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Original Article

Antitumor effects of BMS-777607 on ovarian cancer cells with constitutively activated c-MET

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ABSTRACT

Objective: Tyrosine-protein kinase MET (c-MET) has been reported to be a prognostic marker and suitable therapeutic target for ovarian cancer. BMS-777607, a small molecule, can inhibit MET and other protein kinase activities. The present study was conducted to investigate the mechanism of action and antitumor effect of BMS-777607 on ovarian cancer cells with constitutively activated c-MET.
Materials and methods: Ovarian cancer cells with constitutively activated c-MET were first identified through Western blot analysis. Bio-behaviors, including signal transduction, proliferation, apoptosis, and migration, of the cells with constitutively activated c-MET were evaluated after BMS-777607 treatment. Liu's stain and immunological staining of α-tubuline were performed to evaluate the ploidy of the cells. A xenograft mouse model was also used to evaluate the antitumor effects of BMS-777607 on ovarian cancer cells with constitutively activated c-MET.
Results: BMS-777607 could induce the highest inhibition of cell growth in ovarian cancer cells constitutively expressing c-MET. Treating SKOV3 cells with BMS-777607 could reduce c-MET activation and inhibit downstream cell signaling, thus causing cell apoptosis and polyploidy as well as cell cycle and cell migration inhibition. This molecule also inhibited tumor growth in a mouse xenograft model of SKOV3 ovarian cancer cells in vivo.
Conclusion: BMS-777607 exhibits antitumor effects on ovarian cancer cells that constitutively express c-MET through c-MET signaling blockade and the inhibition of Aurora B activity. Combination treatments to enhance the effects of BMS-777607 warrant investigation in the future.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy worldwide [1]. The current standard treatment for EOC is a combination of optimal cytoreduction and chemotherapy [2]. Because of the absence of specific symptoms during early stages and a shortage of reliable markers, most cases of epithelial ovarian cancer are diagnosed at an advanced stage at which disease recurrence is high and the survival rate is low [3–5]. Thus, the development of novel therapeutic strategies for this recalcitrant disease is necessary.

The use of small molecules, such as tyrosine kinase inhibitors (TKIs), to block the signaling cascade involved in tumorigenesis is a robust targeted therapy for cancer treatment [6]. For example, Erlotinib and Gefitinib, which target epidermal growth factor receptors (EGFRs), were approved by the Food and Drug Administration for the treatment of non-small-cell lung cancer [7] and HER2/neu blockade was approved for breast cancer therapy [8]. However, there had not been a promising target therapy in treating ovarian cancer and TKIs have not been used for the treatment of ovarian cancer. In this study we aimed to design a pre-clinical study to experiment on this potential target to scrutinize its antitumor effect. c-MET is a receptor tyrosine kinase that plays a crucial role in tumorigenesis [9]. c-MET over expression has been considered a prognostic marker for ovarian cancer [10]. The receptor is activated on binding with its ligand, hepatocyte growth factor (HGF), and activates the ras-mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and signal transducers and

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activators of transcription signaling pathways [11,12]. All these signals collectively induce cell proliferation and dissemination. Thus, targeting c-MET by blocking the interaction between HGF and c-MET is considered as an effective strategy for the management of ovarian cancer. Aberrant activation of c-MET may be due to the overexpression of its ligand. However, a clinical trial using humanized IgG2 directed against HGF did not reveal any antitumor effect of this strategy on ovarian cancer [13]. HGF is expressed at high levels in normal ovarian stromal fibroblasts but not in cancer-associated fibroblasts [14]. These results indicate that c-MET activation may be independent of HGF in ovarian cancer. Furthermore, point mutations of the *MET* gene [15] or activation of other receptors [16], such as EGFR, semaphorin 4D receptor, or α_5-integrin, can activate Met. Thus, targeting c-MET directly may be suitable for ovarian cancer treatment.

BMS-777607 is an ATP-competitive MET kinase inhibitor that primarily targets several MET family members, including RON, c-MET, Axl, and Tyro3 [17]. This molecule has been demonstrated to suppress HGF-stimulated prostate cancer metastasis [18]. BMS-777607 also suppressed c-MET auto-phosphorylation and downstream signaling in PC-3 cells with constitutively activated c-MET [19]. This implies that BMS-777607 could be used for inhibiting HGF-independent c-MET auto-phosphorylation for treating ovarian cancer. In addition to the MET family, BMS-777606 has been reported to block Mer, Flt-3, Aurora B, Lck, and vascular endothelial growth factor (VEGF) receptor 2 at higher concentrations [17]. The inhibition of Aurora B by BMS-777607 was also reported to induce polyploidy in breast cancer cell lines [20]. These results demonstrate that BMS-777607 is a multikinase inhibitor that can inhibit carcinogenesis through different mechanisms.

In this study, we used a c-MET constitutive phosphorylation tumor model to mimic HGF-independent activation and evaluated the antitumor effects of BMS-777607 on ovarian cancer cells. Ovarian cancer cells with constitutively activated c-MET were first screened. The viabilities of cells with constitutively activated c-MET and overexpressed c-MET as well as of c-MET-negative cells were compared after BMS-777607 treatment. The phenotypes of BMS-777607-treated ovarian cancer cells, including cell apoptosis, migration, and polyploidy, were further evaluated to determine the effects of BMS-777607. In addition, the antitumor effects of BMS-777607 were further demonstrated in xenographic mouse tumor systems. In summary, this study demonstrated that BMS-777607 affects tumorigenesis through the inhibition of multikinases.

Materials and methods

Reagents and antibodies

BMS-777607 was purchased from MedChemExpress (Monmouth Junction, NJ). Moreover, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DAPI, propidium iodide (PI), Liu'sstain solution A and B were purchased from Sigma. Antibodies against c-Met, p-c-Met (Y1234/Y1235), Gab1, p-Gab-1, Gab2, p-Gab2, PI3K, p-PI3K, AKT, p-Akt, p-c-raf, cyclinA2, cyclinB1, cyclinD1, p-histone H3, Aurora A, Aurora B, α-tubulin, and β-actin were purchased from Cell Signaling Technologies (Beverly, MA, USA). An Annexin V apoptosis detection kit with PI was purchased from Biolegend (San Diego, CA).

Mouse lines and cell lines

NU/NU female mice were purchased from BioLASCO, Taiwan. The animals were housed under specific pathogen-free conditions. All procedures were conducted in accordance with approved protocols and recommendations for the appropriate care and use of laboratory animals. Human ovarian cancer cell lines, namely SKOV3, ES-2, TOV112D, TOV21G, A2780, OVCAR3, and OVCAR8, were obtained from the American Type Culture Collection. The cell lines were cultured in RPMI medium supplemented with 10% (v/v) fetal bovine serum, 100 units/mL of penicillin, and 100 units/mL of streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Western blot analysis

Western blot analysis for specific protein detection was performed according to a previously mentioned protocol. Briefly, 50 μg of cell total lysate protein was resolved using a 7.5% SDS-PAGE gel and electrotransfered to a polyvinylidene difluoride membrane. The membrane was blocked in blocking buffer with 5% skim milk, after which it was probed with the primary antibodies diluted according to the manufacturer's instructions and incubated overnight at 4 °C. The membrane was further incubated using relevant secondary antibodies with conjugated horseradish peroxidase. Subsequently, the membrane was incubated with an enhanced chemoluminescence substrate, and the signal was detected using a chemoluminator.

Cell viability analysis

The cells were seeded in a 96-well plate at a density of 5×10^3 cells/well and exposed to various concentrations of BMS-7776007 for 72 h. At the end of the treatment, the cells were incubated in MTT reagent for 3 h and the precipitated dye was solubilized in 100% DMSO. The absorbance of each well was determined using a microplate reader at 570 nm, with the reference wave length being 690 nm. The viability of the cells was calculated as the ratio of the absorbance from treated samples to that of the untreated control sample.

Apoptosis assay

The cells were treated with 2×10^{-5} M BMS-7776007 for 24, 48, and 72 h and stained using the Annexin V apoptosis detection kit with PI. The proportion of apoptotic cells was determined using flow cytometry.

Cell migration assay

Cell migration was determined using an in vitro “wound-healing” assay. Briefly, the cells were seeded in ibidi chambers (culture inserts) (2×10^4 cells/window) and cultured overnight. The culture inserts were removed the next day, and the cells were treated with 2×10^{-5} M BMS-7776007. Wound closure was monitored using a light microscope. Images were recorded at 0 and 24 h by using a light microscope fitted with a charge-coupled device camera.

Cell cycle analysis

The cells were treated with 2×10^{-5} M BMS-7776007 for 48hand fixed in chilled ethanol overnight. After washing with 1 × phosphate-buffered saline (PBS), the cells were further incubated in 1 × PBS with 20 mg/mL of PI and 20 mg of RNase A for 30 min at room temperature (RT). The DNA content was analyzed using a FACSC aliburflow cytometer (BD Biosciences, DNA Staining Protocol for Flow Cytometry).

Liu's staining

Ten thousand SKOV3 cells were seeded on a cover slip and cultured overnight for attachment. The cells were then treated with 2×10^{-5} M BMS-777606 for 48 h. The cells were rinsed with PBS and stained with Liu's stain according to the manufacturer's instructions. The slides were then rapidly but gently rinsed under running tap water. They were then dried and examined under a light microscope.

Immunofluorescence staining

Twenty thousand SKOV3 cells were seeded on a cover slide and cultured overnight for attachment. The cells were then treated with 2×10^{-5} M BMS-777606 for 48 h and then fixed in cold methanol (−20 °C).The cells were treated for 30 min at RT with an anti-α-tubulin antibody to stain α-tubulinin of the cells. After rinsing with PBS, the slips were further incubated with PE-conjugated secondary antibodies for 30 min at RT. The cells were counter stained with DAPI, and the cover slips were mounted using antifade mounting media (Sigma). The fluorescent images were visualized and recorded using a Zeiss Axioplan2 imaging microscope (Carl Zeiss, Göttingen, Germany).

SKOV3 tumor model treated with BMS-777607 in vivo

Nu/Nu mice were intraperitoneally injected with 1×10^{6} SKOV3-Luc cells to form tumors. After 5 days, BMS-777607 (50 mg/kg daily) or an equal volume of water (control) was administered orally to these tumor-bearing mice. The tumor growth was monitored in terms of luminescence activity and determined using a noninvasive IVIS system (Xenogen, Grantham, UK).To determine the tumor burden, chemiluminescent images of the tumor areas of each mouse were calculated using ImageJ software.

Results

Inhibition of c-MET phosphorylation by BMS-777607 reduced the viability of ovarian cancer cells

BMS-777607 was first considered an inhibitor of c-MET kinase. Thus, the antitumor effect of constitutive c-MET phosphorylation blockade by BMS-777607 warrants investigation. Fig. 1A shows that the SKOV-3 cells are ovarian cancer cells with constitutively phosphorylated c-MET. These cells were more sensitive to BMS-777607 treatment than were the ES-2 cells with overexpressed c-MET and the c-MET-negative A2780 cells during in vitro cell proliferation (Fig. 1B; $p < 0.0001$, SKOV3 versus ES2 at 1×10^{-5} M BMS-777607; $p < 0.0001$, SKOV3 versus ES2 at 2.5×10^{-5} M BMS-777607; $p = 0.0468$, SKOV3 versus A2780 at 1×10^{-5} M BMS-777607; $p = 0.0392$, SKOV3 versus A2780 at 2.5×10^{-5} M BMS-777607). This result demonstrates that c-MET phosphorylation blockade can reduce the proliferation of ovarian cancer cells with constitutively phosphorylated c-MET.

Effects of BMS-777607 on the c-MET signaling transduction pathway and bio-behavior of SKOV3 cells

Induction of c-MET signaling transduction has been considered to be involved in cell proliferation, cell motility, and cell cycles. For evaluating the effects of BMS-777607 on the SKOV3 cells, we first analyzed the pattern of expression levels and phosphorylation status of c-MET downstream proteins after BMS-777607 treatment. The expression and phosphorylation of immediate c-MET-associated Gab1 and Gab2 proteins decreased (Fig. 2A). The phosphorylation of

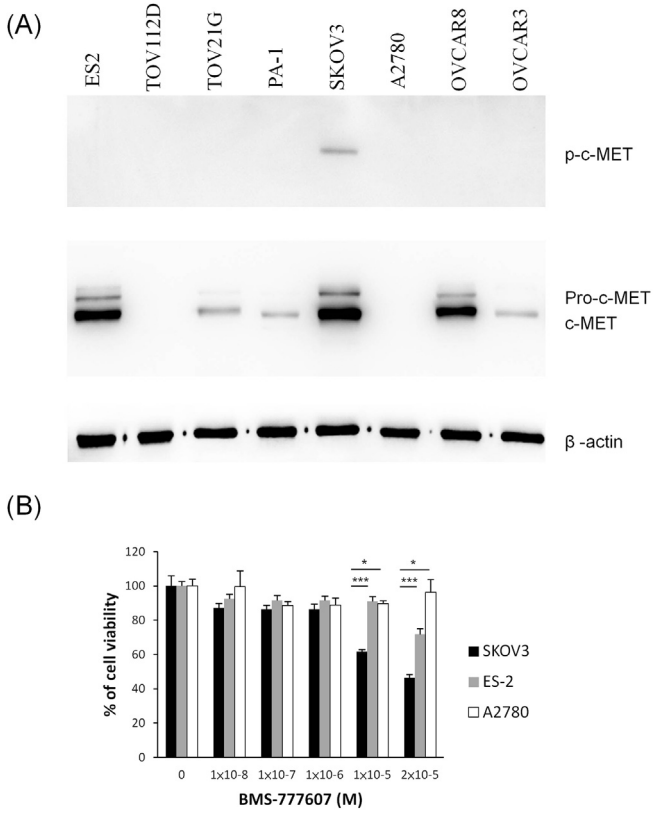

Fig. 1. Relationship between constitutive phospho-c-MET expression and cell viability after treatment with BMS-777607. (A) Western blot analysis of total c-MET and phospho-c-MET expression in various human ovarian cancer cell lysates. (B) MTT assay of SKOV3, ES-2, and A2780 cells co-cultured in media containing various concentrations of BMS-777607 for 72 h. The results demonstrated that the SKOV3 cells with constitutively phosphorylated c-MET were more sensitive to BMS-777607 than the ES-2 cells with overexpressed c-MET and c-MET-negative A2780 cells. Error bars in the figure represents the standard error. *$p < 0.05$, **$p < 0.0001$.

PI3K was also inhibited but not that of AKT or Raf. Cells treated with BMS-777607 also exhibited apoptosis (Fig. 2B; $p < 0.05$, BMS-777607 versus dimethyl sulfoxide (DMSO) at 24 h; $p < 0.01$, BMS-777607 versus DMSO at 48 and 72 h). Migration of the SKOV-3 cells was also inhibited (Fig. 2C; $p < 0.0001$, BMS-777607 versus control at 24 h). These results demonstrate that BMS-777607 can inhibit constitutively phosphorylated c-MET in the SKOV3 cells. Parts of the c-MET downstream cascade, such as Gab1, Gab2, and PI3K, were also blocked. All these effects were observed as the inhibition of the proliferation and migration of the SKOV3 cells.

Effects of BMS-777607 on the cell cycle and mitosis of the SKOV3 cells

Because BMS-777607 inhibited the proliferation of the SKOV3 cells, the effects of this compound on the cell cycle and mitosis in SKOV3 cells were also investigated. Expression of cyclin family proteins was first detected by Western blot analysis after BMS-777607 treatment. The expression of both cyclin A2 and cyclin B1 decreased, where as the protein level of cyclin D1 increased (Fig. 3A). These results demonstrate that the cell cycle regulation of the BMS-777607-treated SKOV3 cells occurred at the G2/M transition.

Apart from c-MET, BMS-777607 is known to inhibit other kinases, particularly Aurora kinases. Thus, the effect of BMS-777607 on the expression and activity of Aurora kinases of SKOV3 cells

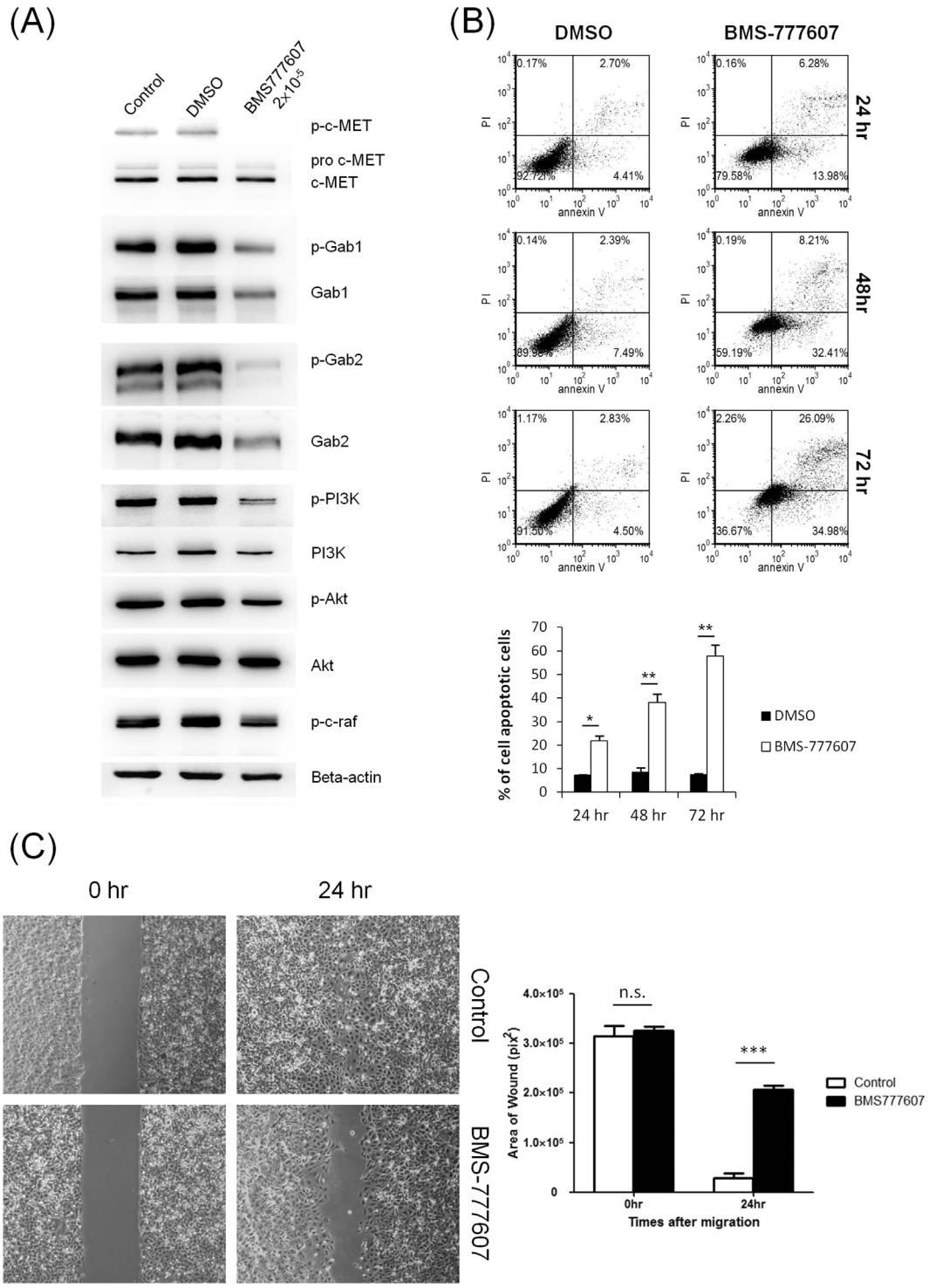

Fig. 2. Effects of BMS-777607 on c-MET-associated signaling pathways and behavior of the SKOV3 cells. (A) Western blot of c-MET signaling-associated proteins of the SKOV3 cells treated with BMS-777607. (B) Cell apoptosis assays. The percentage of apoptotic cells was determined through flow cytometry based on PI and annexin V staining. (C) Wound healing analysis. These results imply that inhibition of c-MET phosphorylation by BMS-777607 can increase apoptosis and inhibit the migration of SKOV3 cells. Bars in each chart in this figure represent the standard error. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

was also investigated. The levels of Aurora A and Aurora B (proteins) decreased (Fig. 3A). This effect also caused a decrease in Histone H3 phosphorylation and led to the formation of highly polyploid cells (Fig. 3B and C) as well as α-tubulin spindle assembling disorder (Fig. 3D). All these results demonstrate that BMS-777607 causes cell cycle arrest and polyploidy through the inhibition of Aurora A and B expression.

Antitumor effects of BMS-777607 on the SKOV3 cells in vivo

Because BMS-777607 inhibited the proliferation and division of the SKOV3 cells with constitutively phosphorylated c-MET, we investigated whether this compound could inhibit tumor growth in vivo. BMS-777607 (50 mg/kg) was orally administered to SKOV3 tumor-bearing mice daily. The tumors in the BMS-777607-treated

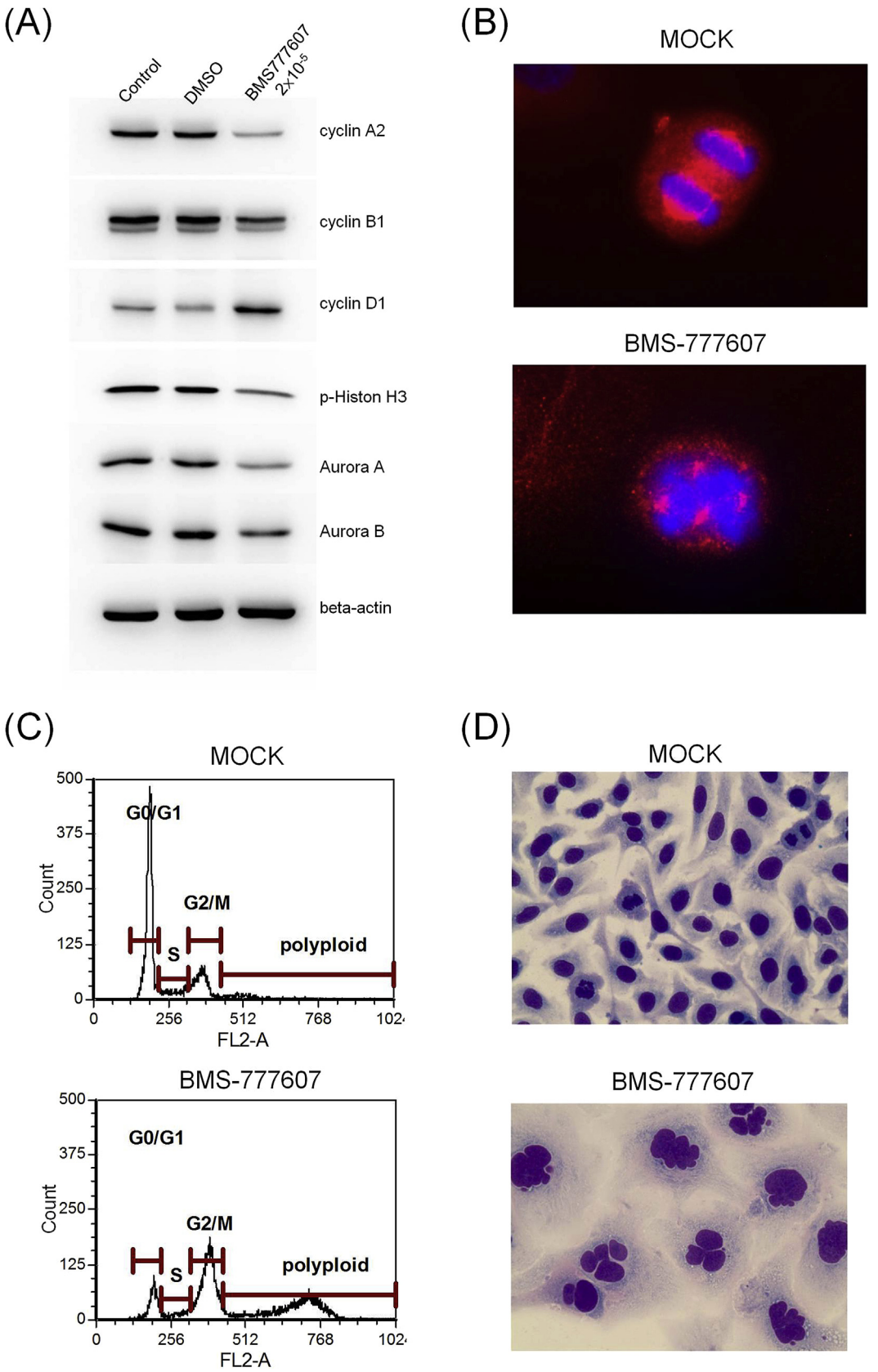

Fig. 3. Effect of BMS-777607 on mitosis in SKOV3 cells. (A) Western blot of cell cycle- and mitosis-associated proteins in SKOV3 cells treated with BMS-777607 for 48 h. (B) Immunofluorescent staining of α-tubulin in SKOV3 cells treated with BMS-777607. The colors red and blue in the figure represent tubulin and DNA, respectively. (C) Flow cytometric analysis of cell cycle with PI DNA staining in SKOV3 cells treated with BMS-777607. (D) Changes in the nuclear morphology of the SKOV3 cells treated with BMS-777607. These data demonstrate that BMS-777607 can affect the cell cycle, mitosis regulation, protein expression, and Histone H3 phosphorylation in SKOV3 cells. The SKOV3 cells treated with BMS-777607 also induced α-tubulin assembling disorder and polyploidy.

group were smaller than those in the control group 30 days after cell inoculation (Fig. 4; p = 0.0439, control group versus BMS-777607 group at day 30). The tumor burden, represented by the tumor dissemination area, was also milder in the BMS-777607-treated group than in the control group (p = 0.0091, control group versus BMS-777607 group at day 30). These results

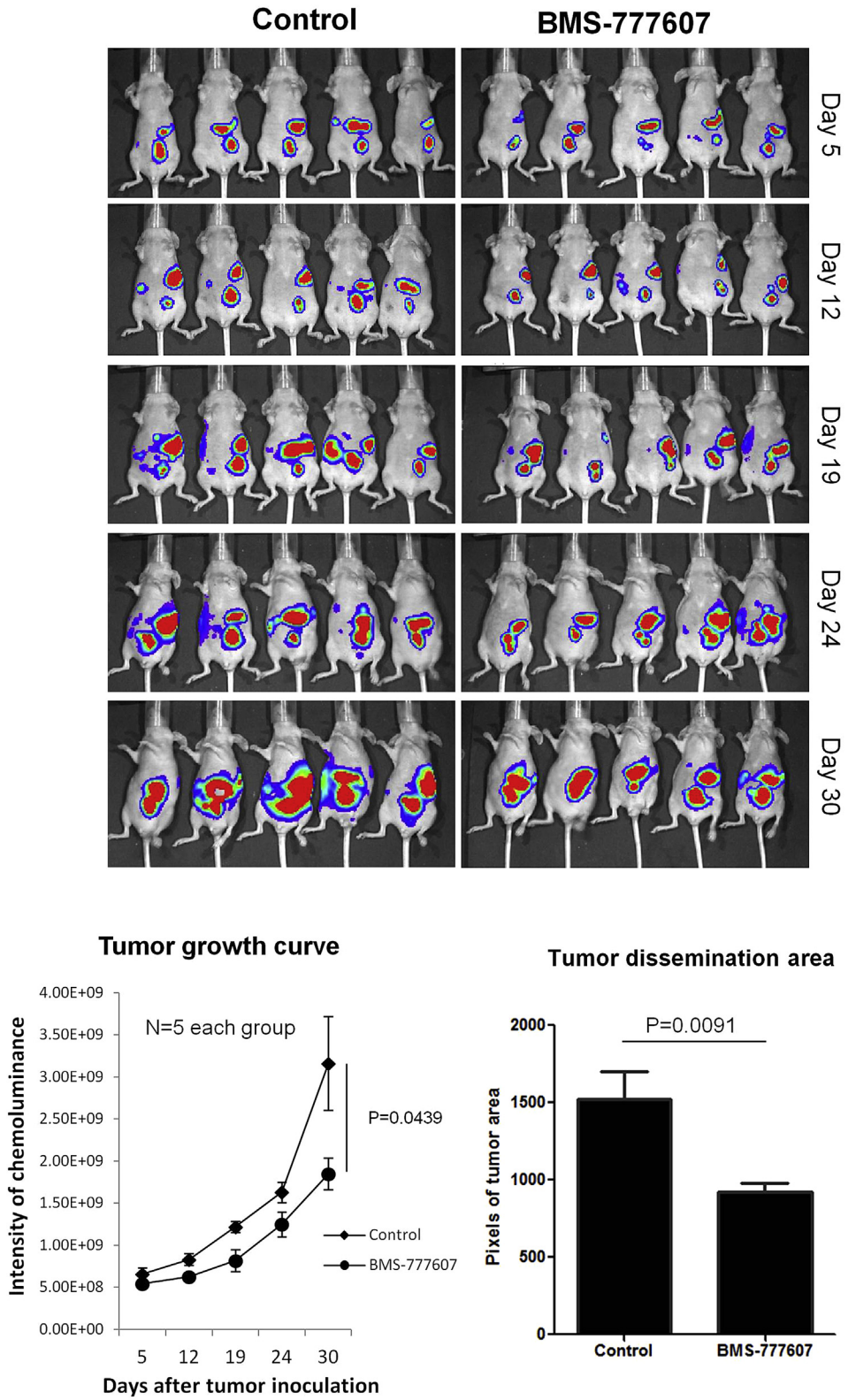

Fig. 4. In vivo antitumor effects of BMS-777607 in SKOV3 cells constitutively expressing phosphorylated c-MET 50 mg/kg of BMS-777607 daily was orally administered to SKOV3 cell xenograft nude mice (water was administered to the control mice). The tumor sizes were monitored using an IVIS system. The tumor burden was proportional to the size of cell dissemination area. The results demonstrated inhibition of tumor growth (p = 0.0439, control versus BMS-777607) and reduction in tumor burden (p = 0.0091, control versus BMS-777607) due to BMS-777607 treatment in the ovarian cancer cells with constitutively phosphorylated c-MET. Error bars in each chart represent the standard error.

demonstrate that the growth of the SKOV3 tumor was inhibited in mice treated with BMS-777607. The tumor burden in mice treated with this compound was also reduced.

Discussion

The design of targeted therapies has improved considerably after the molecular characterization of ovarian cancer [21,22]. Therapies that target multiple tumorigenesis effects are favored in drug development. c-MET was indicated to play a crucial role in tumor growth and metastasis because of crosstalk with multiple signaling pathways. Other evidence also shows that MET can regulate VEGF-A signaling and may be involved in tumor invasion and angiogenesis [23]. All these results suggest that inhibiting c-MET activation could block multiple phenomena to achieve antitumor effects.

Many small molecules that target VEGF receptor or various receptor tyrosine kinases were evaluated in preclinical studies and clinical trials of ovarian cancer [24]. Although they cannot induce an immune response against tumor cells as can monoclonal antibody drugs, small molecular inhibitors can penetrate the cytoplasmic membrane to suppress the phosphorylation of proteins inside the cells [25].Compared with therapeutic monoclonal antibody drugs, small molecular inhibitors generally possess lower specificity. Hence, these inhibitors might inhibit several signaling pathways at a clinically feasible dosage and can reduce the possibility of resistance. This evidence suggests that small molecular inhibitors have high clinical value in cancer therapy.

Our data demonstrate that BMS-777607 has higher cell growth inhibition effects in cells with constitutively activated c-MET than in other cells. However, the growth of cells that did not exhibit c-MET activation or expression was also affected after BMS-777607 treatment. This indicates that c-MET phosphorylation blockade was not the only method to inhibit ovarian cancer cell growth. Aurora B is the other target of BMS-777607 at concentrations higher than IC50 [17]. Aurora B inhibition can reduce the phosphorylation of histone H3, thus disturbing the stability of α-tubulin and inducing polyploidy [26,27]. Whether concomitant inhibition of c-MET and Aurora B could cause synergistic effects and reduce the viability of cells with constitutively activated c-MET requires further investigation.

A combination of targeted therapy and chemotherapy or different signaling inhibitors was previously a trend for tumor treatment. However, the efficacy of combinational treatment with tyrosine kinase inhibitors and chemotherapy is controversial. A combination of EGFR TKI and chemotherapy did not surpass monotherapy in a total of six randomized controlled trials of non-small-cell lung cancer [28]. Although BMS-777607 possesses a moderate antitumor effect alone, research reported that cells treated with BMS-777607 may induce drug resistance to chemotherapy because of the induction of polyploidy [20]. A combination of BMS-777607 and the mTOR inhibitor AZD8055 was revealed to reduce cell polyploidy and achieve a maximal synergistic cytotoxic effect on cancer cells derived from pancreatic cancer and cancer stem cells [29]. Thus, large-scale screening to identify small molecules that can induce synergistic effects with BMS-777607 is necessary.

Constitutive activation of receptor tyrosine kinases was reported to involve a gene mutation. Because the HGF level in the microenvironment of ovarian cancer is low, c-MET constitutive activation in ovarian cancer cells remains unexplained. Active mutation of c-MET in human cancer was first identified in both somatic and inherited forms of human renal papillary carcinomas [30]. Identifying the mutation in the kinase domain of c-MET will facilitate the selection of a strategy for targeted therapy between c-MET and HGF blockade.

In conclusion, BMS-777607 exhibits the highest inhibition of cell growth in ovarian cancer cells constitutively expressing c-MET. Treating SKOV3 cells with BMS-777607 could reduce c-MET activation and inhibit downstream cell signaling, thus causing cell apoptosis and polyploidy as well as cell cycle and cell migration inhibition. This molecule also inhibited tumor growth (induced using the SKOV3 ovarian cancer cells) in a mouse xenograft model in vivo. Examining the synergistic effect of BMS-777607 with other therapeutic agents is warranted for identifying effective therapies for ovarian cancer.

Conflict of interest

There is no interest conflict.

Acknowledgements

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