Average number of fluorescence dots of three images from each treatment group was calculated.

Statistical analysis

The significance of the results was determined by the Student's t test (two-tailed). Values are

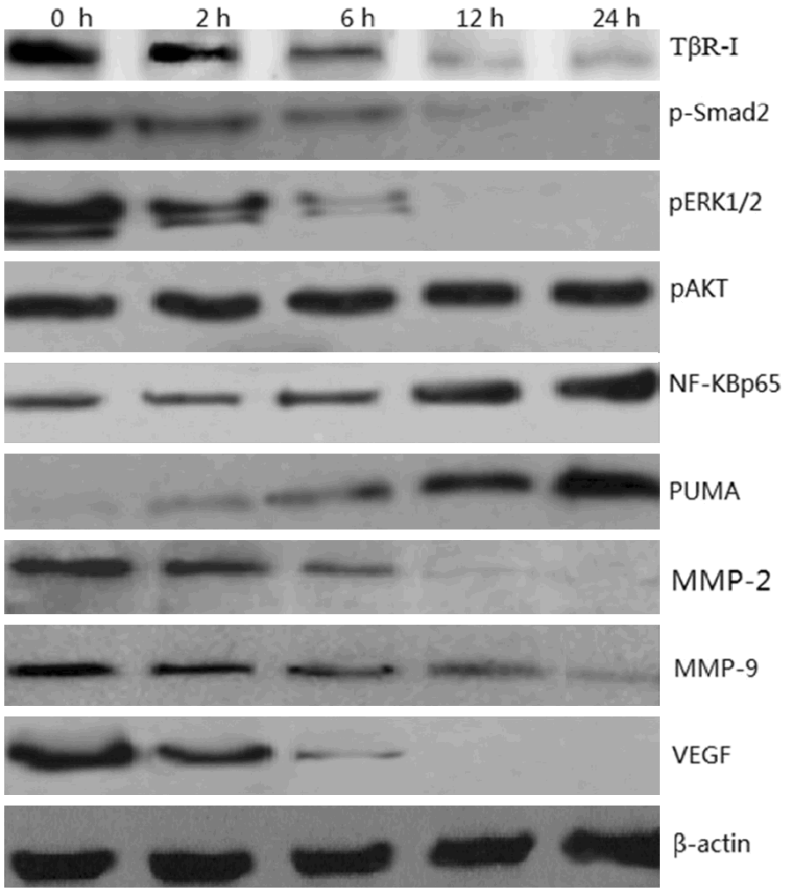

Figure 1. Effects of galunisertib on TGF-β signaling expression. 8505C cells was treated with 10 μM galunisertib for 2-24 hrs. Protein expression was evaluated by Western blot assay.

expressed as mean ± SD from at least three separate experiments and differences were considered significant at $P < 0.05$.

Results

Effect of Galunisertib on TGF-β signaling expression in 8505C cells

To study the effects of galunisertib on intracellular signalling, 8505C cells were treated with 10 μM galunisertib for 2-24 hours. After 24 hrs exposure of 10 μM galunisertib, TβR-I was completely inhibited at 12 hrs. p-Smad2, MMP-2,9, VEGF and pERK1/2 signalling began to inhibit from 2 hours, and completely inhibited at 24 hrs. Expression of p-AKT was unchanged. However, expression of NF-κBp65, PUMA was gradually increased with the treatment of galunisertib, and reached the highest levels at 24 hrs (**Figure 1**).

Galunisertib inhibits invasion of 8505C cells in vitro

Previous studies from our laboratory have shown that knockdown of TGF-β by siRNA transfection inhibited invasion of 8505C cells in vitro. In this study, we assessed the ability of Galunisertib to inhibit invasion of 8505C cells in vitro. Human ATC cell line 8505C cells was seeded in Matrigel-coated invasion chambers in the absence or presence of Galunisertib (10 μM). After 24 h, cells that migrated through the Matrigel barrier were stained and counted. Invasion was significantly inhibited by Galunisertib in the 8505C cells (**Figure 2**).

Effects of Galunisertib on 8505C cell growth and apoptosis

8505C cells were treated with Galunisertib (10 μmol /L) for 3 days, the growth of the 8505C cells was significantly inhibited. In the 3th day, more than 45% of the growth of the 8505C cells was inhibited (**Figure 3A**).

To examine the mechanisms of inhibition of cell growth, cell apoptosis was evaluated in the 8505C cell lines. Flow cytometry analysis shows (**Figure 3B**) that treatment with 10 μmol/L Galunisertib resulted in an approximately 24% increase of cell apoptosis, which was significant increase compare to the control ($P < 0.05$).

The TUNEL assay was also performed to verify the therapeutic effect of Galunisertib. As shown in **Figure 3C**, the cells with nuclei that contained amaranth fluorescence represented apoptotic cells. TUNEL-positive cells. The percentage of apoptotic cells was obtained via counting the number of TUNEL-positive cells. The 8505C cells treated with Galunisertib (10 μmol/L) for 3 days showed the highest level of

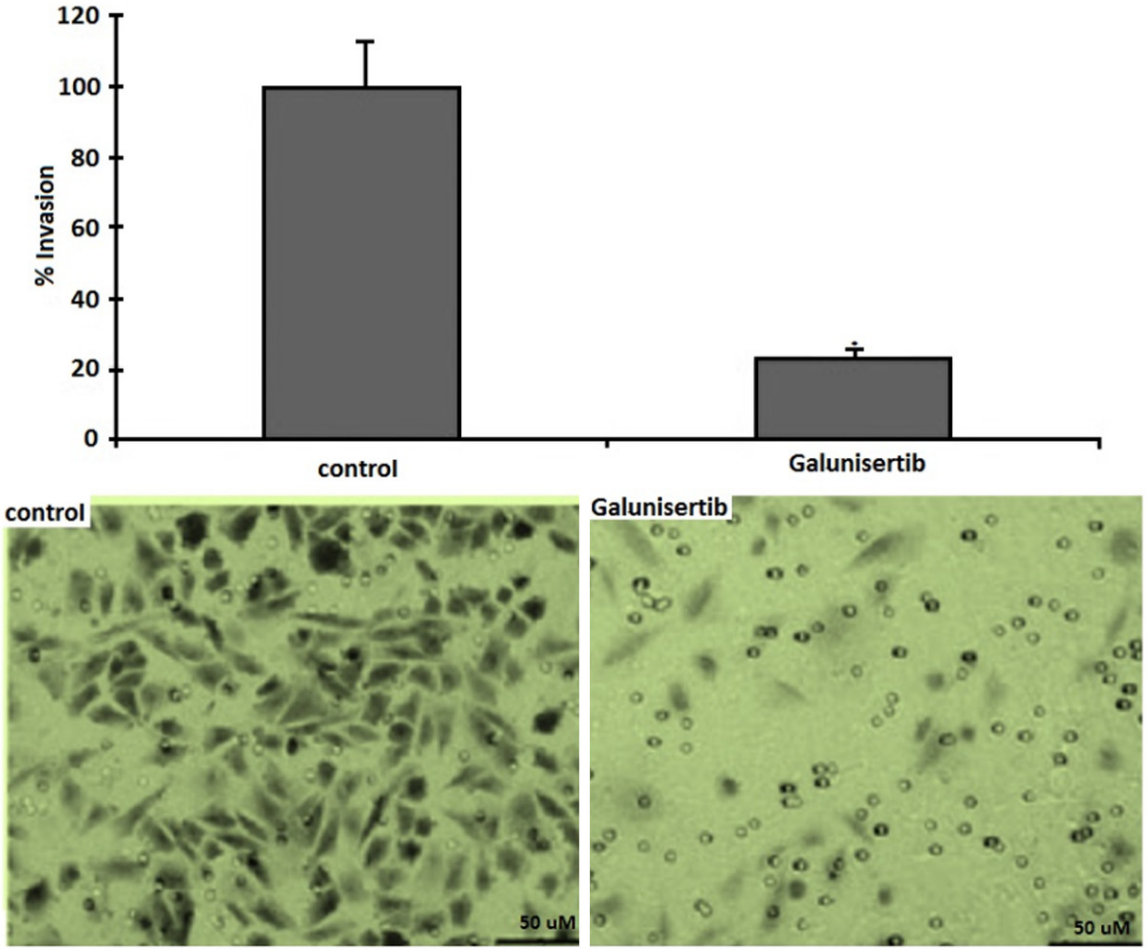

Figure 2. Galunisertib treatment inhibits invasion of human 8505C cells in Matrigel-coated invasion chambers. Cells were seeded in the upper chamber in medium supplemented with 5% FCS, treated with Galunisertib, or left untreated. After 24 h, cells migrated in the lower chamber were stained and counted. In the lower chamber, medium supplemented with 10% FCS was used as chemoattractant. Invasion of the untreated cells was set to 100. Results are reported as percent migration ± SD compared with untreated cells. Experiments were carried out twice in triplicate ($^{*}P < 0.05$).

cell apoptosis (18%, $P < 0.05$) compared with the control cells. The TUNEL assay data are consistent with the MTT, and the flow cytometry analysis.

Effects of galunisertib on tumor growth and metastasis in vivo

Three deaths were noted in the vehicle control and no deaths were found in the Galunisertib treated groups, respectively, before the end of the 6-week treatment period because of large tumors. Xenograft tumor growth and lung metastasis models were obtained as described in the methods section. As shown in **Figure 4A**, tumors grew at a slower rate and had smaller volumes in the Galunisertib treated groups than the non-treated group ($P < 0.05$). In addition, fewer metastatic nodes were formed on the surface of lungs in the Galunisertib treated groups than the control group (**Figure 4B**, $P < 0.05$).

By western blot assay, in the Galunisertib treated groups, p-SMAD2, p-ERK1/2, MMP-9, VEGF was significantly decreased, and NF-κB and PUMA was significantly increased, and no affection was found in pAKT (**Figure 4C**). Significant decrease of Ki67 staining was observed in galunisertib treated groups (9.3%±1.4%), than that of the untreated groups (37.5%±7.2%, $P < 0.05$) (**Figure 4D**). TUNEL staining of the tumor tissues found that the average number of fluo-

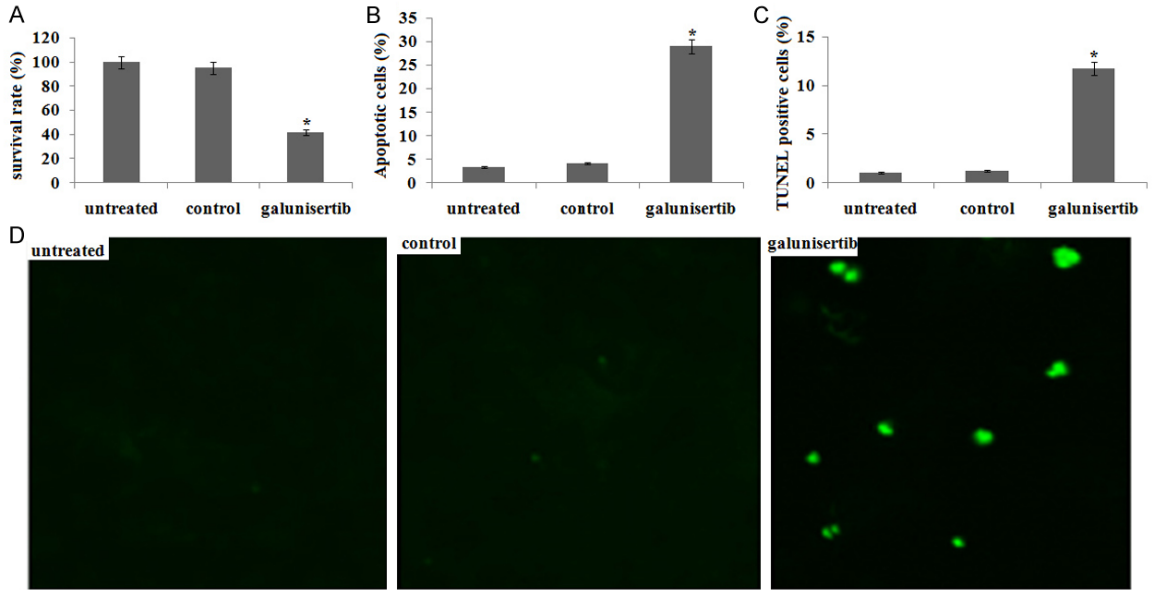

Figure 3. Effects of Galunisertib on 8505C cell growth and apoptosis. 8505C cells were treated with Galunisertib (10 μmol/L) for 3 days. A. Cell viability was detected by MTT assay. B. Cell apoptosis was detected flow cytometry assay. C. Fluorescence images of the cells following TUNEL staining. Green are the apoptotic cells. Percentage of apoptotic cells was detected by TUNEL analysis. *P* values were calculated with the Student's t-test. Vs control, $^{*}P < 0.05$.

rescence dots of images from galunisertib treated groups (11.3%±2.4%) was much more than the non-treated groups (0.9%±0.2%, $P < 0.05$) (**Figure 4E**).

Discussion

Galunisertib (LY2157299 monohydrate), a small-molecule inhibitors targeting the TGF-βRI serine/threonine kinase [17] is now in clinical evaluation and has shown antitumor effects in patients with glioblastoma, hepatocellular carcinoma [14], pancreatic and lung cancer [18]. Studying the antitumor activity in vitro and in vivo remains a challenge for Galunisertib. Using in vivo models, Bueno et al. found that Galunisertib inhibited growth of human xenografts of Calu6 (non-small cell lung cancer) and MX1 (breast cancer) implanted subcutaneously followed by downregulation of phosphorylated pSmad in tumour [19]. In a hepatocellular carcinoma models and in ex vivo whole tumor tissue samples from patients, Galunisertib could inhibit proliferation and induce apoptosis [15]. Using in vitro assays, Fransvea et al. characterized the role of TGF-β signaling in the migration and invasion of hepatocellular carcinoma cells. These studies suggested that Galunisertib did not have an apoptotic effect at the intended pharmacologic levels for patients, but rather blocked invasion and migration of hepatocellular carcinoma cells [20]. Galunisertib also reduces secretion of matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9), which modulates endothelial cells (ECs) angiogenesis [21].

Maier et al. has investigated the effect of Galunisertib in a series of experiments on a panel of 66 tumors derived xenografts (PDX) models [22]. They found that Galunisertib inhibited colony growth in 1/66 samples (1.5%) in a concentration-dependent manner when tested up to 10 μM. Growth stimulation was observed in 15/66 (22.7%) of the samples and no response was seen in 50/66 (75.8%) of the samples [22]. No correlation between responses and histopathological characteristics of the different samples was observed.

In the present study, we found that treatment with Galunisertib (10 μM) for 72 hrs significantly inhibited survival and induced apoptosis of the ATC 8505C cells in vitro. Furthermore, cell invasion was significantly inhibited by Galunisertib in the 8505C cells. Although the main molecular target of Galunisertib is TGF-β/TβR-I, several TGF-β/TβR-I-dependent and independent mechanisms to inhibit cell growth have been reported, such as suppression of Smad

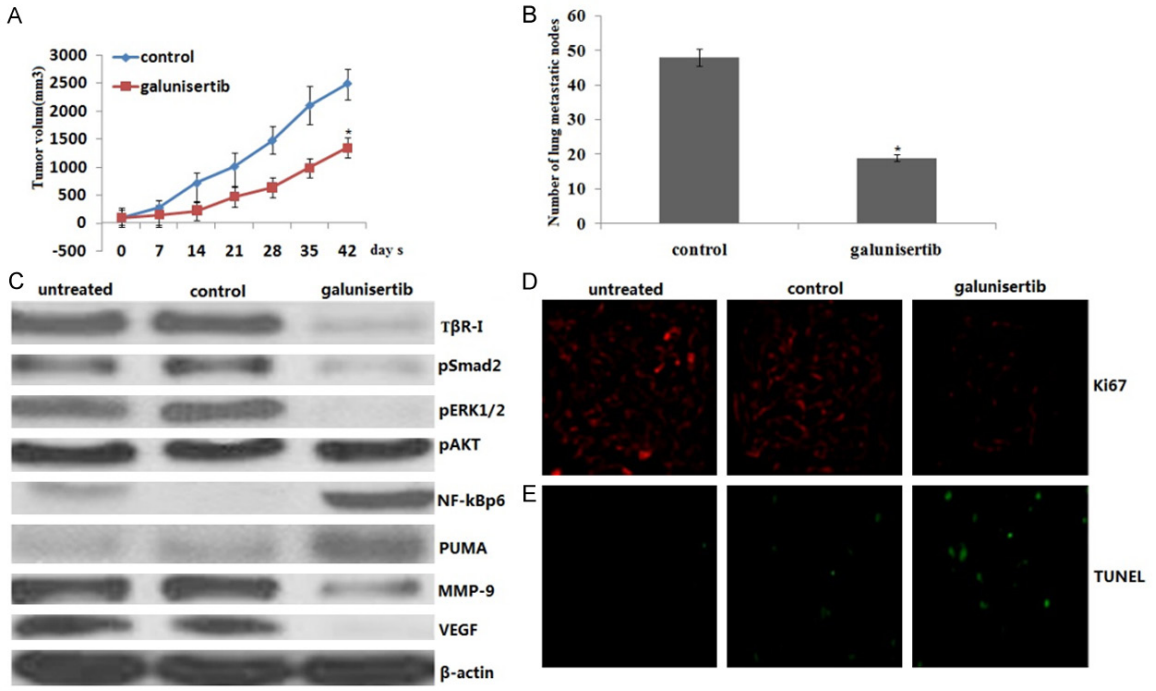

Figure 4. Effects of galunisertib on tumor growth and metastasis in vivo. A. 8505C ATC cells (5×10^6) were inoculated subcutaneously into the right flank of 4- to 6-week-old female athymic nude (nu/nu) mice. After tumors reached approximately 50-100 mm^3 in average size, animals were treated as the methods depicted in the "material sand methods". B. The number of metastatic nodes in each group. Tumors were counted with the naked eye. The data represent the mean and the standard deviation (n = 6). C. Protein expression of p-SMAD2, p-ERK1/2, MMP-9, VEGF, NF-κB, PUMA and pAKT was detected in the tissues in vivo. D. Ki67 immunohistochemical staining of the tumor tissues. E. TUNEL staining of the tumor tissues. Data are expressed as the mean ± SEM (*, $P = 0.01$).

[23], inhibition of ERK1/2-mTORC1 [24] and inhibition of phosphoinositide 3-kinase/AKT/survivin pathway [25]. Additionally, TGF-β/TβR-I pathway activation could induce apoptosis by NF-kappaB activation [26] or p53-up-regulated modulator of apoptosis (PUMA) activation [27].

Recent study found the targeting Ras/Raf/MEK/ERK signaling promoted PUMA-dependent apoptosis of tumor cells [28, 29], suggesting PUMA was negatively regulated by ERK signaling. Wang et al. has demonstrated that PUMA is a direct target of NF-kappaB and mediates TNF-alpha-induced apoptosis in vitro and in vivo [30]. Our results demonstrate that Galunisertib treatment induced NF-kappaB and PUMA upregulation, followed by increased apoptosis and tumor growth inhibition. We suggested that Galunisertib induce apoptosis by NF-kappaB and PUMA regulation. However, the mechanisms in detail need further investigation.

PI3K/AKT signaling pathways were associated with proliferation, apoptosis, and migration in tumor cells. The pro-cell survival response exerted by TGF-β activation of PI3K/AKT has been linked to stimulation of tumor formation [31]. In our study, Galunisertib did not affect pAKT level, suggesting pAKT did not take part in the apoptosis and growth regulation of Galunisertib to 8505C cells.

We performed *in vitro* experiments using 10 μmol/L Galunisertib to substantially induce PUMA and apoptosis and inhibit survival in 8505C cells. The results of these experiments were confirmed by those in vivo xenograft tumor experiments. It was indicated that Galunisertib could effectively inhibit growth and induce apoptosis by PUMA inducing in vivo and in vitro of ATC 8505C cells. These observations suggest that induction of PUMA is likely to be involved in the effects of Galunisertib on ATC 8505C cells.

TGF-β has emerged as a promising new target for treatment of cancer metastasis. Most of the studies to date have examined the effects of TGF-β receptor inhibition on tumor metastasis

[16, 32-35]. The TGF-β/Smad signaling pathway has been linked to the activation of mitogen-activated protein kinase signaling pathways in several cancer models [36-38]. ERK1/2 activation was associated with invasion and metastasis of many cancer cells [39-41].

In our in vitro experiment, treatment of 8505C cells with Galunisertib inhibited ERK1/2 activation, MMP-9 and VEGF expression, and inhibited cell invasion. We performed *in vitro* experiments using 10 μmol/L Galunisertib to inhibit invasion of 8505C cells. The results of these experiments were confirmed by those in vivo lung metastasis experiments. We also found in vivo that Galunisertib inhibited ERK1/2 activation, MMP-9 and VEGF expression. These observations might have some relation between ERK1/2-MMP-9 and VEGF and metastasis.

In conclusion, we demonstrate targeting TGF-β receptor (TβR)-I with Galunisertib in 8505C tumor cells may be effective in inducing apoptosis and inhibiting cell growth and decreasing metastasis.

Disclosure of conflict of interest

None.

Address correspondence to: Kejun Zhang, Department of Thyroid Surgery, The Affiliated Hospital of Qingdao University, Qingdao, China. E-mail: zkejun@yahoo.ca

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