Antitumor activity of the PD-1/PD-L1 binding inhibitor BMS-202 in the humanized MHC-double knockout NOG mouse

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ABSTRACT

Recently, the first series of small molecule inhibitors of PD-1/PD-L1 were reported by Bristol-Myers Squibb (BMS), which were developed using a homogeneous time-resolved fluorescence (HTRF)-based screening investigation of the PD-1/PD-L1 interaction. Additional crystallographic and biophysical studies showed that these compounds inhibited the interaction of PD-1/PD-L1 by inducing the dimerization of PD-L1, in which each dimer binds one molecule of the stabilizer at its interface. However, the immunological mechanism of the antitumor effect of these compounds remains to be elucidated. In the present study, we focused on BMS-202 (a representative of the BMS compounds) and investigated its antitumor activity using *in vitro* and *in vivo* experiments. BMS-202 inhibited the proliferation of strongly PD-L1-positive SCC-3 cells (IC₅₀ 15 μM) and anti-CD3 antibody-activated Jurkat cells (IC₅₀ 10 μM) *in vitro*. Additionally, BMS-202 had no regulatory effect on the PD-1 or PD-L1 expression level on the cell surface of these cells. In an *in vivo* study using humanized MHC-double knockout (dKO) NOG mice, BMS-202 showed a clear antitumor effect compared with the controls; however, a direct cytotoxic effect was revealed to be involved in the antitumor mechanism, as there was no lymphocyte accumulation in the tumor site. These results suggest that the antitumor effect of BMS-202 might be partly mediated by a direct off-target cytotoxic effect in addition to the immune response-based mechanism. Also, the humanized dKO NOG mouse model used in this study was shown to be a useful tool for the screening of small molecule inhibitors of PD-1/PD-L1 binding that can inhibit tumor growth via an immune-response-mediated mechanism.

Novel anticancer immunotherapies based on immune checkpoint blockade (ICB) have achieved remarkable success in the last several years. ICB-based cancer immunotherapy using monoclonal anti-

bodies (mAbs) shows durable antitumor effects and produces long-lasting remission in patients with a variety of cancers (7, 12–14, 17, 21, 25, 26, 28). However, mAb therapies, such as anti-programmed death (PD)-1/programmed death-ligand (PD-L)1 Abs, are expensive and still have a number of disadvantages, such as immunogenicity, which is typical of human mAbs, low oral bioavailability, poor solid-tumor tissue uptake, a limited half-life and, most importantly, mAb-related toxicity (immune-related adverse effects, irAEs) (10, 33). In contrast,

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small-molecule compound-based therapeutics can have a greater affinity and specificity for targeted molecules than antibodies. Additionally, small molecule inhibitors have been shown to lack immunogenicity and to be good orally bioavailable and inexpensive. However, the development of small molecule inhibitors of the PD-1/PD-L1 pathway is currently far behind antibody development.

Recently, Zak *et al.* revealed the molecular features of the human PD-1/PD-L1 interaction based on the X-ray structure of the complex, and several hot spots located on the PD-L1 molecule were shown to be involved in the formation of the complex (15, 30).

To date, several small molecules, macrocyclic peptides and peptide mimetics targeting the PD-1/PD-L1 interaction have been reported (8, 18, 19), primarily in patent applications, but almost no fully validated and qualified therapeutics exist. Recently, the binding action and biological activities of potent small molecule inhibitors of PD-1/PD-L1 have been reported by Bristol-Myers Squibb (BMS) (1, 31, 32). The first series of small molecule inhibitors of PD-1/PD-L1 interaction identified using a homogeneous time-resolved fluorescence (HTRF) binding assay-based screening method was reported in a patent document (31). In the crystallographic study, BMS-202 was shown to inhibit the interaction by inducing the dimerization of PD-L1. Gunik *et al.* demonstrated that BMS-202 is located at the center of the PD-L1 homodimer in a deep hydrophobic pocket between the two-PD-L1 molecules (11).

In the present study, we focused on BMS-202 (a representative BMS compound with biological activity) and investigated its antitumor activity using *in vitro* and *in vivo* experiments. In particular, for the *in vivo* studies, we used humanized major histocompatibility complex (MHC)-double knockout (dKO) NOD/Shi-*scid*-IL2 γ^{null} (NOG) mice (29), and investigated the immunological effect of the PD-1/PD-L1 inhibitor BMS-202 on human lymphoma SCC-3 cells.

MATERIALS AND METHODS

Chemicals. The BMS-202 (Fig. 1A) compounds were purchased from Selleck Chemicals (Houston, TX, USA) and Ark Pharm, Inc. (Arlington Heights, IL, USA). The former was used for the *in vitro* (cell proliferation assay and flow cytometry) experiment. The latter was used for the cell proliferation assay and *in vivo* experiments. The biological activities of both compounds were compared in the cell prolifer-

ation assay. These compounds were suspended in a sterile 0.5% methyl cellulose 400 cp solution (Wako, Tokyo, Japan) or dissolved in dimethyl sulfoxide (DMSO) (Wako).

Cell lines and reagents. The human lymphoma SCC-3 and Jurkat cell lines were purchased from JCRB (Saito, Osaka, Japan) and ATCC (Manassas, VA, USA), respectively, and maintained in RPMI 1640 (SIGMA, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA). Anti-programmed death-ligand 1 (PD-L1, CD274)-APC and anti-PD-1 (CD279)-APC antibodies purchased from BioLegend Inc. (San Diego, CA, USA) were used for flow cytometric analysis. The anti-CD3 antibody was purified from the supernatant of the anti-human CD3 (OKT3) hybridoma cell line (ATCC) and used for the stimulation of Jurkat cells.

Cell proliferation assay. Cell proliferation was examined using the WST-1 assay (Dojin Kagaku Corp., Kumamoto, Japan) as described previously (2). Briefly, 1×10^4 SCC-3 cells were seeded in a 96-well microculture plate (Corning Inc., Corning, NY), and compounds at concentrations ranging from 0.25 to 100 μM were added. A total of 1×10^4 Jurkat cells were seeded in a 96-well microculture plate coated with anti-human CD3 antibody (2 $\mu\text{g}/\text{mL}$) at 4°C overnight and incubated with the BMS-202 compound. After 4 days, the WST-1 substrate was added to the culture, and the optical density (OD) was measured at 450 and 620 nm using an immuno-reader (Nivo, PerkinElmer Inc., Waltham, MA, USA).

Flow cytometry analysis. SCC-3 or Jurkat cells treated with 25 μM BMS-202 compound for 24 h were washed and stained with anti-PD-1 or anti-PD-L1 antibody for 15 min at 4°C. The staining method was described previously (3). The stained cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences, San Diego, CA, USA).

Development of humanized NOG-dKO mice. Six-week-old NOG-dKO mice were kindly supplied by Dr. Mamoru Ito, the Central Institute for Experimental Animals (Kawasaki, Japan). All animals were cared for and treated humanely according to the Guidelines for the Welfare and Use of Animals in Cancer Research, and the experimental procedures were approved by the Animal Care and Use Committee of Shizuoka Cancer Center Research In-

Fig. 1 Chemical structure of BMS-202 and effect of BMS-202 on cell proliferation. **(A)** Formula, $\text{C}_{25}\text{H}_{29}\text{N}_3\text{O}_3$. Molecular weight: 419.52. **(B)** The proliferation of SCC-3 and anti-CD3 antibody-stimulated Jurkat cells was inhibited by BMS-202. Each point represents the mean $\pm$ SD of triplicates. Open circle; BMS-202 (Selleck), closed circle; BMS-202 (Ark Pharm).

stitute.

The experimental procedures used were described previously (3). Briefly, eight-week-old NOG-dKO mice were irradiated with X-rays (2.5 Gy) and 1×10^7 human peripheral blood mononuclear cells (PBMCs) with the human leukocyte antigen (HLA)-A*0201 genotype were intravenously (i.v.) administered to each mouse on day 0. A total of 2×10^5 SCC-3 cells with the HLA-A*0201 genotype were subcutaneously transplanted into three mice in each group on day 1. The daily injection of BMS-202 at a dosage of 20 mg/kg was started on day 14 and administered a total of 9 times. The tumor volume was calculated based on the National Cancer Institute formula as follows: tumor volume (mm^3) = length (mm) $\times$ [width (mm)]² $\times$ 1/2.

Immunohistochemistry (IHC). Anti-CD4 and anti-CD8 antibodies (Thermo Fisher Scientific), anti-granzyme B antibody (DAKO), anti-CD204 antibody (TransGenic Inc.), and anti-PD-L1 antibody (BioLegend Inc) were purchased and used for IHC analysis as described previously (3).

Statistical analysis. Significant differences were analyzed using Student's *t*-test. Values of $P < 0.05$ were considered to be indicative of statistical significance.

RESULTS

BMS-202 inhibited cell proliferation of SCC-3 and Jurkat cells

The IC_{50} value for the cytotoxic activity of the PD-1/PD-L1 binding inhibitor BMS-202 in SCC-3 cells and anti-CD3 Ab-activated Jurkat cells was 15 μM and 10 μM , respectively (Fig. 1B). BMS-202 showed moderate cytotoxicity similar to other BMS-series small compounds of 2-methyl-3-biphenyl-methanol moiety-containing compounds (BMS-8, -37 and -242), had EC_{50} values between 3 and 10 μM (22).

BMS-202 had no effect on PD-1 or PD-L1 expression

Flow cytometry analysis of BMS-202-treated SCC-3 cells or activated Jurkat cells demonstrated that BMS-202 had no regulatory effects on the PD-1 or PD-L1 expression level at the cell surface (Fig. 2).

BMS-202 inhibited the tumor growth of SCC-3 cells in humanized NOG-dKO mice

The experimental design and treatment schedule used for BMS-202 therapy are shown in Fig. 3A. BMS-202 showed 41% growth inhibitory activity against humanized mouse-transplanted human lymphoma SCC-3 cells (Fig. 3B). In addition, the body weight-reducing effect, which was a measure of the

Fig. 2 PD-1/PD-L1 expression levels on the cell surface. BMS-202 showed no significant effect on the PD-1/PD-L1 expression level. The PD-1 and PD-L1 expression levels measured by flow cytometry on SCC-3 and anti-CD3 antibody-stimulated Jurkat cells treated with various doses of BMS-202, are shown. Red line: isotype control, blue line: anti-PD-1 or anti-PD-L1 antibody.

Fig. 3 Inhibitory effect of BMS-202 on the growth of SCC-3 tumors *in vivo*. **(A)** Experimental design and treatment schedule used for BMS-202 therapy of SCC-3 tumors in humanized MHC-dKO NOG mice. Beginning on day 14, BMS-202 was administered intraperitoneally for 9 days. **(B)** V/V0 values of BMS-202-treated SCC-3 tumors ($n = 3$) are shown. The efficacy of BMS-202 treatment is expressed as the mean V/V0 value, where V is the tumor volume on the day of evaluation and V0 is the tumor volume on the day of treatment. **(C)** Body weight change in BMS-202-treated mice bearing SCC-3 tumors. Closed circle; control, open circle; BMS-202-treated group. Each point represents the mean $\pm$ SD value derived from the measurement of SCC-3 tumors in three mice.

Fig. 4 Effect of BMS-202 on SCC-3 tumors and infiltrating immune cells in SCC-3 tumors. Images of control and BMS-202-treated tumors stained with H&E and anti-CD8, anti-granzyme B, CD204 and anti-PD-L1 antibodies. Magnification: $\times 200$.

systemic toxicity, was stronger in the BMS-202-administered group than in the control group (Fig. 3C)

BMS-202 did not promote TIL accumulation in tumors

SCC-3 tumors treated with BMS-202 demonstrated increased necrosis and poor viability (Fig. 4) compared with the control group. Generally, the induction of CTL (cytotoxic T lymphocyte) activity killing tumor cells and promotion of intratumoral infiltration of immune effector cells like CTLs are immune responses frequently observed in patients with can-

cer treated with antibodies targeting immune checkpoint molecule (27). However, in the present study, the numbers of tumor-infiltrating lymphocytes (TILs), which consisted of $CD8^+$ and granzyme B^+ T cells in the central area of the tumor, was markedly diminished compared to that in the control (Fig. 5). These results might suggest that a direct cytotoxic effect rather than immune response was likely to be involved in the antitumor mechanisms. Furthermore, PD-L1 protein staining was reduced by damage to the tumor tissue. Tumor-associated macrophages (TAM), which infiltrate into tumor tissues, are well

Fig. 5 Effect of BMS-202 on the number of infiltrating immune cells in SCC-3-tumors. More than 10 areas of tumor in each tumor section stained with various antibodies were analyzed at high magnification (×200) using Winroof image analysis software. The positive cell counts per field were compared between the control and BMS-202-treated groups. Each column shows the mean±SD value. * $P < 0.05$, statistically significant.

known to be involved in cancer progression. CD204 is a representative pro-tumor marker for M2-type TAMs (24). There was a tendency towards a decrease in the CD204-positive cell number in BMS-202-treated tumors compared to control tumors (Fig. 5).

DISCUSSION

Recently, several small molecule inhibitors targeting the PD-1/PD-L1 pathway that are based on peptidomimetics (9, 23) and macrocyclic peptides (16) have been reported. The small molecule inhibitors developed by BMS are structural derivatives of (2-methyl-3-biphenyl)methanol. The capability to block PD-1/PD-L1 complex formation was evaluated using the HTRF assay. Among the most potent inhibitors, BMS-202 inhibited the formation of the PD-1/PD-L1 complex, with an IC_{50} value of 0.018 μ M (31). Despite the fact that BMS-202 has poor druglike properties and is unlikely to become a lead compound, the results show that targeting the PD-1/PD-L1 interaction surface is feasible not only by using anti-PD-1/PD-L1 antibodies, but also by using anti-PD-1/PD-L1 small molecule compounds (11). However, a substantial evidence that BMS-202 can regulate tumor growth based on specific immunological mechanisms has not been proven at the pre-clinical level, including animal models.

Our study shows the *in vitro* and *in vivo* activity of the small molecule PD-1/PD-L1 inhibitor BMS-202 in preclinical studies. To define the highest concentrations of BMS-202 that could be used *in vitro* assays, the cytotoxicity of BMS-202 was evaluated by a proliferation assay using strongly PD-L1-positive SCC-3 cells and WST-1. Compared with the most cytotoxic derivatives of (2-methyl-3-biphenyl)methanol, such as BMS-37 and BMS-242, BMS-202 showed moderate cytotoxicity, with an IC_{50} value

of 13.9 μ M.

Two optimized (2,3-dihydro-1,4-benzodioxine-based inhibitors, BMS-1001 and BMS-1166, presented significantly improved cytotoxic properties (22), unlike the other three compounds tested (BMS-202, BMS-37 and BMS-242). Additionally, BMS-1001 and BMS-1166 restored the activation of effector Jurkat T cells; however, their potency was significantly weaker than that shown by anti-PD-1 and anti-PD-L1 antibodies (22). Further optimization based on the structural data may lead to the development of more potent PD-1/PD-L1 inhibitors. However, with regard to these compounds, no biological experiments indicating the immunological activity have been published to date.

Interestingly, a novel small molecule dual inhibitor of immune checkpoint PD-L1 and V-domain Ig-containing suppressor of T-cell activation (VISTA), CA-170, was recently investigated in a phase 1 trial of advanced solid tumors and lymphomas (6, 10).

In the present study, we used humanized NOG-dKO mice transplanted with human PBMCs and SCC-3 human lymphoma cells and found that BMS-202 treatment efficiently inhibited the growth of transplanted tumors. Moreover, the promotion by BMS-202 of tumor-infiltrating lymphocytes (TIL) accumulation within the tumor was not observed; rather, TIL numbers were diminished in BMS-202-treated tumors compared with control tumors as shown in Fig. 4. These results suggest that the anti-tumor effect of BMS-202 might be partly mediated by a direct off-target cytotoxic effect in addition to the immune response-based mechanism. Previously, we demonstrated in humanized NOG-dKO mice that anti-PD-1 antibody inhibited the growth of SCC-3 tumors through the promotion of active effector T cell accumulation in the tumor and reduced weight loss induced by SCC-3 tumor progression (4). In

contrast, BMS-202 administration resulted in remarkable TIL depletion and substantial weight loss in the current study.

Recently, BMS-202 has been reported to show a potent PD-1/PD-L1 protein binding inhibition, but result in weak cell-based PD-1/PD-L1 blockade activity and strong cytotoxicity activity (5), which might support our observation *in vivo* study using humanized dKO NOG mouse. Therefore, based on these observations, we are not aiming at developing BMS-202 as a PD-1/PD-L1 binding inhibitor.

The humanized NOG-dKO mouse model has clear advantages over other immune-deficient mouse models that use patient-derived immune cells and tumor cells. In *in vivo* study investigating the effects of a STAT3 inhibitor (STX-0119) and anti-PD-1 antibody on human immune cells (3, 4), the humanized NOG-dKO model was more successful compared to a conventional nude mice model. This demonstrated that this model will be a good tool for screening small molecule inhibitors of PD-1/PD-L1 binding and possibly lead to the development of potent novel immune-therapeutics for malignancies.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All animals were cared for and treated humanely according to the Guidelines for the Welfare and Use of Animals in Cancer Research, and the experimental procedures were approved by the Animal Care and Use Committee of Shizuoka Cancer Center Research Institute. The clinical experiments using peripheral blood mononuclear cells (PBMCs) derived from glioma patients were approved by the Institutional Review Board of Shizuoka Cancer Center (Authorization Number: 24-29).

COMPETING INTERESTS

The authors declare that they have no competing interests.

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