INTRODUCTION

The inflammatory tumor microenvironment (TME) is known to promote oncogenic transformation and malignant progression through genetic or epigenetic mechanisms.\(^3\) This pro-tumor role of cancer-associated inflammation is mediated by a variety of inflammatory immune cells through their production of cytokines and chemokines.\(^3\) Among these pro-inflammatory cytokines, members of the interleukin (IL)-6 family are known to play essential roles in tumor progression through the activation of signal transducer and activator of transcription 3 (STAT3).\(^2\)−\(^7\) STAT3 is a latent cytosolic transcription factor activated by the phosphorylation of tyrosine705 in its SH2 domain.\(^6\) Binding of IL-6 to its receptor leads to the activation of Janus kinase (JAK) family of protein tyrosine kinases, which results in the phosphorylation and activation of STAT3.\(^7\) In addition to STAT3 activation by IL-6, the over-activation of STAT3 plays an important role in different type of cancer, including breast cancers.\(^8\) It plays an essential role in selectively inducing and maintaining a pro-carcinogenic inflammatory microenvironment, both at the initiation of malignant transformation and during cancer progression\(^11\)−\(^18\); therefore, STAT3 is considered a promising target for cancer therapy.

Natural products are used for medical treatment and as valuable resources for drug discovery and development. Baicalein is an active constituent of the dried root of Scutellaria baicalensis Georgi, which is widely used as a traditional medicine in many countries. Baicalein is a flavonoid compound and has a numerous biological activities.\(^19\) In this study, we identified that baicalein suppresses STAT3 activity and has anti-metastatic effects in the breast cancer cell lines.

MATERIALS AND METHODS

Reagents Baicalein and baicalin were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). JSI-124 (cucurbitacin I hydrate) was purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). These compounds were dissolved in dimethyl sulfoxide (DMSO, FUJIFILM Wako Pure Chemical Corporation) and stored at \(-20^\circ\)C. The stock solution diluted in the relevant assay medium and 0.1% DMSO served as vehicle controls. \(\beta\)-Luciferin was obtained from Promega (Madison, WI, U.S.A.). The primary antibodies against STAT3 and p-STAT3 were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.), and the antibody against \(\beta\)-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cells The murine breast cancer cell line 4T1 (ATCC) and human breast cancer cell line MDA-MB-231 (ATCC) were maintained in RPMI-1640 medium containing 10% fetal bovine serum 1% antibiotics (penicillin and streptomycin) in 5% CO\(_2\) at 37°C. 4T1 cells stably expressing an STAT3-mediated luciferase gene (4T1-STAT3-C4) were established previously.\(^2\)\(^0\) Briefly, the tandem-repeated binding sites for STAT3 were subcloned into the pGL4.26 vector (Promega) and 4T1 cells were transfected with the reporter plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.). The cells were selected with Hygromycin B (100 \(\mu\)g/mL) and cloned by limiting dilution.

Cell Viability Assay Cell viability was quantified using the WST-8 Cell Counting kit (FUJIFILM Wako Pure Chemical Corporation) according to the manufacturer’s instructions. Cells (2 × 10\(^4\) cells/well) were seeded onto 96-well plates and

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co-cultured with test compounds. After the incubation with test compounds (48 h), WST-8 reagent was added and the absorbance at 450/620 nm was measured using a microplate reader.

**Western Blotting** Cells (10⁶ cells/well) were treated with tested compounds varying concentrations for 24 h. The treated cells were trypsinized and collected by adding phosphate buffered saline (PBS) and centrifuged for 10 min at 2000 rpm and 4°C. Then, the supernatant was discarded and the cells were lysed in whole-cell lysis buffer (25 mmol/L N-(2-hydroxyethyl)piperezine-N′-2-ethanesulfonic acid (HEPES), pH 7.7, 300 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 20 mmol/L β-glycerophosphate, 1 mmol/L Na₂VO₃, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 10 mg/mL aprotinin, and 10 mg/mL leupeptin). Cell lysates were subjected to electrophoresis by 7.5–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to Immobilon-P nylon membranes (Millipore, Bedford, MA, U.S.A.). The membranes were treated with Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) for at least 2 h, and probed with the indicated primary antibodies overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:1000 dilutions). Bands were visualized using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, Piscataway, NJ, U.S.A.). Primary antibodies used (at a dilution of 1:1000) were specific to STAT3 (79D7, #4904), p-STAT3 of 1:1000), and also specific to β-actin (C4, sc-47778, Santa Cruz).

**Wound Healing Assay** Cells were seeded on 24-well plates (2 x 10⁶ cells/well) and when they reached confluency, the cell monolayer was scraped by sterile 200-µL pipette tips, and fresh medium was added containing different concentrations of baicalein and JSI-124. After a 24-h incubation, cells were fixed and photographed. Images were acquired using inverted microscope and the percentage of migrated cells was expressed using 100% as the value of the untreated group.

**Transwell Migration Assay** A total of 10⁶ cells/well were pre-treated with tested compounds at varying concentrations for 24 h. A 100-µL aliquot of cell suspension containing 3 x 10⁶ cells were added to Transwell cell culture insert with filter (8-µm pore size; Whatman Japan K K, Tokyo, Japan) and incubated for 6 to 8 h. Non-migrated cells in the upper chamber was discarded using a cotton swab. The migrated cells were fixed in methanol and stained with hematoxylin and eosin. Migrated cells in five randomly selected fields were counted and photographed under an inverted microscope. The results were expressed as the percentage relative migrated cells compared with the untreated group.

**Enzyme-Linked Immunosorbent Assay (ELISA) Assay** Cells seeded in 6-well plates were pre-treated with test compounds at varying concentrations for 24 h. A 100-µL aliquot of cell suspension containing 3 x 10⁶ cells was added to Transwell cell culture insert with filter (8-µm pore size; Whatman Japan K K, Tokyo, Japan) and incubated for 6 to 8 h. Non-migrated cells in the upper chamber was discarded using a cotton swab. The migrated cells were fixed in methanol and stained with hematoxylin and eosin. Migrated cells in five randomly selected fields were counted and photographed under an inverted microscope. The results were expressed as the percentage relative migrated cells compared with the untreated group.

**Experimental Metastasis Model** BALB/c mice (6-week old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). All experiments were approved (A2016INM-7, A2019INM-5) and performed according to the guidelines of the Care and Use of Laboratory Animals of University of Toyama. Cells were inoculated intravenously (6 x 10⁵ cells/100-µL PBS/mouse) into mice. In Vivo Glo, Promega) was intraperitoneally injected into mice at 24 h after the tumor inoculation. After 10 min, the lungs were removed for bioluminescence assay using an in vivo imaging system (IVIS Lumina II, Caliper Life Sciences, Hopkinton, MA, U.S.A.).
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Statistical Analysis All data are expressed as the mean ± standard deviation (S.D.) of at least two or three independent experiments unless otherwise stated. Significance was analyzed using the Mann–Whitney U-test or one-way ANOVA followed by Bonferroni’s post-hoc comparisons tests. p < 0.05 was considered significant.

RESULTS

Baicalein to Suppressed STAT3 Activity in Breast Cancer Cells In order to identify a novel anti-cancer agent from natural products by targeting STAT3 activity, we used 4T1-STAT3-C4 cells expressing the firefly luciferase gene under the control of the STAT3 reporter. By screening the
both compounds in the STAT3 reporter assay. As baicalein demonstrated much stronger STAT3 inhibition than baicalin (0.01, 0.1, 1, 10 µM). After 24 h cells were harvested, and equal amounts of protein in cell lysates were analyzed by Western blotting. The actin protein levels were used to confirm that equal amounts of protein were subjected to electrophoresis.

![Biacalein (µM)](image)

**Fig. 3. Effects of Baicalein on STAT3 Phosphorylation in 4T1 Cells**

4T1 cells (1 × 10^6 cells/well) were seeded in 6-well plates and treated with baicalein (0.01, 0.1, 1, 10 µM). After 24 h cells were harvested, and equal amounts of protein in cell lysates were analyzed by Western blotting. The actin protein levels were used to confirm that equal amounts of protein were subjected to electrophoresis.

![Biacalein (µM)](image)

**Fig. 4. Effects of Baicalein on the Growth of 4T1 and MDA-MB-231 Cells**

(A) 4T1 cells or (B) MDA-MB-231 cells (2 × 10^4 cells/well) were treated with baicalein for 24 and 48 h at the indicated dose, and cell viability was evaluated as the relative cell viability to untreated control. The data are presented as the mean ± S.D. (*p < 0.01).

![Biacalein (µM)](image)

**Fig. 5. Effects of Baicalein on IL-6 Production by 4T1 Cells**

4T1 cells (2 × 10^5 cells/well) were seeded in 24-well plates, and treated with baicalein and JSI-124. After 24 h, culture supernatants were collected and cytokine IL-6 was quantified using the ELISA kit according to the manufacturer’s instructions. The data are presented as the mean ± S.D. (*p < 0.01).
and its ability to control their metastatic potential.

**DISCUSSION**

Breast cancer is highly malignant because of its aggressive metastatic potential. In general, metastasis is one of the key...
Bioactive compounds obtained from the dried roots of *Scutellaria baicalensis* Georgi, a species of flowering plant in the family Lamiaceae. Baicalein and baicalin were reported to have numerous pharmacological actions, including anti-cancer activity, 21–26) STAT proteins have dual roles: they transduce signals through the cytoplasm and function as transcription factors in the nucleus. Although some STAT proteins, such as STAT1, increase anti-tumor immunity, STAT3 is known to be involved in cancer-promoting inflammation. The activation of STAT3 increases tumor cell proliferation, survival, and invasion, while suppressing anti-tumor immunity. 27) STAT3 can be activated by inflammatory mediators, such as cytokines and chemokines, as well as by oncopgenic proteins, and its target genes are involved in multiple steps of metastasis. 27–29) In particular, the pro-inflammatory cytokine IL-6 drives cancer cell proliferation, survival and metastasis through the activation of the STAT3 pathway while also suppress the anti-tumor immune responses. 30) There are several reports regarding the anti-inflammatory effect of baicalein and their relationship with STAT3 inhibition in different types of cells, 22,31–33) including hematological malignancy. 34) Of note, Ke et al. recently reported that baicalein and baicalin decreased STAT3 activity to downregulate interferon-γ (IFN-γ)-induced programmed cell death-ligand 1 (PD-L1) expression and restored anti-tumor T cell activity. 35) However, we newly demonstrated the anti-metastatic action of baicalein through the inhibition of cancer-intrinsic STAT3 activity in breast cancer cells. Our study suggests baicalein as a potential therapeutic agent against breast cancer growth and metastasis through the inhibition of STAT3 activity.

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**Conflict of Interest** The authors declare no conflict of interest.

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