AS602801, an Anti-Cancer Stem Cell Drug Candidate, Suppresses Gap-junction Communication Between Lung Cancer Stem Cells and Astrocytes

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Abstract. *Background/Aim:* Cancer stem cells (CSCs) are associated with tumorigenesis, recurrence, and metastasis. Cell–cell communication via gap junctions (GJs) between metastatic cancer cells and astrocytes is necessary for brain metastasis. Agents targeting communication between CSCs and astrocytes are expected to suppress brain metastasis. *Materials and Methods:* Using the A549 CSC, a cancer stem-like cell derived from A549, we examined the effect of AS602801, an anti-cancer stem cell agent whose safety has been confirmed in a phase 2 clinical trial, on GJ communication and connexin expression using a dye-transfer assay and immunoblot analysis, respectively. *Results:* AS602801 specifically suppressed cell–cell communication in A549 CSCs without any suppression of GJ communication in astrocytes; it also decreased the expression of connexin 43, a constituent of GJs, in A549 CSCs. *Conclusion:* The anti-cancer stem cell agent, AS602801, is a potential drug candidate against brain metastasis.

Metastatic brain tumors are one of the most intractable tumors and are frequently found at metastatic sites. Several types of cancers including lung cancer, breast cancer, and melanoma can spread to the brain (1-3). Among them, the lung is the most common (40-50%) primary site with 25-40% of non-small cell lung carcinoma (NSCLC) patients showing brain metastasis (4). Cancer metastasis proceeds

through a multistep process. When cancer cells metastasize to the brain, cross-talk between the metastatic cancer cells and astrocytes plays a pivotal role. The metastatic cancer cells communicate with astrocytes through gap junctions (GJs) that directly link the cytoplasm of the two cells (5, 6). Cell–cell communication via GJs has been reported to regulate brain metastasis at various steps (6-9). It has recently been reported that GJs consisting of connexin 43 (Cx43), the principal GJ constituent protein in epithelial lung cancer cells and astrocytes, promote brain metastasis of cancer cells via cGAMP transfer between cancer cells and astrocytes (5). Cancer stem cells (CSCs) contribute significantly to the establishment of metastasis (10, 11). A GJ inhibitor, oleamide, has been found to suppress metastatic foci of MDA-MB-231 cells intravenously injected into mice, resulting in increased survival (12). The oral drugs tonabersat, a neurogenic inflammation inhibitor and meclofenamate, a non-steroidal anti-inflammatory drug (NSAID), inhibit dye transfer activity, paracrine signal transduction, and brain metastasis in a breast cancer-xenograft model (5). However, the effect of drugs targeting CSCs by inhibiting their GJ-communication and metastasis is not reported. Such drugs are strongly expected to suppress brain metastasis.

AS602801 (also known as bentamapimod) is a novel JNK inhibitor originally developed during a search for drugs to treat neurological disorders (13). The efficacy of AS602801 against endometriosis has been confirmed in animal models (14, 15). A phase 2 clinical trial (NCT 01630252) in patients with inflammatory endometriosis has been completed to confirm its efficacy and safety. We demonstrated that AS602801 inhibits CSCs *in vitro* and *in vivo*, and AS602801 is a candidate anti-cancer drug (16). In the present study, we examined the effects of AS602801 on GJs in CSCs to determine whether it can be a promising drug against brain metastasis.

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Materials and Methods

Reagents and antibodies. AS602801 was purchased from AdooQ BioScience (Irvine, CA, USA) and dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution. Anti-Connexin 43 (#3512), anti-c-Jun (#9165), anti-phospho-c-Jun (Ser 63) (#9261) and anti-GAPDH (#5174) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Cell culture. A549 CSCs used in this study have been previously described (16, 17). A549 CSCs were maintained under monolayer stem cell culture condition (16, 17). Briefly, cells were cultured on collagen type I-coated dishes (IWAKI, Tokyo, Japan) in stem cell culture medium (DMEM/F-12 supplemented with 1% B27 [Thermo Fisher Scientific, Waltham, MA, USA], 20 ng/ml epidermal growth factor (EGF) and fibroblast growth factor (FGF) 2 [Peprotech, Inc., Rocky Hill, NJ, USA], D-(+)-glucose [final concentration 26.2 mM], L-glutamine [final concentration 4.5 mM], 100 units/ml penicillin and 100 µg/ml streptomycin). The stem cell culture medium was changed every three days, and EGF and FGF2 were added to the medium every day. Mouse primary astrocytes were isolated from the cerebral cortex of neonatal mice. Collected cerebral cortical tissues were rinsed with ice-cold phosphate buffered saline (PBS) and mechanically dissociated by gentle pipetting. Dissociated cells, including astrocytes, were plated on collagen I-coated dishes after passing through a 70-µm cell strainer. Thirty min after seeding, the dishes were shaken, and the medium was changed to improve the purity of astrocytes. Medium of cells was replaced with fresh medium every 72 h and used for experiments 7-14 days after seeding. Cultures with more than 70% of cells positive for GFAP were considered pure astrocyte cultures (not shown). Mouse primary astrocytes and parental A549 cells were maintained in DMEM medium supplemented with 10 % FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin.

RT-PCR. Total RNA was isolated from treated A549 CSCs using TRIzol reagent (Thermo Fisher Scientific), following the manufacturer's protocol. For first strand cDNA synthesis, total RNA was reverse transcribed using random hexamer primers and PrimeScript II RTase (Takara Bio, Inc., Tokyo, Japan). Target genes were amplified with Quick Taq HS DyeMix (TOYOBO CO., LTD., Tokyo, Japan) using the following gene-specific primer sets; 5'-TCTTTCATTAGGGG AAGGCG-3' (Forward primer) and 5'-GGAGTTTGCTTAAGG CGCTC-3' (Reverse primer) for Connexin 43; 5'-ACCATCTTCCA GGAGCGAGAT-3' (Forward primer) and 5'-ATGACGAACATGGG GGCATC-3' (Reverse primer) for GAPDH. These gene-specific RT-PCR primers were designed using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The PCR products were resolved *via* electrophoresis in a 1.5% agarose gel containing ethidium bromide. Specific bands were detected using a ChemiDoc Touch (Bio-Rad, Hercules, CA, USA). The intensity of specific bands was quantified using the software ImageJ (<https://imagej.nih.gov/ij/>).

Dye transfer assay. Dye transfer assay was performed based on a previous report (5) with modifications. Donor cells were labeled with 1 mg/ml calcein-AM (nacalai tesque, Kyoto, Japan), and acceptor cells were labeled with 10 µM DiD (Takara) for 30 min. The donor cells were co-cultured with the acceptor cells at a 1:1 ratio for six hours in stem cell culture medium without any drugs,

and then subjected to analysis of calcein transfer from donor cells to acceptor cells using flow cytometry. At least 1×10^4 acceptor cells were gated using side and forward scatter to identify viable cell populations, and the rate of dye transfer was evaluated. All analyses were run on the FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed using the software FlowJo, version 7.6.5 (Treestar Inc., Ashland, OR, USA).

Immunoblot analysis. Immunoblot analysis was conducted as described previously (18, 19). Cells were washed with ice-cold PBS and lysed in Laemmli buffer (62.5 mM Tris-HCl [pH 6.8], 2 % SDS, 10 % Glycerol). Cell lysates were boiled at 95°C for 5 min, and protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). After measurement of protein concentration, 2-mercaptoethanol and bromophenol blue was added to each sample at 5% and 0.002% concentrations, respectively, and samples were boiled again at 95°C for 5 min. Samples containing equivalent amounts were resolved *via* SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with primary antibodies and subsequent HRP-conjugated secondary antibodies. Specific bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) and detected using a ChemiDoc Touch (Bio-Rad). The intensity of specific bands was quantified using ImageJ.

Statistical analysis. All data are expressed as mean±standard deviation (SD). Differences were compared using 2-tailed Student's *t*-test. $p < 0.05$ was considered statistically significant and is indicated by an asterisk.

Results

AS602801 inhibits the function of gap-junctions in cancer stem cells. To examine whether AS602801 affects cell-cell communication in cancer stem cells, its effects were evaluated A549 CSCs, which are derived from the A549 non-small lung cancer cell line, using a dye transfer assay. The dye transfer assay involves measurement of calcein fluorescence transferred from calcein-labeled cells (calcein (+) donor cells) to DiD-labeled cells (calcein (–) acceptor cells) using flow cytometry to examine GJ activity (Figure 1A). AS602801 treatment significantly decreased the number of cells with transferred calcein fluorescence, suggesting that it suppressed GJs in the A549 CSCs (Figure 1B). Because GJs in astrocytes are responsible for important homeostatic functions such as Ca^{2+} waves which are mediated *via* diffusion of cytoplasmic inositol triphosphate (20), we assessed the effect of AS602801 on GJ communication in primary astrocytes freshly isolated from the mouse cerebral cortex. Unlike A549 CSCs, AS602801 treatment had no effect on the function of GJ-mediated dye transfer rate between the astrocytes (Figure 2). Because communication between cancer cells and the non-tumor astrocytes through GJs plays an important role in brain metastasis (5, 6), we next examined whether AS602801 decreases dye transfer rate between A549 CSCs and astrocytes. AS602801 significantly decreased dye transfer rate in both directions, from A549 CSCs to astrocytes

Figure 1. AS602801 suppressed gap junctions in A549 CSC. (A) Schematic model for the study of cell–cell communication using dye transfer. (B) Histograms and quantification of dye transfer between A549 CSCs. A549 CSCs were incubated without or with 7.5 μ M AS602801 AS for 24 h, then subjected to dye transfer assay. Representative histograms are shown. Data are shown as means $\pm$ S.D. from triplicate samples of a representative experiment repeated with similar results. * p <0.05.

Figure 2. AS602801 treatment did not affect the function of astrocyte–astrocyte gap junctions. (A) Scheme of dye transfer analysis of astrocyte communication. (B) Histograms and quantification of dye transfer between astrocytes. Astrocytes were incubated without or with 7.5 μ M AS602801 AS for 24 h and then subjected to dye transfer assay. Representative histograms are shown. Data are shown as means $\pm$ S.D. from triplicate samples of a representative experiment repeated with similar results.

(Figure 3A and B) and from astrocytes to A549 CSCs (Figure 3C and D). Collectively, these results demonstrated that AS602801 could specifically inhibit cell-cell communication *via* GJs in tumor cells but not in normal astrocytes.

Connexin 43 expression is down-regulated by AS602801. Having shown that AS602801 is a potent inhibitor of GJ function in cancer cells, we next explored the mechanisms by which AS602801 inhibits cell-cell communication in the cells. Recently, it was reported that GJs consisting of connexin 43 (Cx43), the principal GJ constituent protein in epithelial lung cancer cells and astrocytes, promotes brain metastasis of cancer cells *via* cGAMP transfer between cancer cells and astrocytes (5). Because differences in Cx43-expression status and cell-cell communication between CSCs and non-CSCs of non-small lung cancer are unknown, we examined Cx43 expression levels in A549 CSCs and parental A549 cells. The protein expression of Cx43 in A549 CSCs was approximately two-fold higher than that in parental A549 cells (Figure 4A). In addition, previous reports have shown that Cx43 expression level is linked to the function of GJs (5, 9); therefore, we examined whether GJ communication in CSCs is higher than that in A549 parental cells using a dye transfer assay. Indeed, dye transfer activity in CSCs was significantly higher than that in parental cells (Figure 4B). Because AS602801 impaired GJ communication in cancer cells but not in astrocytes, we expected this agent to suppress the expression of Cx43. As expected, AS602801 treatment decreased protein expression level of Cx43 in A549 CSCs in a time- and dose-dependent manner (Figure 4C and D). Twenty-four hours after AS602801 treatment, Cx43 mRNA levels were also significantly decreased (Figure 4E). Thus, the results revealed that Cx43 was expressed at higher levels in A549 CSCs than in non-CSCs, and that its expression was suppressed by AS602801.

Discussion

In NSCLC patients, recurrence and brain metastasis contribute to decreased survival (21, 22). Communication between metastatic tumor cells and astrocytes across GJs is important during the late stages of brain metastasis. Once brain-metastatic cells communicate with astrocytes *via* GJs, the expression patterns of various genes are altered, affecting various signaling pathways which regulate cell growth, stem cell proliferation, migration, cell division, aging, apoptosis, and drug resistance in cancer cells (6, 8). GJs are composed of connexins. Cx43 and Cx30 are the most abundant connexins in the brain (6, 23). In particular, Cx43 is expressed in metastatic tumor tissues at cancer cell-astrocyte interfaces, and its expression level is higher in metastatic tumor tissues than in primary tumor tissues or normal brain tissues (5). CSCs which possess tumorigenic capacity also have a central role in the aggressiveness of tumor metastasis. Indeed, our results in the present study showed that Cx43 expression level in lung CSCs is clearly higher than that

in serum-cultured parental (most likely non-stem) cancer cells. Additionally, CSCs showed high dye transfer activity *via* GJs compared with parental cells. In previous reports, Cx43 expression level has been found to be linked to high GJ-communication function (5, 9). Our results in the present study and previous reports suggest that brain-metastatic potential of CSCs might coincide with high expression of Cx43 in CSCs. Moreover, it was recently reported that transfer of cGAMP from cancer cells to astrocytes through GJs consisting of Cx43 promoted brain metastasis of cancer cells (5). Therefore, GJs between tumor cells and astrocytes could be a good therapeutic target to treat metastatic cancers.

Notably, we identified AS602801 as a candidate drug for inhibiting GJ-dependent cell-cell communication in CSCs. We found that AS602801 suppresses the expression of Cx43 in CSCs. Most importantly, this agent does not impair the molecular transport function of astrocyte-astrocyte communications, and we previously reported that AS602801 does not affect IMR90, normal human fibroblasts, at the concentrations used (16). AS602801 is a therapeutic agent in endometriosis treatment, and its molecular mechanism involves ATP-competitive JNK inhibition; its safety was confirmed through a phase 2 clinical trial (13, 24). Therefore, AS602801 can be considered a promising therapeutic drug targeting cell-cell communication. However, some reports have shown that Cx43 in GJs demonstrated a tumor suppressive role in glioblastoma stem cells (25, 26). Indeed, because other reports show that Cx43 expression positively regulates E-cadherin expression, downregulation of Cx43 might promote the initial migration step in metastasis (26-28). Therefore, timing of treatment may be important in the development of a treatment regimen.

Previously, we reported that AS602801 inhibits CSCs *in vitro* and *in vivo* without significant side effects (16). Additionally, we found that AS602801 suppresses the expression of Cx43 in A549 CSCs. It remains unknown whether other anti-CSC agents suppress CSC capacity, Cx43 expression, and/or GJ-dependent communication. However, because it is possible to reduce CSC capacity in (metastatic) tumor cells and suppress cell-cell communication in late-stage metastasis, AS602801 could be a therapeutic agent against highly brain-metastatic CSCs. AS602801 is a JNK inhibitor and several previous reports have shown that activation of the JNK pathway induces Cx43 expression in several cells including cancer cells and normal somatic cells (2, 29-31). Although the relationship between the JNK signaling pathway and Cx43 in CSCs is not well known, AS602801 was found to suppress Cx43 expression in A549 CSCs in the present study. STRING analysis (<https://string-db.org/>) showed that Cx43 has a putative interaction with JNK pathway genes such as *jun* and *fos*. Future studies might clarify whether the JNK signaling pathway is linked to Cx43/GJ-dependent cell-cell communication.

Figure 3. AS602801 inhibited the function of A549 CSC-astrocyte gap junction. (A, C) Schemes of dye transfer analyses of A549 CSC-astrocyte or astrocyte-A549 CSC communication. (B, D) Histograms and quantification of dye transfer between A549 CSCs and astrocytes. A549 CSCs and astrocytes were incubated without or with 7.5 μ M AS602801 AS for 24 h and then subjected to dye transfer assay. Representative histograms are shown. Data are shown as means $\pm$ S.D. from triplicate samples of a representative experiment repeated with similar results. * p <0.05.

Figure 4. AS602801 suppressed connexin 43 (Cx43) expression in A549 CSCs. (A) A549 CSCs and parental A549 cell lysates were subjected to immunoblot analysis of Cx43 and GAPDH. Immunoblot signals were quantified via densitometry and normalized to the values obtained in CSCs. (B) Histograms and quantification of dye transfer between A549 CSCs and A549 CSCs or between parental A549 and parental A549 cells. (C) A549 CSCs were treated with the indicated concentrations of AS602801 for three days and subjected to immunoblot analyses of the indicated proteins. (D) A549 CSCs were treated with 7.5 μM AS602801 for the indicated time periods and then subjected to immunoblot analyses of the indicated proteins. (E) A549 CSCs were treated without (Control) or with 7.5 μM AS602801 for 24 h and then subjected to RT-PCR. The graph shows the expression levels of Cx43 mRNA, using ImageJ (n=3 per group). Representative histograms are shown. Data are shown as means±S.D. from triplicate samples of a representative experiment repeated with similar results. *p<0.05.

In summary, AS602801 suppressed cell-cell communications *via* GJs and Cx43 expression level. To the best of our knowledge, this is the first such report. The Cx43 expression level and cell-cell communication *via* GJs were higher in CSCs than in the parental cells. Our findings suggest that AS602801 can serve as a novel agent against brain metastasis of lung cancer by inhibiting GJs in CSCs.

Conflicts of Interest

The authors declare no conflict of interest in regard to this study.

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