Blocking Effect of Chaga Mushroom (*Inonotus obliquus*) Extract for Immune Checkpoint CTLA-4/CD80 Interaction

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Abstract: *Inonotus obliquus*, also known as the Chaga mushroom, has been used as a traditional medicine to treat many different diseases in Asia. Ethanol and water extraction were performed to examine the blocking effect of the Chaga mushroom on the CTLA-4/CD80 interaction. The inhibitory activities of the Chaga mushroom/70% EtOH extract (CME) and the Chaga mushroom/water extract (CMW) were confirmed using several cell-based assays. To identify the contents of major compounds CME and CMW, we performed HPLC analysis. The content of lanosterol (1) in CME was 0.41%. Our findings provide experimental evidence that the Chaga mushroom can develop a small-molecule inhibitor that blocks the CTLA-4/CD80 interaction.

Keywords: immune checkpoints; CTLA-4; CD80; *Inonotus obliquus*

1. Introduction

Inonotus obliquus, commonly known as the Chaga mushroom, belongs to the family Hymenochaetaceae, and is a parasite mainly found on the birch tree (*Betula platyphlla* var. *japonica*). It is widely distributed in Russia, China, and Alaska, and has diverse bioactivities, such as anti-inflammatory effects [1], anti-nociceptive effects [2], and inhibition of cell cycle progression [3]. Several constituents, including triterpenoids [4,5], phenolic acids [6], and polysaccharides [7], have previously been reported as contributing to these bioactivities. The Chaga mushroom has been used for a long time as a medicinal mushroom in Asia, Russia, and North America to treat various human diseases, such as gastrointestinal disorders, cardiovascular diseases, and diabetes [8]. Although research on the Chaga mushroom is ongoing, its potential to block immune checkpoints, especially CTLA-4/CD80, is not well understood. We previously reported compounds derived from the medicinal plant that inhibit the immune checkpoint interactions PD-1/PD-L1 and CTLA-4/CD80 [9]. Here, we investigate the blocking effect of Chaga mushroom extract on the CTLA-4/CD80 interaction, by using in vitro assay and HPLC analysis.

When a T cell is exposed to a pathogen, an immune checkpoint-regulated immune response and an immune reaction by immune checkpoints are both essential for establishing immunological equilibrium. In lymph nodes, the interaction between CD80 on antigen-presenting cells (APCs) and CD28 on the naive T cell leads to T cell activation. Activated T cells are the key lymphocytes of the immune response, and cytotoxic T cells kill cancer cells. Among several immune checkpoints, CTLA-4 is expressed on naive T cells and interacts with CD80 molecules on cancer cells. The binding of CTLA-4 with CD80 then produces an inhibitory signal during the primary phase of T cell activation, resulting in the inhibition of T cell activation and a decreased immune response [10]. Cancer cells use this reaction to avoid the immune system and, consequently, survive in the body. Blocking the CTLA-4/CD80 interaction is one of the key targets for cancer immunotherapy, and has become a successful therapeutic strategy. The first

immune checkpoint inhibitor to be developed, CTLA-4 mAb (ipilimumab), was approved by the US Food and Drug Administration (FDA). However, patients treated with Ipilimumab suffered side effects, such as systemic, dermatologic, and respiratory issues. Additionally, challenges exist with monoclonal antibodies (mAbs) that are yet to be overcome, such as their high cost, long half-life, large molecular weight, and the possibility of immune-related adverse events [11,12]. Thus, the development of small-molecule inhibitors that interfere with the CTLA-4/CD80 interaction offers a solution to the disadvantages of drug discovery. In this study, we investigated an immune checkpoint inhibitor that blocks the CTLA-4/CD80 interaction, using a cell-based assay and HPLC analysis.

2. Materials and Methods

2.1. Plant Materials

Dried *Inonotus obliquus* was purchased from the Bomyeong Herbal Market, Seoul in 2019. Its identity was confirmed (by Dr. Wei Li). A voucher specimen (KIOM-26) was deposited at the Herbarium of Korean Medicine-Application Center, Korea Institute of Oriental Medicine, Republic of Korea.

2.2. Extraction

The dried *Inonotus obliquus* (50.0 g) was chopped and extracted with 70% EtOH using reflux, and the extract was filtered; then, the filtrate was evaporated to dryness (6.81 g). The dried *Inonotus obliquus* (50.0 g) was extracted by water, and the water solution was frozen and dried (40.0 g).

2.3. Instruments

The HPLC system used was a Thermo Scientific Dionex UltiMate 3000 system equipped with a binary pump, auto-sampler, diode array UV/VIS detector DAD, and column oven (Dionex Corp., Sunnyvale, CA, USA). The Corona CAD detector (Thermo Fisher, Couteauboeuf, France) was linked to the HPLC system. Highly pure nitrogen for the CAD detector was produced by a nitrogen generator at a nitrogen pressure of 63.8. Chromeleon 7 software (Thermo Fisher, Couteauboeuf, France) was used for data acquisition and processing.

2.4. Preparation of Standard and Sample Solutions

The 70% EtOH and Chaga mushroom water were extracted using an ultrasonicator (JAC Ultrasonic JAC-3010). Each sample was accurately weighed (300 mg) and dissolved in 10 mL of methanol for 30 min. After extraction, each solution was filtered through a 0.2 µm membrane. A 10 µL aliquot of the filtrate was analyzed using HPLC. A standard solution of lanosterol, betulin, and betulinic acid was prepared at 1.0 mg/mL (1000 ppm) with acetonitrile, and stored at 4 °C until use. A standard curve of the solution, diluted using methanol at each concentration, was created.

2.5. Chemicals and Antibodies

The CTLA-4/CD80 Inhibitor Screening Assay Kit (#72009) was purchased from BPS Bioscience (San Diego, CA, USA). Anti-CTLA-4 antibody (#A2001) was purchased from Selleck (Houston, TX, USA). Lanosterol, betulin, and betulinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from JT Baker (Phillipsburg, NJ, USA). ACS reagent-grade formic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA, purity of ≥99.0%). Water was prepared using a Puris-Evo RO water system (Mirae ST Co., Ltd., Anyang-si, South Korea). All solutions prepared for HPLC were filtered through 0.2 µm membrane filters before use.

2.6. Competitive ELISA

Competitive ELISA was performed using a CTLA-4/CD80 Inhibitor Screening Assay Kit, according to the supplier's instructions. Briefly, Fc-tagged human CTLA-4 (BPS Bioscience, #71149) was coated

onto a 96-well plate overnight. After washing, vehicle or test samples were diluted in solvent, and then added to each coated well. As a positive control, anti-CTLA-4 neutralizing antibodies were used. After 1 h of reaction with the compound, biotinylated hCD80 (BPS Bioscience, #71114) was added to each well, and the plates were incubated for 2 h at room temperature. After three washes with PBS-T, diluted streptavidin horseradish peroxidase (HRP) was added to each well, and the plates were reacted for 1 h while shaking at a low speed. After the reaction, HRP substrates A and B were added. Relative chemiluminescence was measured on a SpectraMax L luminometer (Molecular Devices, San Jose, CA, USA) and expressed as the relative binding activity. The results were normalized to the relative percentage of the vehicle control group. The half-maximal inhibitory concentration (IC_{50}) was calculated using the Prism “log[inhibitor] vs. normalized response” equation. All results are presented as the means of three independent biological replicates, with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, and compared with the vehicle control group [9,13,14].

2.7. CTLA-4 Blockade Bioassay

The bioluminescent cell-based assay was performed using a CTLA-4 blockade bioassay (Promega, Madison, WI, USA, #JA4001), according to the supplier’s instructions. To identify CMW and CME for inhibitory activity, 5×10^3 CD80/aAPC CHO-K1 cells were pre-incubated with F-12 (10% FBS) into a 96-well plate. After 24 h, CTLA-4 antibodies or CMW and CME were added by co-culture to the 1×10^4 CTLA-4 Jurkat T cells. Following mixing with Bio-Glo™ reagent (Promega, #G7940), the luminescence was measured using a GloMax® explorer multimode microplate reader (Promega). The fold induction of nuclear factor of the activated T cell (NFAT) luciferase reporter expression was calculated from the sample treated wells/untreated control wells.

2.8. HPLC Analytical Methods

We performed HPLC profiling of the Chaga mushroom/70% EtOH extract (CME) and the Chaga mushroom/water extract (CMW) for the mushroom’s three major compounds: lanosterol, betulin, and betulinic acid. HPLC analysis was performed on a Phenomenex Luna C18 column (250 mm $\times$ 4.6 mm, 5 μ m) coupled to a C18 guard cartridge (4.0 mm $\times$ 3.0 mm). The mobile phase was applied at a flow rate of 1 mL/min, with a gradient of 0.1% formic acid in water (eluent A) and acetonitrile (eluent B), which included the gradient elution as follows: 0–20 min, 90–100% B; 20–50 min, 100% B; 50–60 min, 90% B. The chromatogram was monitored using a CAD detector. The column temperature was set to 30 °C, the auto-sampler was set to 20 °C, and the injection volume was 10 μ L. Calibration curves, assessed by standard solution and the limits of detection (LOD) and quantification (LOQ) under the chromatographic conditions, were determined by injecting a series of standard solutions until the signal-to-noise (S/N) ratio for each compound was 3 for LOD and 10 for LOQ. Each sample was analyzed as three preparations from three parallel determinations. Data were collected and analyzed using Chromeleon 7 software (Thermo Fisher, Counteaboeuf, France).

2.9. Cell Viability in CTLA-4 Jurkat T Cells and CD80/aAPC CHO-K1 Cells

The cell viabilities of CME and CMW were measured using the CCK-8 assay (#CK04, Dojindo Molecular Technologies, Inc., Rockville, MD, USA), according to the manufacturer’s instructions. Briefly, cells were placed in 96-well plates at a density of 1×10^4 cells/well and were cultured for 24 h. After incubation, CME (0–200 μ g/mL) and CMW (0–200 μ g/mL) were treated and incubated for 24 h. At this point, the cells were treated with 10 μ L of CCK solution, and the mixture was kept for 2 h at 37 °C. A microplate reader (Molecular Devices i3, San Jose, CA, USA) was used to measure the absorbance at 450 nm.

3. Results

3.1. Chaga Mushroom Extract-Blocked CTLA-4/CD80 Interaction

To test whether the Chaga mushroom extract blocked the CTLA-4/CD80 interaction, we performed a binding inhibition assay using a competitive CTLA-4/CD80 enzyme-linked immunosorbent assay (ELISA) kit. When well-coated CTLA-4 binds to the biotinylated CD80, it produces chemiluminescence, which can then be measured using a chemiluminescence reader. The presence of our sample can block the CTLA-4/CD80 interaction so that it does not produce chemiluminescence. We observed that anti-CTLA-4 neutralizing antibodies completely inhibited the CTLA-4/CD80 binding (IC_{50} 0.29 $\mu\text{g/mL}$). Chaga mushroom/70% ethanol extract (CME) and Chaga mushroom/water extract (CMW) showed a concentration-dependent inhibition of CTLA-4/CD80 association with IC_{50} values of 24.05 $\mu\text{g/mL}$ and 45.24 $\mu\text{g/mL}$, respectively. CMW and CME at 50 $\mu\text{g/mL}$ showed inhibitory effects similar to those of the CTLA-4-blocking antibodies at 0.1 $\mu\text{g/mL}$ and 1.0 $\mu\text{g/mL}$, respectively (Figure 1).

Figure 1. Blocking effect of the CTLA-4/CD80 interaction of CMW and CME by competitive enzyme-linked immunosorbent assay (ELISA). Effect of anti-CTLA-4 neutralizing antibody (A); water extract (B); and 70% ethanol extract (C) of Chaga mushroom on the CTLA-4/CD80 binding activity. The relative binding activity was normalized to the relative percentage of the vehicle control group. The half-maximal inhibitory concentration (IC_{50}) was calculated using the Prism “log[inhibitor] vs. the normalized response” equation. All results are presented as the mean of three independent biological replicates, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the vehicle control group.

3.2. NFAT Reporter Activity of the Chaga Mushroom Extract

In order to evaluate the immunological activity of the CMW and CME, we tested the nuclear factor of the activated T cell (NFAT) assay. The CTLA-4 Jurkat T cells have a NFAT response when CTLA-4/CD80 is blocked by anti-CTLA-4 antibodies or small molecules sent to signal expression of luciferase. Once luciferase is expressed, it produces light in an enzymatic assay, and the amount of light can be measured. CMW and CME were treated to a four-fold dilution (1.56 $\mu\text{g/mL}$ to 12.5 $\mu\text{g/mL}$) on a co-culture well plate with CTLA-4 Jurkat T cells: CD80/aAPC CHO-K1 cells (1:1). We observed that the CMW and CME blocked CTLA-4/CD80 interaction. The luminescence of the CTLA-4/CD80 reporter assays that were treated with CMW and CME significantly increased (~3 fold and ~4.5 fold, respectively) at 12.5 $\mu\text{g/mL}$, higher than the negative control “cell only”, with EC_{50} values of 3.12 $\mu\text{g/mL}$ and 3.95 $\mu\text{g/mL}$, respectively. These results supported our observation of the ELISA assay results (Figure 2).

Figure 2. Nuclear factor of the activated T cell (NFAT) luciferase reporter activity by CTLA-4/NFAT Reporter-Jurkat T cells treated with Chaga mushroom/water extract (CMW) and Chaga mushroom/70% EtOH extract (CME). Fold luminescence of Jurkat T cells treated with CMW (A) and CME (B) at several concentrations (1.56 to 12.5 µg/mL). The half-maximal effective concentration (EC_{50}) was calculated using the Prism “log[agonist] vs. normalized response” equation. All results are presented as the mean of three independent biological replicates and compared with the vehicle control group.

3.3. Evaluation of Cytotoxicity of the Chaga Mushroom Extract in CTLA-4 Jurkat T and CD80/aAPC CHO-K1 Cells

To identify how CMW and CME can affect the cell viability of both CTLA-4 Jurkat T cells and CD80/aAPC CHO-K1 cells, they were used in the cell-based assay for CTLA-4/CD80 inhibitor screening. Several concentrations of CMW and CME were treated on CTLA-4 Jurkat T and CD80/aAPC CHO-K1 cells for 24 h. Then, the cell viability was assessed using a Cell Counting Kit-8 (CCK) assay. In all tested concentrations, neither the CMW nor the CME showed an effect on the cell viability of CTLA-4 Jurkat T cells or CD80/aAPC CHO-K1 cells. These results showed no cytotoxicity at the concentrations used in the previously described NFAT reporter assay (Figure 3).

Figure 3. Cell viability effects of the Chaga mushroom/water extract (CMW) and Chaga mushroom/70% EtOH extract (CME) on CTLA-4 Jurkat T and CD80/aAPC CHO-K1 cells. CME (A) and CMW (B) were treated with various concentrations (6.25 to 200 µg/mL) on CTLA-4 Jurkat T and CD80/aAPC CHO-K1 cells for 24 h, and the cell viability was assessed using a CCK8 assay. All results are presented as the mean of three independent biological replicates, with * $p < 0.05$, ** $p < 0.01$, compared with the vehicle control group.

3.4. Content Analysis of Lanosterol (1)

We investigated the amount of lanosterol (1) in the 70% EtOH extract of the Chaga mushroom that showed blocking of the CTLA-4/CD80 interaction via HPLC–CAD analysis. The calibration curve of the lanosterol (1) was $y = 0.2003x + 3.9707$ with good linearity ($r^2 = 0.9981$) in the concentration range tested (25–200 $\mu\text{g/mL}$). The LODs and LOQs of the lanosterol (1) were 0.1192 and 0.0477 $\mu\text{g/mL}$, respectively. To confirm the lanosterol in the sample chromatogram, we compared the results with the standard solution retention time, 29.14 min, and the UV spectrum (Figure 4). The content of lanosterol (1) in the 70% EtOH extract of Chaga mushroom was 0.41% (Figure 4). Consistent with the results of Chaga mushroom extract (Figure 1), lanosterol also interfered the interaction of CTLA-4 and CD80 in a dose-dependent manner (Figure S1). Considering this result, lanosterol might be suggested as one of active compound that blocked CTLA4/CD80 interaction in CME.

Figure 4. Structure of lanosterol (1) (A); UV spectra of the standard compound of lanosterol (1) and 70% ethanol extract (B); regression equations, LODs, and LOQs of lanosterol (C); and HPLC–CAD chromatograms (D).

4. Discussion

In this study, we performed an ELISA-binding assay of CME and CMW on the blocking of the CTLA-4/CD80 interaction in a concentration-dependent manner. Additionally, to evaluate whether the CME and CMW can promote T cell functional activity using the CTLA-4/CD80 blockade bioassay, both extracts were used to enhance T cell activation and increase the expression of luciferase. Moreover, we confirmed the constituents of the Chaga mushroom/70% ethanol extract via HPLC–CAD analysis.

Chaga mushroom (*I. obliquus*) has been used as a traditional therapy in Asia for a long time [1]. The anticancer effects of *I. obliquus* extract on various types of cells, including melanoma cells, human lung carcinomas, and human liver cancer cells, have been researched and reported [15–17]. However, the blocking effect of the immune checkpoint against Chaga mushroom extract is not understood.

The CTLA-4/CD80 inhibitory effect and T cell activation effects of CME and CMW extracts were confirmed using several cell-based assays. Competitive ELISA results showed that CME and CMW blocked CTLA-4/CD80 interaction in a concentration-dependent manner. It was also confirmed through CTLA-4 blocking assays that CMW and CME interfere with CTLA-4/CD80 interaction and activate T cells. CMW and CME were concentration-dependent, and did not show cell cytotoxicity in CTLA-4 Jurkat T and CD80/aAPC CHO-K1 cells. We conducted HPLC–CAD analysis to confirm the content of lanosterol. According to the standard curve solution, the amount of lanosterol (1) was diluted with methanol at each concentration. The established HPLC–CAD method was suitable for analysis of lanosterol (Figure 4). Lanosterol is present in the Chaga mushroom/ethanol extract at 0.41%. Lanosterol is one of the tetracyclic triterpenoids, and is the precursor to all animal and fungal steroids. Steroids

are hydrophobic compounds that have very low solubility in water, and we showed that lanosterol was detected only in the ethanol extract but not the water extract. As shown in Supplementary Material Figure S1, lanosterol tested competitive ELISA and it also blocked the interaction of CTLA-4/CD80. Although lanosterol has been suggested as one of active compound for Chaga mushroom ethanol extract, however, we will investigate other immune checkpoint inhibitors in the CMW extract in a future study.

Chaga mushrooms stimulate the immune system and show antitumor activity against diverse types of cancer [15–20]. In addition, the Chaga mushroom blocks CTLA-4/CD80 interaction and stimulates T cell activity. Terpenoids isolated from the herb medicinal plant showed immunological activity. [21] Triterpenoids isolated from the Chaga mushroom have been reported to show a variety of bioactivities, including those mentioned in ref. [22,23]. Thus, we suggest that the blocking effect of CME on the CTLA-4/CD80 interaction can be attributed to the abundant triterpenoid constituents in the CME. According to a previous study [24], adverse effects, such as oxalate nephropathy, can occur after the long-term usage of Chaga mushrooms. Administration of Chaga mushroom should be carefully monitored in patients with kidney function disorders, due to contraindications and side effects. In addition, Chaga mushrooms may interact with drugs such as aspirin and clopidogrel, given that the Chaga mushroom inhibits platelet aggregation [25].

5. Conclusions

We have investigated the immune check point inhibitor of herbal medicines. The blocking of the immune check point CTLA-4/CD80 interaction by the Chaga mushroom was confirmed using several cell experiments and HPLC analysis. The Chaga mushroom extracts did not show cell cytotoxicity, blocking of the CTLA-4/CD80 interaction, or activation of T cells. Our findings suggest that the Chaga mushroom can be developed as a new immune check point inhibitor. In future research, we will conduct an in vivo study to test the inhibitory effect of another triterpenoid compound.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/10/17/5774/s1>, Figure S1: Blocking effect of CTLA-4/CD80 interaction of lanosterol.

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