A new signal transducer and activator of transcription 3 inhibitor, BBI608, in malignant mesothelioma cell lines

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ABSTRACT Chronic inflammation plays a key role in the pathogenesis of malignant pleural mesothelioma as a result of asbestos exposure. Signal transducer and activator of transcription 3 (STAT3) signaling is a major pathway for cancer inflammation. The purpose of this study was to investigate the effectiveness of a new STAT3 inhibitor, BBI608, on mesothelioma cells in vitro and in vivo. The results showed that BBI608 blocked STAT3 phosphorylation, with and without suppression of IL-6. The effects of BBI608 on STAT3 downstream target genes such as cyclin D1 and survivin involved a decrease in cell viability with induction of apoptosis and cell cycle arrest. Nuclear translocation of phosphorylated STAT3 was blocked by BBI608. In addition, the agent showed significant antitumor activity in xenograft mouse models implanted with mesothelioma cells. These findings suggest that BBI608 may be a novel agent for malignant mesothelioma.

INTRODUCTION Malignant pleural mesothelioma (MPM) is a rare malignant tumor arising from mesothelial cells of the pleura. It accounts for less than 0.3% of all cancers. It is generally accepted that asbestos exposure is responsible for MPM carcinogenesis. Although asbestos has been banned in most industrialized countries, the highest incidence of MPM is expected to occur in the coming decades because of the long latency between exposure and diagnosis. Most patients present with unresectable disease at diagnosis or are deemed inoperable due to age or medical comorbidities. Although several treatment options have been introduced, the median survival time is approximately 12 months. The standard first-line treatment for advanced MPM, cisplatin plus pemetrexed chemotherapy, has a short and insufficient efficacy, and no validated treatment beyond first-line therapy is available. Novel therapeutic strategies are therefore needed.

Systemic inflammatory responses play an important role in cancer progression, and chronic inflammation contributes to almost every aspect of tumor development. Although the mechanisms of MPM carcinogenesis are not completely understood, chronic inflammation is involved in
Inhibitors of STAT3 signaling can be divided into agents that act either directly or indirectly\(^{19}\). Several compounds, including Static (6-Nitrobenzo[b] thiophene-1,1-dioxide), BBI608 (napabucasin), and STA-21 inhibit STAT3 by directly binding to the protein\(^{20-22}\). Indirect inhibition of STAT3 can be achieved by interrupting the kinases upstream of STAT3 signaling using JSI-124 (cucurbitacin I), which is a small molecule inhibitor that targets the JAK/STAT3 pathway\(^{23}\). Although the role of STAT3 signaling in mesothelioma cell lines is still unknown, this pathway may represent an attractive therapeutic target. Thus, it is important to determine the role of STAT3 activation in mesothelioma cells. In this study, we determined the antitumor activities of a new STAT3 inhibitor, BBI608, on mesothelioma cells in vitro and in vivo.

**MATERIALS AND METHODS**

1. **Cell lines and culture conditions**

Four human mesothelioma cell lines (MESO-1, H2452, H28, and MSTO-211H) were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were incubated in a humidified 5% CO\(_2\) environment at 37°C.

2. **Agent**

BBI608 was purchased from Selleck Chemicals (Houston, TX, USA). Stock solutions of the agent (1 mM) prepared in dimethyl sulfoxide (DMSO) were stored at -20°C.

3. **Cell viability assay**

Cells (3,000-4,000 cells/well) were seeded into 96-well plates and continuously exposed to each drug for 72 h. Cell viability was measured using the modified 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl...
tetrasolium bromide (MTT) assay. IC_{50} was defined as the concentration resulting in a 50% reduction in growth compared with the control cell growth.

4. Western blot analysis and antibodies

Mesothelioma cells were treated with different concentrations of DMSO (control) or BBI608 for 4 h and then harvested. For IL-6 stimulation experiments, the cells were serum-starved for 24 h and pretreated with DMSO (control) or BBI608 for 4 h. Then, 40-60 ng/mL recombinant human IL-6 (Abcam, Cambridge, MA, USA) was added, and the cells were collected 30 min later. The collected cells were washed with cold phosphate-buffered saline (PBS) and lysed in ice-cold Mammalian Protein Extraction Reagent lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Cell lysates were clarified by centrifugation (14,000 rpm for 20 min), and the protein concentrations were determined by BCA protein assay (Thermo Fisher Scientific). Whole cell extracts were resolved using SDS-PAGE, transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA), blocked with 5% nonfat milk, and probed with primary antibodies overnight at 4°C. Antibodies targeting phosphorylated STAT3 (Tyr705), STAT3, cyclin D1, PARP, Bcl-xL, survivin, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Cell Signaling Technology (Danvers, MA, USA). Membranes were then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and visualized using enhanced chemiluminescence reagent (GE Healthcare).

5. Cell cycle and apoptosis assays using flow cytometry

Cells were seeded in a 6-well plate and treated with DMSO (control) or BBI608 for 24 h. After removing the culture medium, the cells were harvested and washed with PBS. For cell cycle detection, the cells were resuspended in PBS containing 0.2% Triton X-100 and 1 mg/mL RNase in the dark and stained with 50 μg/mL propidium iodide (PI). For the apoptosis assay, apoptotic cells were monitored using the annexin V–FITC/PI apoptosis detection kit (Nacalai Tesque, Kyoto, Japan) and the MEBSTAIN Apoptosis TUNEL Kit Direct (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer’s protocols. Flow cytometric analyses were performed using the FACSVers instrument (BD Biosciences, San Jose, CA, USA).

6. Enzyme-linked immunosorbent assay for IL-6

Cells were seeded in six-well plates in RPMI 1640 containing 10% FBS for 24 h and then were washed with PBS and cultured for an additional 24 h in serum-free medium. To examine the effect of BBI608 on IL-6 secretion, the cells were treated with either DMSO (control) or BBI608 for 4 h in serum-free medium, followed by collection of the medium. The levels of IL-6 in the supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) using a human IL-6 ELISA Ready-SET-GO kit (Thermo Fisher Scientific) according to the manufacturer’s protocols.

7. Immunofluorescence analysis of STAT3 localization

Cells were seeded on glass coverslips in six-well plates. The next day, the cells were cultured in serum-free medium for 24 h and pretreated with BBI608 for 4 h, followed by induction with IL-6 for 30 min. The cells were then fixed with paraformaldehyde, permeabilized with methanol, blocked with PBS containing 0.3% Triton X-100 and 1% bovine serum albumin (BSA), and then incubated with the anti- phosphorylated STAT3
antibody (1:100 dilution) overnight at 4°C. The cells were probed with Alexa 594-conjugated goat anti-rabbit secondary antibody (1:200 dilution, Abcam) in the dark at room temperature for 1 h. Cell nuclei were stained with 4', 6'-diamino-2-phenylindole, and the coverslips were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were captured using the Nikon Eclipse 600 microscope and NIS-Elements D30 software (Nikon, Tokyo, Japan).

8. Immunohistochemical staining

Immunohistochemical (IHC) staining was performed on 4-μm-thick sections of formalin-fixed, paraffin-embedded tumor tissues. Sections were deparaffinized using a series of xylene, graded ethanol, and water immersion steps. After microwave antigen retrieval using Dako Target Retrieval Solution (Agilent, Santa Clara, CA, USA), the sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. The slides were blocked with 5% BSA and then incubated with antibodies against Ki-67 (1:400 dilution, Abcam) and phosphorylated STAT3 (1:100 dilution) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies (Agilent). The slides were visualized using 3, 3-diaminobenzidin (Agilent) and counterstained with hematoxylin. Apoptosis in the tumor specimens of the mouse model was examined by TUNEL assay using the MEBSTAIN Apoptosis TUNEL Kit Direct according to the manufacture's protocol. Images of apoptotic cells in the sections were captured using the Nikon Eclipse 600 fluorescent microscope. The percentage of positive cells was determined by counting at least 1,000 cells from multiple randomly selected areas under identical conditions.

9. Mouse xenograft tumor model

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kawasaki University Graduate School of Medicine (Ethics Committee reference number: 17-072). H28 (6×10⁶ cells/mouse) and MSTO-211H (4×10⁶ cells/mouse) were injected subcutaneously into the right flank area of 6-7-week-old female BALB/cAJcl-nu/nu mice, which were purchased from CLEA Japan (Tokyo, Japan). After the tumors reached 100-150 mm³ in size, ten mice were randomly allocated to the following PBS as the vehicle control once daily or BBI608 (40 mg/kg once daily). Each treatment was administered via intraperitoneal injection for 21 days. Tumor growth was determined by measuring the length (L) and width (W) of the tumors three times a week using calipers, and the tumor volume (mm³) was calculated as 0.5×W²×L. After the mice were sacrificed, the tumors were harvested, snap-frozen in liquid nitrogen, and stored at -80°C. Part of the tumor tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The levels of phosphorylated STAT3 and Ki-67 were examined by IHC staining. The TUNEL assay was used to detect the percentage of apoptotic cells in the xenograft tumors.

10. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). All experiments were performed three times, and statistical significance was determined using the paired two-tailed Student’s t-test. A value of P < 0.05 was considered to indicate significance. *P < 0.05; **P < 0.01.

RESULTS

1. BBI608 suppressed the growth of mesothelioma cell lines.

STAT3 activation is important for cell proliferation and survival. We first evaluated the
cytotoxic effect of BBI608 in mesothelioma cell lines using the MTT assay. Treatment with BBI608 for 72 h resulted in a dramatic decrease in cell viability in a dose-dependent manner (Fig. 1). The IC₅₀ values (µM; mean ± SEM) from three separate experiments were 0.40 ± 0.01 for MESO-1, 0.37 ± 0.02 for H2452, 0.36 ± 0.01 for H28, and 1.24 ± 0.05 for MSTO-211H.

2. BBI608 induced cell cycle arrest.

To determine whether BBI608-mediated growth suppression was mediated by induction of cell cycle arrest or apoptosis, we performed cell cycle analysis. It was reported previously that the growth inhibitory effects of BBI608 are mediated by cell cycle arrest at the G₀/G₁ phase[25]. Mesothelioma cells were treated with increasing concentrations of BBI608 for 24 h, and the percentages of cells in different phases of the cell cycle were determined. The distributions of cells in each phase are shown in Fig. 2. Treatment with BBI608 resulted in cell cycle...
arrest at the G0/G1 phase in MESO-1 and H2452 cells and in increased percentages of sub-G1 phase in H28 and MSTO-211H cells.

3. BBI608 induced apoptosis.

To evaluate the apoptosis-inducing effect of BBI608 in mesothelioma cells, the cells were treated with the inhibitors for 24 h, stained with annexin V–fluorescein isothiocyanate (FITC)/PI, and subjected to the TUNEL assay. The apoptotic cells were evaluated by flow cytometry, which showed that treatment with BBI608 induced apoptosis in a dose-dependent manner. The early and late apoptotic cells are shown in the lower and upper right quadrants, respectively. The TUNEL assay showed significantly increased apoptosis in the groups treated with 10 μM BBI608 for 24 h. The percentages of apoptotic cells after BBI608 treatment were counted, and the cumulative data from three independent experiments are expressed as means ± SEM. **P < 0.01 compared with the control group.

4. BBI608 suppressed STAT3 phosphorylation with the related proteins.

Abnormally activated STAT3 has been implicated in the development of various human cancers. STAT3 activation relies on the phosphorylation at Tyr705, and this phosphorylation event induces its dimerization, nuclear translocation, and DNA binding. We first examined whether BBI608 suppresses constitutive STAT3 activation in MESO-1, H2452, MSTO-211H, and H28 cells, all of which show constitutive STAT3 activation (Fig. 4A). Western blot analysis showed that treatment with BBI608 decreased phosphorylation of STAT3 at Tyr705, without affecting the total STAT3 level, in each mesothelioma cell line in a dose-dependent manner after 4 h of treatment (Fig. 4A). As mentioned previously, STAT3 activated the transcription of a variety of genes responsible for
cell cycle regulation, anti-apoptotic effects, and other cancer hallmarks\textsuperscript{10}. In a time course study, we found that BBI608 suppressed the transcription of STAT3 downstream target proteins such as cyclin D1 and survivin in a time-dependent manner, but had minimal effect on Bcl-xL in many cells (Fig. 4B). The level of phosphorylated STAT3 was upregulated in some cell lines after prolonged exposure (8-12 h) to BBI608. Our results also showed that BBI608 induced the cleavage of poly (ADP-ribose) polymerase (PARP), indicating apoptosis in mesothelioma cells.

5. \textit{BBI608 suppressed IL-6-induced STAT3 phosphorylation.}

IL-6 has been reported to induce STAT3 phosphorylation and play a role in cancer development\textsuperscript{26}. We found that exogenous IL-6 (40 or 60 ng/mL) stimulated STAT3 phosphorylation in MESO-1, H2452, and H28 cells (Fig. 5A). MSTO-211H cells did not respond to IL-6 stimulation at up to 100 ng/mL (data not shown). The induction of STAT3 phosphorylation by IL-6 was inhibited with BBI608 and the level of total STAT3 was not affected by treatment with BBI608 or IL-6 (Fig. 5A). These results showed that BBI608 inhibited IL-6-induced STAT3 phosphorylation.

6. \textit{BBI608 suppressed secretion of IL-6.}

Because the IL-6 gene is under the control of STAT3\textsuperscript{27}, we determined whether BBI608 blocked the secretion of IL-6. Basal IL-6 secretion levels in culture supernatants were determined by ELISA. Although all cell lines exhibited detectable basal production of IL-6 (15.3 ± 0.6 pg/mL in MESO-1, 4.5 ± 1.0 pg/mL in H2452, 35.3 ± 2.8 pg/mL in...
H28, and 14.6 ± 0.8 pg/mL in MSTO-211H cells), the levels of IL-6 production varied widely depending on the cell type. After treatment with BBI608 for 4 h, the secretion of IL-6 was inhibited in MESO-1, H2452, and H28 cells, but in MSTO-211H cells (Fig. 5B).

7. **BBI608 suppressed nuclear translocation of STAT3.**

As a transcription factor, the translocation of STAT3 from the cytoplasm to the nucleus is important. To determine whether BBI608 affects STAT3 nuclear translocation, we performed an immunofluorescence assay. In cells at rest without IL-6 stimulation, the activated form of STAT3 was localized predominantly in the nucleus and BBI608 suppressed nuclear translocation of phosphorylated STAT3 in all cell lines (Fig. 6). After IL-6 stimulation in MESO-1, H2452, and H28 cells, the nuclear level of phosphorylated STAT3 increased and BBI608 suppressed this nuclear accumulation of phosphorylated STAT3 in stimulated cells (Fig. 6). IL-6 stimulation did not increase nuclear accumulation of phosphorylated STAT3 in MSTO-211H cells (data not shown). Together, these results showed that BBI608 blocked STAT3 nuclear translocation. Furthermore, in mesothelioma cells responding to IL-6 stimulation, the nuclear accumulation of phosphorylated STAT3 by IL-6 was also blocked BBI608.

8. **BBI608 suppressed the growth of H28 and MSTO-211H xenograft tumors.**

To examine the potential of using BBI608 as mesothelioma therapy, we next evaluated the inhibitory effect on tumor growth in vivo. H28 and MSTO-211H xenograft models were created, although MESO-1 and H2452 xenograft models...
Yamagishi T, et al.: A new STAT inhibitor in malignant mesothelioma cells

Could not be generated despite multiple attempts.

Following 21 days of BBI608 (40 mg/kg once per day) treatment, the tumor volumes and weights were estimated. BBI608 significantly suppressed tumor growth compared with the vehicle control group in H28 and MSTO-211H xenograft mice (Fig. 7A, B). The body weights of the mice in both groups showed no significant difference over the experimental period (Fig. 7C). IHC analyses showed decreased STAT3 phosphorylation and Ki-67 expression, indicating the anti-proliferative effects of BBI608 (Fig. 8A). TUNEL assays showed that BBI608 induced tumor cell apoptosis in the mice (Fig. 8B).
DISCUSSION

We demonstrated significant inhibitory effects by BBI608 on STAT3 phosphorylation, nuclear translocation, and transcriptional activity in mesothelioma cell lines. The inhibitors also showed significant antitumor activities in xenograft mouse models.

Several genetic alterations and critical molecular signaling pathways have been identified as contributing to MPM development and progression. These include the p53/DNA repair, cell cycle, mitogen-activated protein kinase, and phosphoinositide 3/AKT pathways and the immune-checkpoint gene VISTA. Growing evidence has also shown that inflammatory signals from the surrounding microenvironment promote tumor growth and progression. In addition, inflammatory signaling has been associated with survival pathways and immunosuppression. It has been suggested that chronic inflammation due to asbestos exposure plays a critical role in the development and progression of MPM. The hallmarks of asbestos fiber inhalation include sustained inflammation, linked to the release of numerous cytokines, and generation of reactive oxygen species, which cause oxidative DNA damage. Several proinflammatory cytokines released by immune cells have been shown to promote cancer cell proliferation and progression, and increased expression of IL-6 is thought to act as a link between chronic inflammation and tumor development. In addition, IL-6/STAT3 inflammatory cytokine signaling is a protumorigenic factor in breast, lung, colon, prostate, ovary, skin, and hematological malignancies. MPM has been reported to be an IL-6-secreting tumor. The increased expression of IL-6 in cancer cells suggests that IL-6 is an important autocrine growth factor that promotes tumorigenesis. Furthermore, activated STAT3 can also upregulate IL-6 induction via a positive IL-6 autocrine loop. Adachi et
al. reported that IL-6, in an autocrine manner, is involved in MPM growth via STAT3 signaling\(^2\). One of the most important downstream target genes of IL-6 is STAT3, which is constitutively activated in various types of cancers, including mesothelioma. IL-6 induces STAT3 phosphorylation at Tyr705 via the JAK pathway\(^3\). We found no altered activity of JAK1 or JAK2, which are not phosphorylated in MESO-1, H2452, H28, or MSTO-211H cells, after treatment with BBI608 (data not shown).

STAT3 activation results in the expression of downstream targets, such as cyclin D1, survivin, Bcl-xl, and Bcl-2\(^10\), which promote cell proliferation and resistance to apoptosis\(^35\). Numerous novel molecular inhibitors of STAT3, such as BBI608, LLL12, and LY5, have been reported to suppress cancer cells and tumor growth\(^21,36,37\). Although the function of IL-6 has been investigated extensively, little is known about the biological function of STAT3 in MPM. Among novel STAT3 inhibitors, BBI608 is well examined in the patients with refractory advanced colorectal cancer as a phase 3 study. In the patients harboring phosphorylated STAT3 in the tumor, overall survival was longer in the BBI608 group than in the placebo group, although there was no difference in overall survival between groups in the overall unselected population\(^38\). We therefore investigated whether STAT3 is a druggable target in MPM cells. BBI608 blocked constitutive STAT3 phosphorylation in all cell lines, as well as IL-6-induced STAT3 phosphorylation in MESO-1, H2452, and H28 cells. The effects of BBI608 on STAT3 downstream target proteins included decreased cell viability and increased apoptosis with cell cycle arrest. The immunostaining results showed that BBI608 effectively inhibited phosphorylation of STAT3 in the nucleus. We found that both IL-6 production and STAT3 phosphorylation were suppressed in most cells treated with BBI608.

Concerning the toxicity of BBI608, the body weights of the mice treated with the drug and with vehicle were similar (Fig. 7C). In the phase 3 study comparing BBI608 to placebo in the patients with colorectal cancer, more patients who received BBI608 had any grade of treatment-related diarrhea (79% vs 19%), nausea (51% vs 24%), and anorexia (38% vs 16%) than did patients who received placebo\(^38\). Grade 3 or worse adverse events of BBI608 and placebo were abdominal pain (4% vs 3%), diarrhea (15% vs 1%), fatigue (10% vs 6%), and dehydration (4% vs 1%). Thus, adverse events of BBI608 were generally mild.

In conclusion, BBI608 was effective in our preclinical MPM models and might suppress the IL-6/STAT3 signaling axis by reducing autocrine IL-6 production. Further studies are required to elucidate other mechanisms such as blocking cancer stemness\(^39\).

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DISCLOSURE STATEMENT.

The authors have no conflicts to disclose.

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