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BIIB021, a novel Hsp90 inhibitor, sensitizes esophageal squamous cell carcinoma to radiation

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ABSTRACT

BIIB021 is a novel, orally available inhibitor of heat shock protein 90 (Hsp90) that is currently in phase I/II clinical trials. BIIB021 induces the apoptosis of various types of tumor cells in vitro and in vivo. The aim of this study is to investigate the effect of BIIB021 on the radiosensitivity of esophageal squamous cell carcinoma (ESCC). The results indicated that BIIB021 exhibited strong antitumor activity in ESCC cell lines, either as a single agent or in combination with radiation. BIIB021 significantly downregulated radioresistant proteins including EGFR, Akt, Raf-1 of ESCC cell lines, increased apoptotic cells and enhanced G_2 arrest that is more radiosensitive cell cycle phase. These results suggest that this synthetic Hsp90 inhibitor simultaneously affects multiple pathways involved in tumor development and progression in the ESCC setting and may represent a better strategy for the treatment of ESCC patients, either as a monotherapy or a radiosensitizer.

1. Introduction

Esophageal carcinoma is the eighth most common cancer and the sixth leading cause of cancer deaths in the world [1]. In China and other East Asian countries, more than 90% of cases are ESCC, whereas adenocarcinoma is more common in western developed countries. The standard treatments of ESCC include surgery, radiotherapy and chemotherapy. Radiotherapy is generally used as preoperative and postoperative treatment that has a crucial role in improving local control and survival of ESCC. However, intrinsic tumor radioresistance accounts for the high recurrence and poor 5-year survival of ESCC patients [2]. Therefore, identification of reliable radiosensitizers to ESCC cells would be urgently desirable. Targeted biological therapies that selectively interfere with cancer cell growth signals may improve patient survival by enhancing the effects of radiation with little damage to normal tissue.

Hsp90 is a molecular chaperone that assists the conformational maturation, folding and refolding of client proteins during stress and protects them from degradation [3,4]. More than 200 "client proteins" covering almost all cellular processes such as cell growth, proliferation and cell survival have been identified so far [5], including tyrosine kinases, transcription factors, structural proteins and hormone receptors [6,7]. Hsp90 is exploited by cancer cells to support activated oncoproteins that are essential for oncogenic transformation. In addition, Hsp90 can also help cancer cells to buffer unavoidable stress and avoid attack by the host immune system. Therefore, cancer cells could survive and flourish in an inhospitable environment [8,9]. Wu et al., showed that Hsp90 is abundantly expressed in esophageal cancer as well as in esophageal cancer cell lines [10]. Therefore, Hsp90 represents a potential therapeutic target in the treatment of patients with ESCC. EGFR, Raf-1, Akt and HER2 are referred to as "clients" of Hsp90 [11]. It has been confirmed that all of them are associated with the radioresponse, thereby protecting radiation-induced cell death [12–16]. Inhibition of Hsp90 leads to degradation of these radioresistant proteins thereby enhancing tumor cell death in a variety of cell lines and tumor models, including ESCC [10,17,18]. All these findings suggest that inhibition of Hsp90 may not only provide a unique therapeutic pathway, but also promote the efficacy of radiotherapy. Lots of encouraging results in multiple malignant tumors demonstrate that Hsp90 inhibitors have strong anti-cancer effects as single anticancer agents, as well as combination with other chemotherapy drugs or radiation [19,20].

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Preclinical studies have shown that 17-allylamino-17-demethoxygeldanamycin (17-AAG) significantly sensitized the ESCC cell lines for γ-photon irradiation [10]. However, 17-AAG is poor in pharmaceutical properties and is difficult to be formulated, which significantly affects its therapeutic efficacy in clinical practice [21]. In this report, we investigated a fully synthetic and bioavailable Hsp90 inhibitor, BIIB021 in ESCC cell lines, either as single agent or in combination with radiation. We found that BIIB021 showed significantly antitumor activity. BIIB021 enhanced the in vitro radiosensitivity of these cell lines with reduced expression of radioresistant proteins, increased apoptotic cells and enhanced G_2 arrest.

2. Materials and methods

2.1. Reagent, cell culture and antibodies

BIIB021 (Selleck, USA) was stored as 10 mM stock solutions in DMSO at −80 °C, and diluted in the appropriate cell culture medium for use, such that the final DMSO concentration did not exceed 0.01%.

Two established human ESCC cell lines (Eca109 and Eca9706) were used for this study. Both of the two cell lines were cultured in DMEM (Hyclone, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and streptomycin in a 37 °C incubator with humidified atmosphere and 5% CO_2.

Antibodies used were anti-c-Raf-1 rabbit polyclonal antibody, anti-phospho-EGFR^{Tyr1173} rabbit monoclonal antibody, anti-phospho-Akt^{Ser473} rabbit monoclonal antibody, anti-cleaved PARP rabbit monoclonal antibody (Cell signaling technology, Beverly, MA,USA) and anti-β-actin mouse polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.2. CCK-8 assay

Logarithmically growing cells were counted and seeded in 96-well plates at 2000 cells per well, in triplicate and incubated overnight. The next morning, all plates were aspirated and fresh medium were added in a final volume of 200 μl with increasing concentrations of BIIB021 (0 nM, 65 nM, 125 nM, 250 nM, 500 nM, 1 μM, 2 μM). Following drug addition, the plates were incubated for 5 days. Cell growth inhibition was examined by CCK-8 assay (BestBio, China). 10 μl CCK-8 labeling reagent was added to each well with 100 μl fresh medium, the plates were incubated at 37 °C for 4 h. The absorbance of each well was measured at 450 nm using Thermo Scientific Varioskan Flash (Thermo Scientific, Finland). Percentage of viable cells = (OD_{450} 0f treated sample − OD_{450} of blank sample)/(OD_{450} of control sample − OD_{450} of blank sample) × 100. The results shown were mean values of 3 independent experiments.

2.3. Clonogenic assay

Clonogenic assay was used to evaluate the effect of BIIB021 in combination with radiation. Cells were trypsinized to generate a single cell suspension and seeded in 6-well plates at 1500 cells per well. After allowing cells to attach, BIIB021 was added about the IC_{25} of each cell line (Eca109: 97 nM; Eca9706: 33 nM). For BIIB021 in combination with radiation, 16 h after adding BIIB021, cells were irradiated with a single dose of 2, 4, 6, 8 Gy from a medical linear accelerator (varian 23 EX, USA). Four hours after radiation, all plates were aspirated and fresh medium were added. 14 days after seeding, colonies were stained with crystal violet, and the number of colonies containing at least 50 cells was counted. The colony survival fraction was calculated for each treatment and data were presented as log plot. The results shown were mean value of 3 independent experiments with triplicate setting in each experiment.

2.4. Flow cytometry analysis of apoptosis and cell cycle

To test if cells were undergo apoptosis, Eca109 and Eca9706 cells were plated and exposed to either 1 μM BIIB021 or 6 Gy radiation for 24 h. For combination, cells were treated with 1 μM BIIB021 for 16 h, and then treated with a single dose of 6 Gy of radiation. Cells were collected 24 h after radiation without washing (both floating and attached cells were collected by centrifugation). Apoptosis analysis was performed according to the manufacturer's instructions (Annexin V-FITC Apoptosis detection kit; BestBio, China). Approximately 1×10^5 cells were incubated with FITC-conjugated annexin V in the presence of PI and then analyzed by flow cytometry (FACS, Becton Dickson). Annexin V positive PI negative cells scored as early apoptotic, Annexin V positive PI positive cells corresponded to late apoptotic cells.

Cell cycle distribution was measured before and after the same treated as above. Cells were digested by trypsin, washed with PBS, fixed with 75% cold ethanol at 4 °C overnight and dyed with PI, and then analyzed by flow cytometry.

2.5. Protein extraction and Western blotting

Protein were extracted 24 h after the same treatment as above. Cells were washed with PBS, and added to RIPA Lysis Buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin) and PMSF (Phenylmethanesulfonyl fluoride, Beyotime, China),that the ratio is 100:1. Total protein concentration was determined using the BCA Protein Assay Kit (Beyotime, China). In addition, a time course for the effect of BIIB021 on client protein expression was also done and cells were collected at 12, 24, 48 h after treatment.

25 μg of protein extract was electrophoresed through 10% SDS polyacrylamide gels under denaturing conditions and transferred to PVDF membranes. The membranes were blocked in 5% non-fat dry milk that was dissolved with 1 × TBST, and incubated with primary antibodies at 4 °C overnight, after washing with 1 × TBST 3 times, the membranes were incubated with anti-rabbit or anti-mouse second antibody. Specific antigen–antibody interactions were detected with enhanced chemiluminescence.

2.6. Statistical analysis

All data were shown as mean ± SD. Statistical significance was assessed by *T*-test for two-group comparison. Differences with p value <0.05 were considered statistically significant.

3. Results

3.1. Hsp90 blocked by BIIB021 inhibited the proliferation and survival of ESCC cell lines

Both Eca109 and Eca9706 cell lines displayed a dose-dependent reduction in cell proliferation. The effects of BIIB021 on cell proliferation and survival in both of the two cell lines were separately shown as bar graphs (Fig. 1A). IC_{50} was defined as the concentration of BIIB021 that resulted in 50% inhibition of cell growth. Graphpad Prism 5.0 software was used for the calculation of IC_{50}. The IC_{50} for BIIB021 was 661.1 nM in Eca109 cells and 53.31 nM in Eca9706 cells. From the results we concluded that BIIB021 significantly inhibited proliferation and survival of ESCC cell lines in

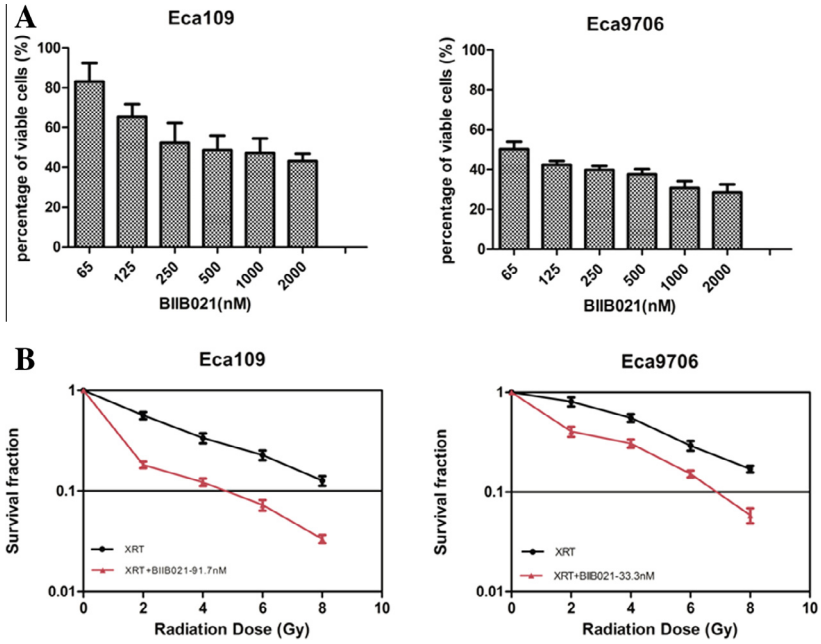

Fig. 1. (A) BIIB021 inhibited the growth of Eca109 and Eca9706 in a dose-dependent manner. Eca9706 was more sensitive to BIIB021 than Eca109. (B) BIIB021 radiosensitized ESCC cell lines. Eca109 and Eca9706 cells were irradiated at a single dose of 0, 2, 4, 6 or 8 Gy in the presence or absence of BIIB021 (IC_{25} concentration for each cell line). Data were shown as mean ± SD of three experiments. $p < 0.05$ for each radiation dose in combination with BIIB021 versus each radiation dose alone for both of the two cell lines.

a dose-dependent manner, and Eca9706 was more sensitive to BIIB021 than Eca109.

3.2. Hsp90 inhibition by BIIB021 can radiosensitize ESCC cells

Many studies have indicated that Hsp90 inhibition may radiosensitize tumor cells [22]. We performed clonogenic cell survival assays to address the same issue in ESCC cell lines. Eca109 and Eca9706 were treated with BIIB021 for 16 h followed by a single dose of radiation. The impact of radiation alone or combined with BIIB021 was shown as survival curves (Fig. 1B). Eca9706 were relatively resistant to radiation compared to Eca109, 50% killing dose is approximately 4 Gy versus 2 Gy for Eca109 cells. When cells were pretreated with BIIB021 prior to radiation, a significant growth inhibition was observed in both of the two cell lines (Eca109: $p = 0.008$ at 2 Gy, $p = 0.013$ at 4 Gy, $p = 0.01$ at 6 Gy and $p = 0.006$ at 8 Gy; Eca9706: $p = 0.02$ at 2 Gy, $p = 0.003$ at 4 Gy, $p = 0.01$ at 6 Gy, and $p = 0.001$ at 8 Gy). BIIB021 overcomed the resistance of Eca9706 to radiation, and significantly increased the antitumor effect of radiation at 4 Gy, 6 Gy and 8 Gy. Inhibition of Hsp90 by BIIB021 possibly cause the degradation of radioresistant proteins therefore improving the efficacy of radiation in sensitive or insensitive cell lines. These results indicated that BIIB021 may be used as radiosensitizer in the radiotherapy of ESCC patients to improve the antitumor effect of radiation, especially the patients who are insensitive to radiation.

3.3. BIIB021 sensitized ESCC cells to radiation by increasing apoptotic cell death

Apoptosis is a mode of cell death in response to radiation. Annexin V stain followed by flow cytometry analysis was performed for Eca109 and Eca9706 cells treated with radiation (6 Gy) or BIIBB021 (1 μM) alone or the combination. As depicted in Fig. 2, radiation alone only induced a small amount of cell apoptotic in both Eca109 ($p = 0.024$) and Eca9706 cells ($p = 0.077 > 0.05$, consists with the XRT relative resistance phenotype of Eca9706) compared with control. Apoptosis rate for Eca109 cells was approximately 26% and for Eca9706 cells was approximately 32% when treated with BIIB021 alone. When BIIB021 was combined with radiation, the apoptosis rate increased to 57% for Eca109 and 59% for Eca9706, significantly higher rate compared with radiation or BIIB021 treatment alone ($p < 0.05$). These results indicated that BIIB021 inhibited cell growth and enhanced radiation effect by inducing apoptosis.

3.4. BIIB021 alone or in combination with radiation induced G_2 arrest in ESCC cell lines

Inhibition of Hsp90 could change cell cycle distribution and increase the drug sensitivity of tumor cells [23]. We studied the effects of all treatments alone or the combination on cell cycle distribution of ESCC cell lines. The G_2/M population increased, and S population decreased in both cell lines when treated with radiation or BIIB021 alone (Fig. 3), but little change happened in the population of G_1/G_0 phase in addition to Eca109 was exposed to radiation of 6 Gy (Fig. 3A: decreased by 10%). When cells were treated with BIIB021 followed by radiation, the G_2/M population significantly increased by 42% for Eca109 and 39% for Eca9706, and obvious reduction in S phase cells was observed. BIIB021 alone or plus radiation caused G_2/M arrest, therefore inhibiting cell mitosis and increasing radiation-induced apoptosis in both ESCC cell lines.

3.5. BIIB021 induced degradation of Hsp90 key client proteins associated with radioresistance

Hsp90 has numerous client proteins that play critical roles in tumor cell growth and survival [24]. EGFR, Akt and Raf-1 were of particular interest because have been reported to be associated with radiosensitivity [25–27]. What's more, EGFR was suggested to predict the radiosensitivity and/or prognosis of human esophageal squamous cell carcinoma [28], and pAkt is a good marker for a radiation resistant phenotype [29]. BIIB021 alone or in

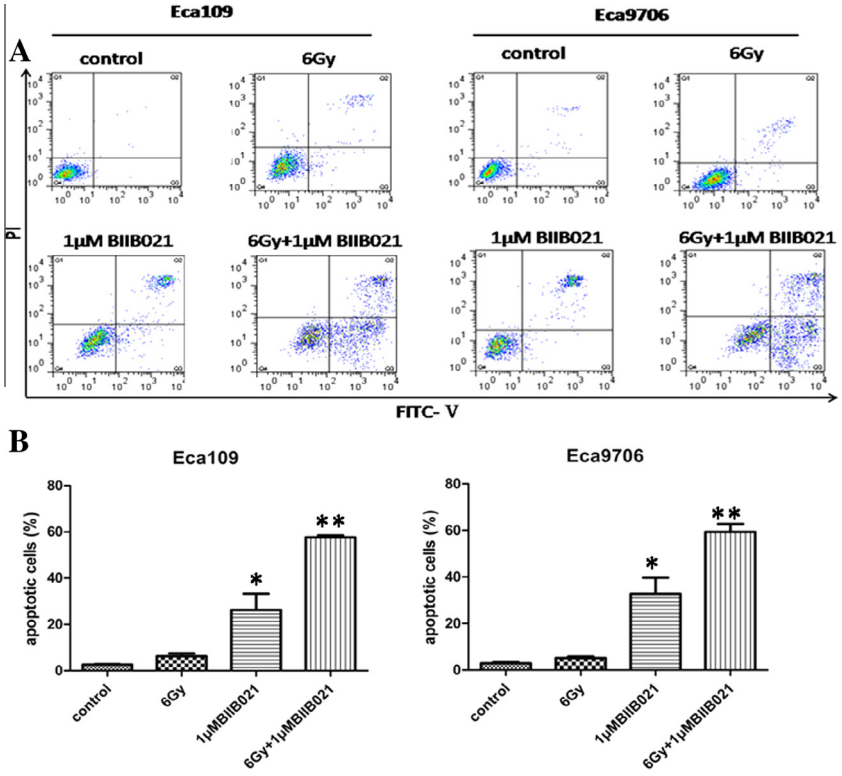

Fig. 2. BIIB021 induced ESCC cell death via apoptosis. (A) Apoptosis for all treatments of Eca109 and Eca9706 cells. Combination of radiation and BIIB021 induced significant apoptosis in both of the two cell lines. (B) Bar graphs represent the total apoptosis of all conditions. The total amount of apoptosis were the results of early apoptosis plus late apoptosis. Data were shown as mean ± SD of three experiments. $^{*}p < 0.05$ versus control or radiation alone; $^{**}p < 0.05$ versus control or other treatment alone.

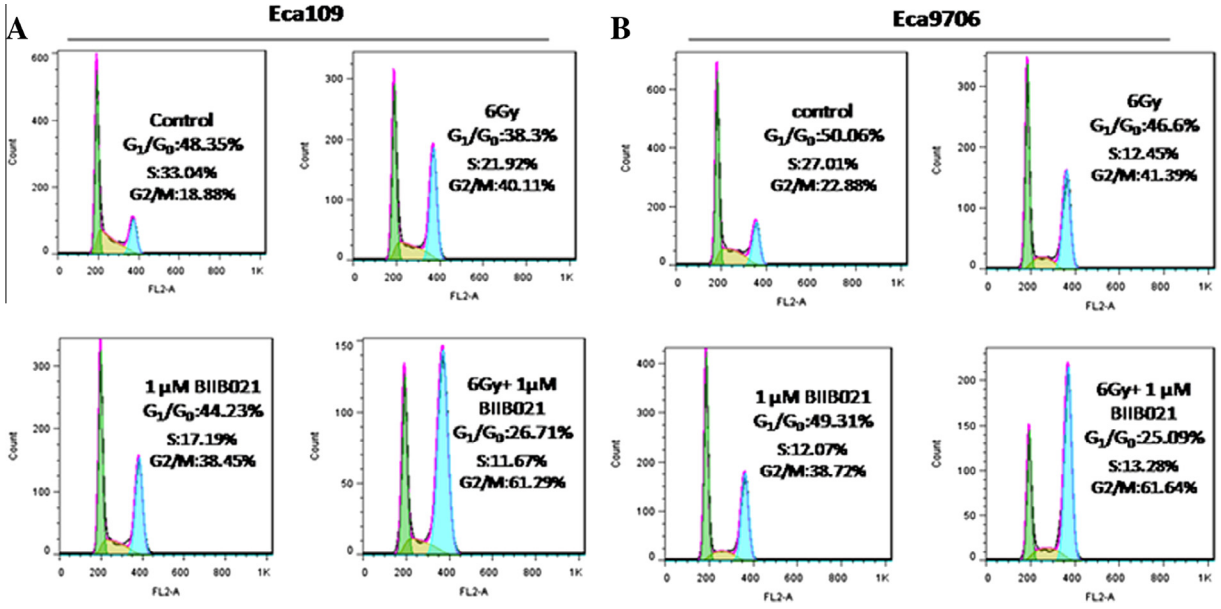

Fig. 3. BIIB021 induced cell cycle arrest in ESCC cell lines. Both Eca109 and Eca9706 were arrested at G_2/M phase after BIIB021 treatment alone or combined with radiation.

combination with radiation induced degradation of these proteins therefore increasing the apoptosis of ESCC cells.

The time course (treated with 1 μM BIIB021 for 12 h, 24 h and 48 h) for the effect of BIIB021 on phosphorylation/activation of EGFR and Akt was done. BIIB021 caused a decrease in the expression of pEGFR and pAkt in a time-dependent manner (Fig. 4A).

To explore the degradation of these key client proteins, western blotting was performed on extracts of Eca109 and Eca9706 cells following 24 h exposure to either 1 μM BIIB021 or 6 Gy radiation alone, or in combination. As shown in Fig. 4B, radiation alone had a limited effect on the level of pAkt and c-Raf-1. Conversely, the expression level of pEGFR even increased after radiation treatment alone, consistent with previous studies [30,31]. However, BIIB021 treatment alone or in combination with radiation significantly decreased the level of pAkt, c-Raf-1 and pEGFR in both cell lines. PARP is the substrate of Caspase-3 and the cleavage of PARP

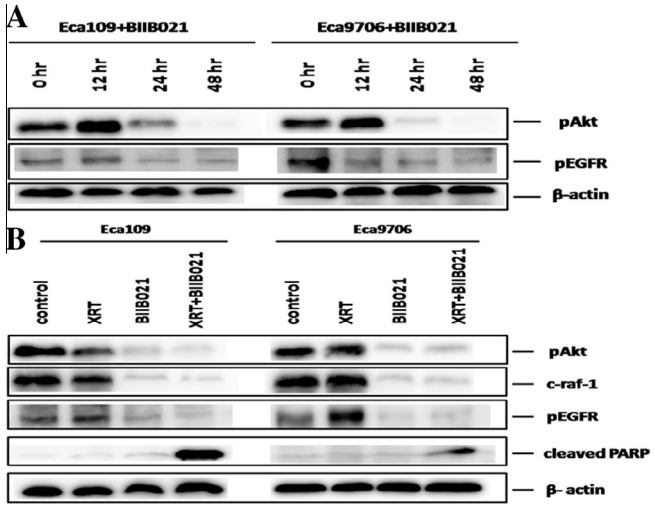

Fig. 4. (A) BIIB021 induced degradation of pAkt and pEGFR in a time-dependent manner in Eca109 and Eca9706 cell lines. (B) BIIB021 downregulated the expression of radioresistant proteins. Eca109 and Eca9706 were either treated with 1 μM BIIB021, or 6 Gy radiation or BIIB021 plus radiation.

is regarded as an important indicator of apoptosis. Radiation alone only minimally increased the level of cleaved PARP compared to control in both cell lines. Moreover, moderative PARP cleavage was seen when treated with BIIB021 alone. However, it is encouraging that significant effect was seen when BIIB021 plus radiation. The results indicated that BIIB021 induced degradation of these radioresistant proteins therefore improving the sensitivity of radiotherapy.

4. Discussion

BIIB021 is a novel, full synthetic and orally bioavailable inhibitor of Hsp90. The results of this study demonstrated the potent ability of BIIB021 to radiosensitize ESCC cell lines in vitro via multiple mechanisms: (1) downregulation of radioresistant proteins involved in critical radioresistance pathways, (2) increasing apoptotic cells, (3) reassortment of ESCC cells into more radiosensitive phases of the cell cycle through G_2/M arrest. Our study indicates that BIIB021 can significantly improve the efficacy of radiotherapy.

Currently, unresectable ESCC patients is primarily treated by chemoradiotherapy. However, due to the widely different susceptibility to radiotherapy exhibited in ESCC patients, the rate of locoregional recurrence and distant metastasis remain unacceptably high [32]. Current efforts have been focused on the development of clinically relevant radiosensitizers that selectively enhancing radiation-induced tumor cell death with as little damage to normal tissue as possible. Towards the development of such a multitarget approach to radiosensitization, the molecular chaperone Hsp90 has been the focus. Clearly, the therapeutic potential of Hsp90 inhibitor as a radiation modifier will ultimately depend on a selective increase in the radiosensitivity of tumor cells over normal cells. Several Hsp90 clients including EGFR, ErbB2, Akt, Raf-1 and VEGF are known to be associated with radioresistance [28,33], which positions Hsp90 inhibitors as good candidates for radiosensitizers. Hsp90 inhibition provides an approach for the simultaneous targeting of these proteins so that significantly improving the efficacy of radiotherapy.

In this report, we described the effect of BIIB021, alone and in combination with radiation, on two ESCC cell lines in vitro. The expression level of EGFR, Akt and Raf-1 that are associated with ESCC cells survival and radioresistance significantly decreased when treated with BIIB021 alone or in combination with radiation. The results indicated that BIIB021 sensitized ESCC cells to radiation by increasing the degradation of these radioresistant proteins that involved in multiple signaling pathways. Therefore the efficacy of radiation was significantly enhanced via disrupting those multiple signaling pathways simultaneously. Interestingly, we found that the expression level of pEGFR even increased after exposure to radiation in both ESCC cell lines tested. This observation consists with previous studies, exposure of human breast cancer cells or xenografts to ionizing radiation increased the level of tyrosine phosphorylated EGFR, a measure of EGFR activation. This phenomenon may underlie the clinical observation of "accelerated repopulation" in which tumor cells undergo stimulated proliferation during a fractionated course of radiation [30,31].

Besides, Annexin V stain followed by flow cytometry analysis demonstrated that BIIB021 sensitized ESCC cells to radiation by increasing apoptotic cell death. Cell cycle analysis of our study also showed that both Eca109 and Eca9706 cells were arrested at G_2/M phase when treated with BIIB021 alone or in combination with radiation, significant effect was observed in the cotreatment. Using HNSCC cell lines, Yin et al. have found that BIIB021 alone induced G_1/G_0 arrest and significantly enhanced radiation-induced G_2 arrest [18]. The effect of BIIB021 alone on cell cycle distribution may be not the same via different mechanisms in different cell lines. But in the two studies, BIIB021 in combination with radiation induced significant G_2 arrest by activating G_2 checkpoint, followed by apoptotic cell death. Therefore, further investigation perhaps can be extended to the effect of BIIB021 and radiation on cell cycle checkpoint kinases which are the principle components of the DNA damage response to ionizing radiation.

Several previous studies have shown that Hsp90 inhibitors such as 17-AAG, 17-DMAG and GA can sensitize a variety types of tumor cell lines and xenografts to radiation. Those tumor types include glioma, prostate carcinoma, pancreatic carcinoma, colon adenocarcinoma, head and neck squamous cell carcinoma and esophageal carcinoma, and so on [20]. However, the antitumor activity of 17-AAG and other ansamycin derivatives are significantly curtailed by the expression of multidrug resistance (MDR) protein that is a significant obstacle in cancer therapy [34]. P-glycoprotein (P-gp) and MRP1 (MDR-related protein 1) are two major members of MDR family. 17-AAG and other ansamycin derivatives are inactive in p-gp and/or MPR-1 expressing cell lines. Unlike 17-AAG, BIIB021 is not a substrate for MDR and exhibits no MDR dependency, so MDR mechanisms will not compromise its therapeutic application [35]. All of these determined that BIIB021 has strong antiproliferative activity in either engineered or natural P-gp and MRP1 overexpressing cells including ESCC cell lines [36,37]. These data indicate that BIIB021 may have broader application against tumors. In order to make better use of BIIB021 in clinical, our further studies will investigate the effect of BIIB021 on ESCC xenografts in vivo.

In conclusion, our studies have shown that BIIB021, a full synthetic, orally available Hsp90 inhibitor exhibited strong antitumor effects in ESCC cell lines as a single agent and sensitized ESCC cell lines to radiation, significantly increased the efficacy of the latter. All of these data and the favorable pharmacokinetic profile of BIIB021 making it a good candidate for clinical relevant radiosensitizer in the radiotherapy of ESCC patients.

Conflict of interest

The authors have no conflict of interest.

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