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A Novel Small-molecule WNT Inhibitor, IC-2, Has the Potential to Suppress Liver Cancer Stem Cells

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Abstract. *Background/Aim: The presence of cancer stem cells (CSCs) contributes to metastasis, recurrence, and resistance to chemo/radiotherapy in hepatocellular carcinoma (HCC). The WNT signaling pathway is reportedly linked to the maintenance of stemness of CSCs. In the present study, in order to eliminate liver CSCs and improve the prognosis of patients with HCC, we explored whether small-molecule compounds targeting WNT signaling pathway suppress liver CSCs. Materials and Methods: The screening was performed using cell proliferation assay and reporter assay. We next investigated whether these compounds suppress liver CSC properties by using flow cytometric analysis and sphere-formation assays. A mouse xenograft model transplanted with CD44-positive HuH7 cells was used to examine the in vivo antitumor effect of IC-2. Results: In HuH7 human HCC cells, 10 small-molecule compounds including novel derivatives, IC-2 and PN-3-13, suppressed cell viability and WNT signaling activity. Among them, IC-2 significantly reduced the CD44-positive population, also known as liver CSCs, and dramatically reduced the sphere-forming ability of both CD44-positive and CD44-negative HuH7 cells. Moreover, CSC marker-positive populations, namely CD90-positive HLF cells, CD133-positive HepG2 cells, and epithelial cell adhesion molecule-positive cells, were also reduced by IC-2 treatment. Finally, suppressive effects of IC-2 on liver CSCs were also observed in a xenograft model using CD44-positive HuH7 cells. Conclusion: The novel derivative of small-molecule WNT inhibitor, IC-2, has the potential to suppress liver CSCs and can serve as a promising therapeutic agent to improve the prognosis of patients with HCC.*

A wide range of tumor types are reportedly composed of multiple cell populations (1). Tumor heterogeneity arises due to clonal evolution, effects of the tumor microenvironment, and the existence of cancer stem cells (CSCs). CSCs possess the capacity for unlimited self-renewal, as well as giving rise to progeny cells (2, 3). The aberrant differentiation into various types of cancer cells from CSCs provides a mechanism for generating phenotypic and functional tumor heterogeneity (1). In addition, we reported that the presence of CSCs serves as a prognostic marker for overall survival in patients with hepatocellular carcinoma (HCC), suggesting that undifferentiated CSCs are responsible for tumor growth and disease progression (4). Recent studies suggested the existence of an intimate relationship between liver stem cells and primary liver cancer according to the following CSC hypothesis. Firstly, a large amount of DNA damage accumulates in liver stem cells over a long life-span because these cells have long-term repopulating capability and longevity (5, 6). Secondly, the heterogeneity of liver cancer can be explained by the differentiation of liver cancer stem cells into various types of cancer cells (6, 7). Thirdly, the capacity for self-renewal and proliferation of liver stem cells are enhanced by damage to mature hepatocytes. As a result, the risk of mutation that facilitates the malignant transformation is significantly increased in liver stem cells.

CSCs are a small cell population that can self-renew and differentiate into various types of cancer cells; therefore, CSCs can accelerate and maintain tumor formation and progression. In HCC, the presence of liver CSCs is also reportedly associated with tumor heterogeneity, metastasis, recurrence, poor

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prognosis, and resistance to chemo/radiotherapy (8-10). Since CSCs behave similarly to normal stem cells, it is expected that WNT, Notch, and, Hedgehog signaling pathways are critically involved in the acquisition and maintainance of stem cell-like properties of CSCs (11). Moreover, it is interesting that these pathways are frequently dysregulated in a wide variety of cancer types, and especially in CSCs. In general, these signaling pathways are thought to be potential targets for developing more effective antitumor drugs, because CSCs are responsible for tumor recurrence, metastasis, and resistance to conventional chemo/radiotherapy (12). Among them, a growing body of evidence supports the critical role of the WNT signaling pathway in CSCs (13-15). The WNT signaling pathway, which regulates crucial aspects of migration, proliferation, polarity, cell fate specification, and survival, is frequently up-regulated in HCC (16). Yamashita and colleagues reported that epithelial cell adhesion molecule (EpCAM)-positive cancer cells isolated from HuH7 HCC cell line exhibit WNT signal activation and liver CSC traits (17). Genetic and pharmacological suppression of the WNT signaling pathway inhibits CSC properties, including self-renewal, proliferation, and cell invasion, in several types of cancer (18, 19). Based on these findings, we focused on the WNT signaling pathway as a target for eliminating liver cancer and examined the effect of 10 existing WNT signal inhibitors and three newly synthesized derivatives of these inhibitors on liver CSCs.

Materials and Methods

Cell culture and reagents. Human HCC cell lines, HuH7, HepG2, and HLF, were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka Japan) and were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; MBL, Nagoya, Japan), glucose, and L-glutamine in a humidified atmosphere of 5% CO_2 at 37˚C. Three derivatives of WNT signal inhibitors, namely HC-1, PN-3-13, and IC-2, were synthesized in-house (20).

Cell viability assay. In order to determine the effects of small-molecule compounds on the proliferation of HCC cells, HuH7 cells were seeded in 96-well plates and were incubated for 24 h. After incubation, cells were treated with different concentrations of small-molecule compounds for 24 to 96 h. 5-Fluorouracil (5-FU) was used as a positive control. Cell viabilities at 24, 48, and 96 h were assessed using 10% Cell Counting Kit-8 (Dojin Kagaku, Kumamoto, Japan) and calculated by measuring absorbance at 450 nm and 600 nm as reference wavelength.

Luciferase reporter assay. HuH7 cells were stably transfected with pTCF4-CMVpro-GL4.2 containing t-cell factor 4 (TCF-4) motif as reported previously (20), and cultured with 2 μg/ml puromycin. The cells were incubated in a 96-well plate for 12 h and treated with different concentrations of small-molecule compounds. After treatment for 48 h, relative luciferase activity was measured using the Steady-Glo Luciferase Assay System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The luciferase activity of dimethyl sulfoxide (DMSO)-treated cells was defined as 100%.

Flow cytometry. Flow cytometric analysis was performed as previously reported (4). HCC cell lines were treated with small-molecule compounds for 48 h. After incubation, harvested cells were incubated with blocking solution containing 0.5% bovine serum albumin, 0.5% FBS, and 2 mM EDTA for 15 min at 4˚C. HuH7 and HepG2 cells were incubated with the primary antibodies to CD44 (1:100; Cell Signaling Technology, Danvers, MA, USA), EpCAM (1:1,600; Cell Signaling Technology), and CD133 (1:10; Miltenyi Biotec, Gladbach, Germany) for 10 min at 4˚C, and then with the secondary, goat anti-mouse IgG Alexa Flour 488 (Life Technologies Corp, Carlsbad, CA, USA) for 10 min at 4˚C. HLF cells were incubated with allophycocyanin (APC)-conjugated anti-CD90 (1:100; BD Biosciences, Franklin Lakes, NJ, USA) for 10 min at 4˚C. Propidium iodide (PI) was used to remove non-viable cells. CD44, EpCAM, CD133, and CD90 expression were analyzed and cells were sorted by Moflo XDP cell sorter (Beckman Coulter, Brea, CA, USA). Data analysis was performed by Summit Software (Beckman Coulter) and FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Sphere-formation assay. The sphere-forming ability of cancer cells was determined as previously reported (4) . The CD44-positive and CD44-negative cells fractions from HuH7 cells (5.0×10^4 cells/well) were seeded onto ultra-low attachment 24-well plate (Corning, Corning, NY, USA), and cultured in DMEM/Nutrient Mixture F-12 Ham (DMEM/F12; Sigma-Aldrich, MO, USA) containing 20 ng/ml human recombinant epidermal growth factor (EGF), 20 ng/ml human recombinant basic fibroblast growth factor (bFGF), 1 × B27 supplement (Gibco, Life Technologies Corp.), and L-glutamine for 24 h. After incubation, cells were treated with 1% DMSO, 0.5 μM 5-FU, 5 μM ionomycin, 200 μM PNU74654, 50 μM IC-2, and 10 μM PN-3-13, respectively. At 1 week after treatment, the number of spheres with ≥100 μm diameter was counted in 10 fields per well using the ImageJ software.

Xenograft model of CD44-positive HuH7 cells in mice. The animal experiment was approved by the Institutional Animal Care and Use Committee of Tottori University (13-Y-42). Male non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Sorted CD44-positive cells were seeded onto ultra-low attachment 24-well plate at 1.0×10^5 cells/well, and cultured with DMEM/F12 supplemented with 20 ng/ml recombinant human EGF, 20 ng/ml recombinant human bFGF, 1 × B27 supplement, and L-glutamine for 1 week to allow the formation of spheres. The spheres derived from CD44-positive cells were mixed with Matrigel matrix (Corning) at a ratio of 1:1 and 0.1 ml of the aliquot was subcutaneously injected into right flank of each NOD/SCID mouse. The mice were randomly divided into following three groups: (i) control group (n=5); (ii) 5-FU-treated group (n=4); and (iii) IC-2-treated group (n=4). At 1 to 3 months after transplantation, DMSO, 5-FU (30 mg/kg), or IC-2 (50 mg/kg) were intraperitoneally administered every 3 days for 18 days while tumor size was measured every 3 days. Tumor volumes were calculated using the following equation: (tumor volume; mm^3)=(length; mm) × (width; $mm)^2 \times 0.5$.

Statistical analysis. Statistical comparisons in this study were performed using Dunnett's test and Student's *t*-test. Differences with a value of $p<0.05$ was considered to be statistically significant.

Results

Screening for small-molecule compounds exhibiting antitumor effect on liver CSCs. Based on our previous study (20, 21), 13 small-molecule compounds were selected and screened to identify agents targeting liver CSCs. Among them, 10 compounds, namely NSC668036, quercetin, CGP049090. ICG-001, imatinib, ionomycin, hexachlorophene, PKF115-584, PKF118-310. and PNU74654, have been reported to inhibit the WNT signaling pathway in colorectal cancer cells (22, 23). Another three small-molecule compounds HC-1, PN-3-13, and IC-2 are newly synthesized derivatives of hexachlorophene, PNU74654, and ICG-001, respectively, reported in our previous study (20). We first examined whether these compounds suppress cell proliferation and WNT-mediated transcriptional activity in the HuH7 cell line. Nine molecules out of 13, with the exception of NSC668036, quercetin, HC-1 and imatinib, suppressed both cell proliferation (Figure 1) and WNT-mediated luciferase activity (Figure 2). Interestingly, 5-FU did not affect luciferase activity up at concentrations up to 10 μM, but rather enhanced it at 50 μM. Considering these data, we selected seven compounds, namely ICG-001, ionomycin, hexachlorophene, PKF118-310. PNU74654, PN-3-13, and IC-2, and then investigated whether these compounds exhibit anti-CSC activity. Since we recently reported that total CD44 is a better prognostic marker than other liver CSC markers, including EpCAM, CD13, and CD44 variant 9, in patients with HCC (4), we used total CD44 as a liver CSC marker in this screening. As shown in Figure 3, PNU74654, IC-2, and PN-3-13 significantly reduced the CD44-positive population, whereas no significant effects were observed with ICG-001, ionomycin, hexachlorophene, and PKF118-310. In contrast, although 5-FU increased the proportion of PI-positive dead or dying cells, the CD44-positive population was increased, suggesting that 5-FU affects only CD44-negative cells and not CD44-positive cells.

Effect of small-molecule compounds on sphere-forming ability. Self-renewal capacity has been reported as a common functional feature of CSCs (2). Therefore, the sphere-formation assay was performed to clarify the effect of each compound on the function of liver CSCs. Representative images of sphere formation in CD44-positive and -negative cells treated with DMSO, 5-FU, PNU74654, IC-2, and PN-3-13 were shown in Figure 4A. The sphere-forming ability of CD44-positive DMSO-treated cells, was higher than that of CD44-negative cells, as previously reported (4). Less sphere formation and smaller spheres were found in cells treated with 5-FU, PNR74654, IC-2 and PN-3-13 (Figure 4A). Furthermore, the number of sphere produced by cells treated with 5-FU, PNU74654, IC-2, and PN-3-13 decreased compared with that of cells treated with DMSO (Figure 4B). However, when compared to CD44-negative cells, the suppressive effects on sphere-forming ability of CD44-positive cells remained smaller in cells treated with 5-FU, PNU74654, or PN-3-13. Interestingly, IC-2 treatment greatly suppressed the sphere-forming ability of both CD44-positive and CD44-negative cells, suggesting that IC-2 potentially suppresses CSC properties.

Effect of IC-2 on cell viability of liver CSCs in comparison to non-CSCs. In order to gain further insights into the effect of IC-2 on liver CSCs, cell viability was assessed after IC-2 treatment of both CD44-positive and CD44-negative populations of HuH7 cells (Figure 5A). Although 5-FU treatment significantly reduced the cell viability of the CD44-negative population, its effect was impaired against CD44-positive cells. Meanwhile, IC-2 treatment reduced cell viability of both CD44-positive and CD44-negative populations with similar efficiency. These data suggest that IC-2 treatment is effective not only against non-CSCs but also against CSCs, in contrast to 5-FU.

We next examined whether IC-2 affects other liver CSCs expressing CD90. CD133, or EpCAM. As shown in Figure 5B, IC-2 reduced the CD90-positive HLF cell population from 21.8% to 13.5%, whereas this was not observed in 5-FU-treated HLF cells. Similarly, CD133-positive or EpCAM-positive HepG2 cell populations were also reduced by IC-2 from 57.1% to 1.45%, and from 76.2% to 51.1%, respectively (Figure 5C and D). These data suggest that IC-2 has the ability to suppress a wide range of liver CSCs.

In vivo effect of IC-2 on liver CSCs. Xenograft study using NOD/SCID mice subcutaneously transplanted with CD44-positive HuH7 cells was performed to investigate the antitumor effect of IC-2 against liver CSCs *in vivo* (Figure 6). In 5-FU-administered mice, tumor growth was significantly suppressed compared to that in the controls. As expected, more potent IC-2 manifested a more potent suppressive effect than 5-FU, suggesting that the suppression of CSCs by IC-2 would provide clinical benefits for patients with HCC.

Discussion

The WNT signaling pathway is often activated in HCC cells in agreement with the fact that this pathway has critical roles for liver CSCs (16, 17). This prompted us to investigate the antitumor effect of WNT signal inhibitors against liver CSCs. In the present study, we demonstrated that IC-2, derivative of ICG-001 recently synthesized in-house, efficiently suppresses liver CSC properties, including sphere-forming ability, cell proliferation, and tumor development *in vivo*.

In our screening experiments, the majority of WNT inhibitors showed suppressive effects on cell proliferation and WNT signaling activity in HuH7 cells. However, although WNT signaling was suppressed by these inhibitors, liver CSC abilities were not affected by the inhibitors,

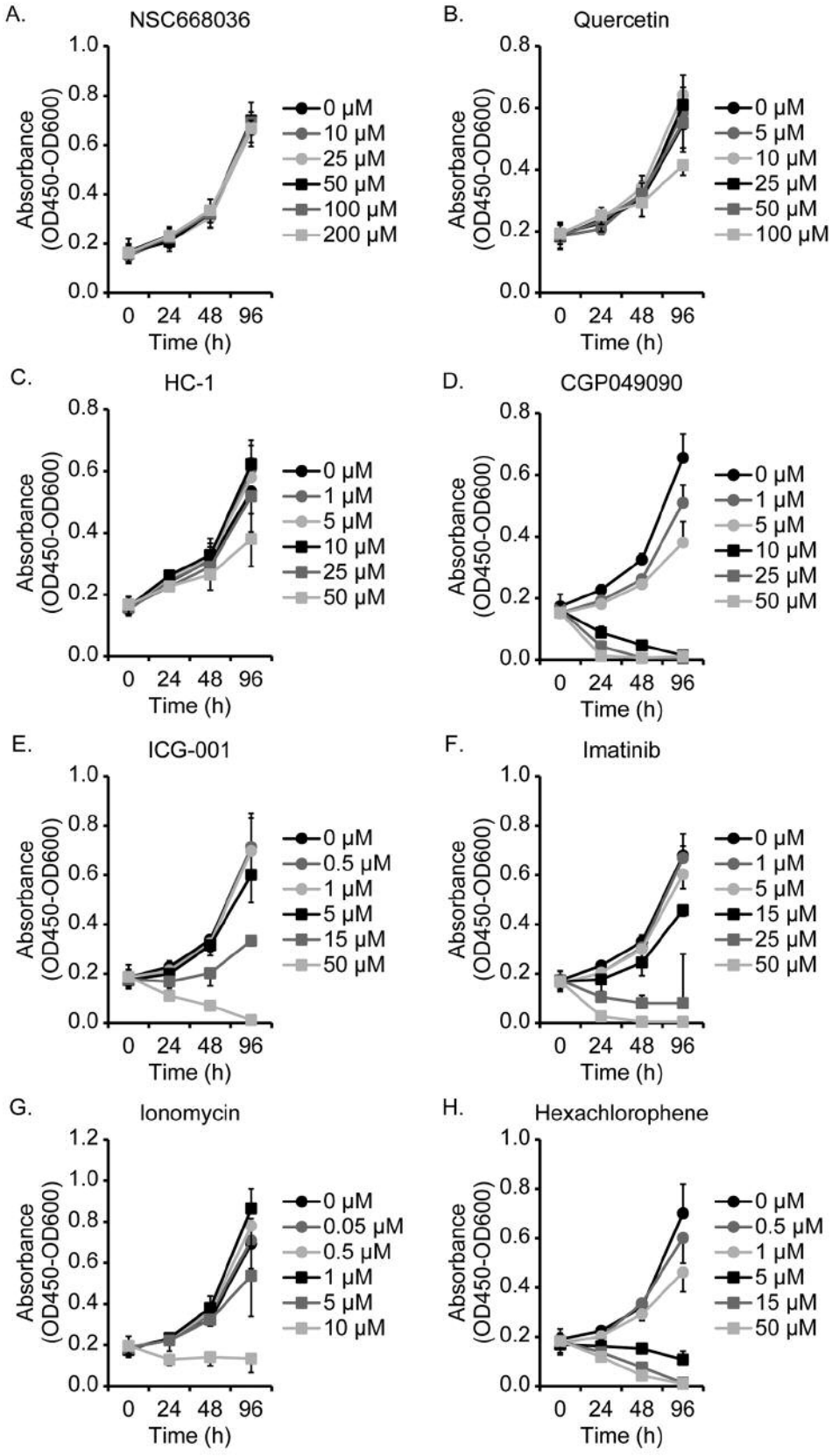

Figure 1. *Continued*

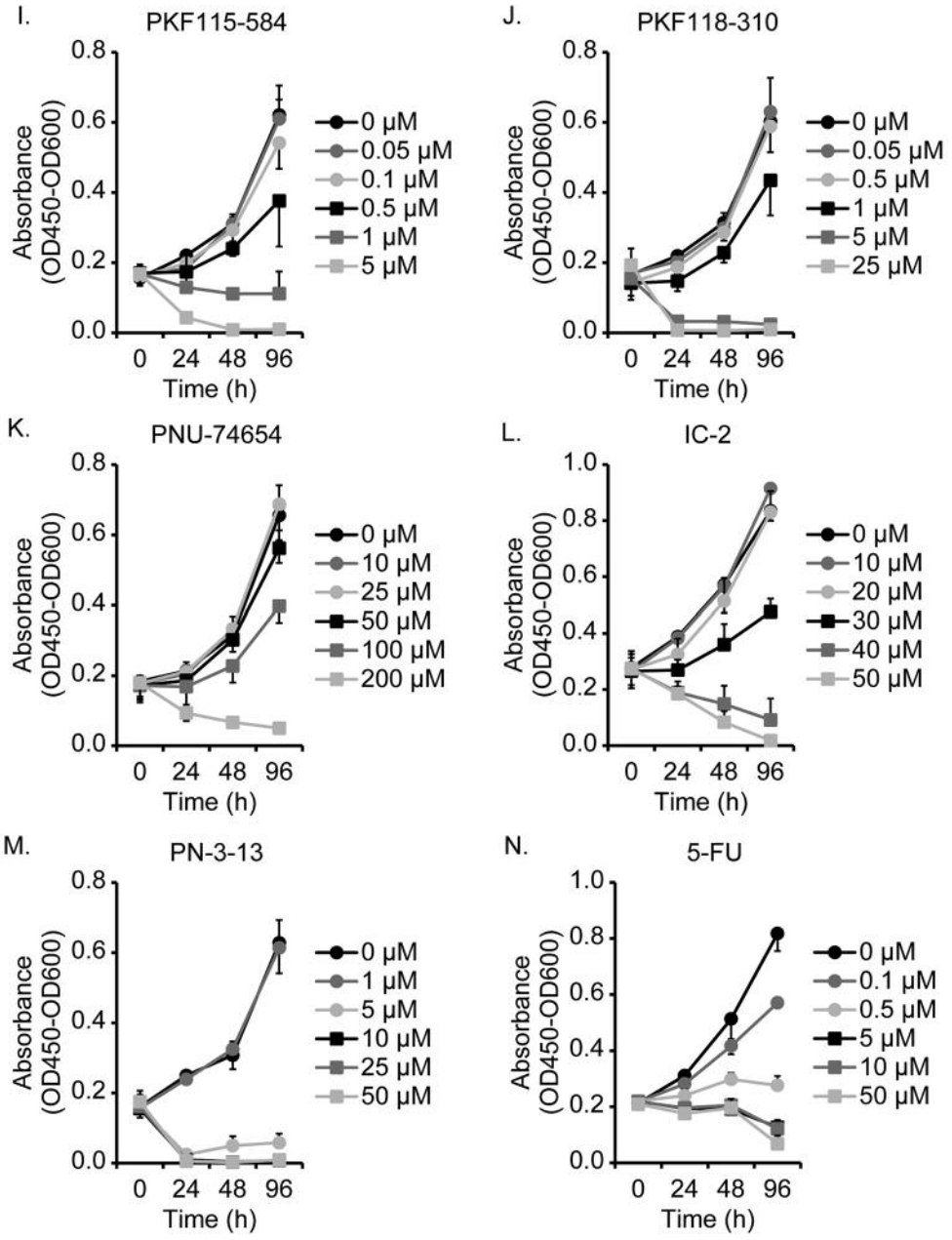

Figure 1. *Time- and dose-dependent antitumor effect of small-molecule compounds on HuH7 cells. Viability of HuH7 cells treated with NSC668306 (A), quercetin (B), HC-1 (C), CGP049090 (D), ICG-001 (E), imatinib (F), ionomycin (G), hexachlorophene (H), PKF115-584 (I), PKF118-310 (J), PNU-74654 (K), IC-2 (L), PN-3-13 (M), and 5-fluorouracil (5-FU) (N). Cell viability was determined using the WST assay at 0. 24, 48, and 96 h. Data are shown as the mean±SD (n=3).*

except for IC-2. The underlying inhibitory mechanisms of these small-molecule compounds have been reported as follows. Hexachlorophene promotes β-catenin degradation *via* a proteasome-dependent manner (24). Ionomycin inhibits nuclear translocation of β-catenin (25). PKF115-584, PKF118-310. CGP049090 and PNU-74654 inhibit the association of β-catenin with TCF of lymphoid enhancer factor (LEF) (22, 26, 27). ICG-001, and possibly IC-2, binds to the transcriptional coactivator cAMP response element-binding protein-binding protein (CBP) to disrupt the interaction of CBP with β-catenin (28). WNT-mediated transcriptional activation in tumor and embryonic stem cells

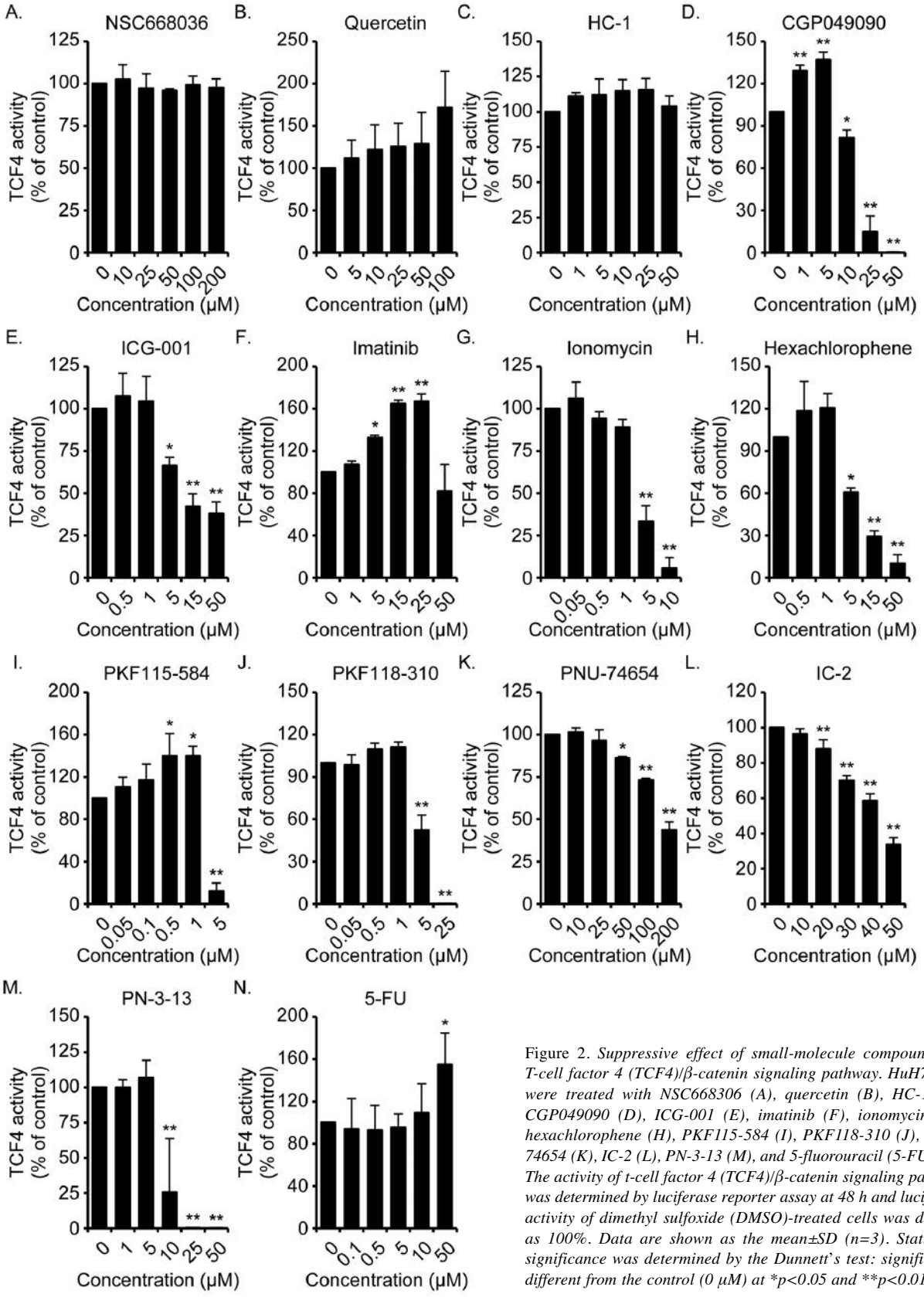

Figure 2. *Suppressive effect of small-molecule compounds on T-cell factor 4 (TCF4)/β-catenin signaling pathway. HuH7 cells were treated with NSC668306 (A), quercetin (B), HC-1 (C), CGP049090 (D), ICG-001 (E), imatinib (F), ionomycin (G), hexachlorophene (H), PKF115-584 (I), PKF118-310 (J), PNU-74654 (K), IC-2 (L), PN-3-13 (M), and 5-fluorouracil (5-FU) (N). The activity of t-cell factor 4 (TCF4)/β-catenin signaling pathway was determined by luciferase reporter assay at 48 h and luciferase activity of dimethyl sulfoxide (DMSO)-treated cells was defined as 100%. Data are shown as the mean±SD (n=3). Statistical significance was determined by the Dunnett's test: significantly different from the control (0 μM) at *p<0.05 and **p<0.01.*

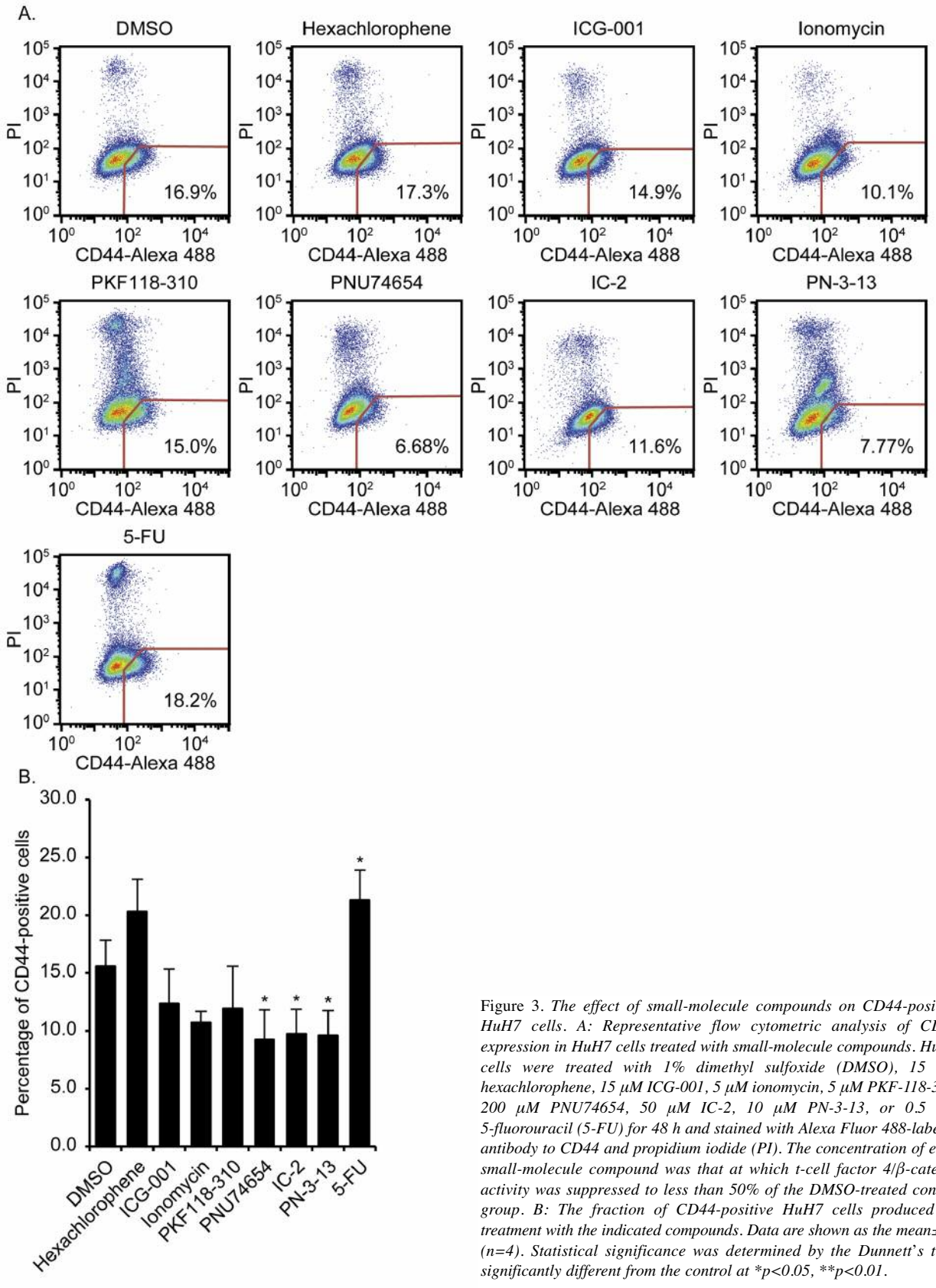

Figure 3. *The effect of small-molecule compounds on CD44-positive HuH7 cells. A: Representative flow cytometric analysis of CD44 expression in HuH7 cells treated with small-molecule compounds. HuH7 cells were treated with 1% dimethyl sulfoxide (DMSO), 15 μM hexachlorophene, 15 μM ICG-001, 5 μM ionomycin, 5 μM PKF-118-310. 200 μM PNU74654, 50 μM IC-2, 10 μM PN-3-13, or 0.5 μM 5-fluorouracil (5-FU) for 48 h and stained with Alexa Fluor 488-labeled antibody to CD44 and propidium iodide (PI). The concentration of each small-molecule compound was that at which t-cell factor 4/β-catenin activity was suppressed to less than 50% of the DMSO-treated control group. B: The fraction of CD44-positive HuH7 cells produced on treatment with the indicated compounds. Data are shown as the mean±SD (n=4). Statistical significance was determined by the Dunnett's test: significantly different from the control at *p<0.05, **p<0.01.*

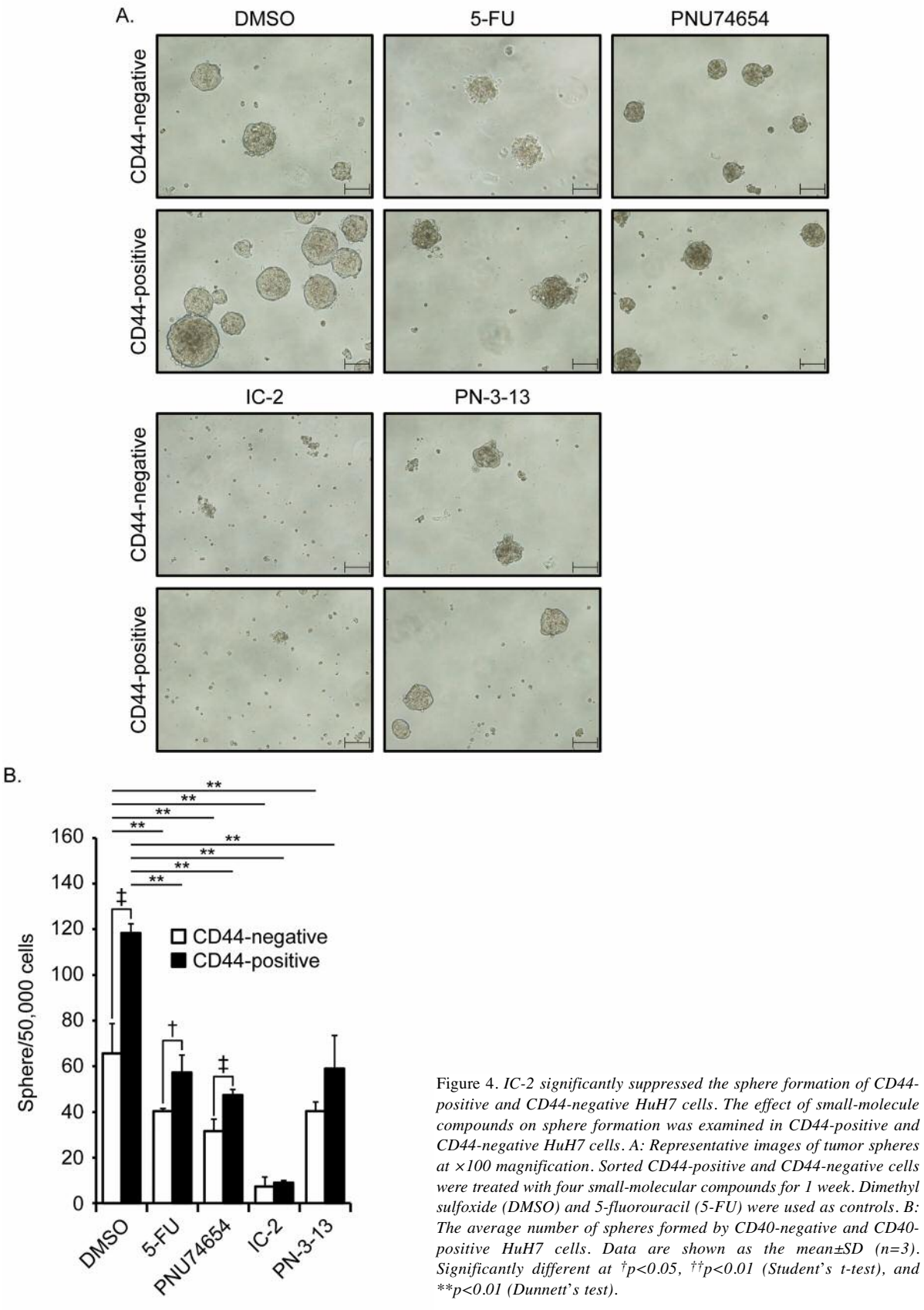

Figure 4. *IC-2 significantly suppressed the sphere formation of CD44-positive and CD44-negative HuH7 cells. The effect of small-molecule compounds on sphere formation was examined in CD44-positive and CD44-negative HuH7 cells. A: Representative images of tumor spheres at ×100 magnification. Sorted CD44-positive and CD44-negative cells were treated with four small-molecular compounds for 1 week. Dimethyl sulfoxide (DMSO) and 5-fluorouracil (5-FU) were used as controls. B: The average number of spheres formed by CD40-negative and CD40-positive HuH7 cells. Data are shown as the mean±SD (n=3). Significantly different at †p<0.05, ††p<0.01 (Student's t-test), and **p<0.01 (Dunnett's test).*

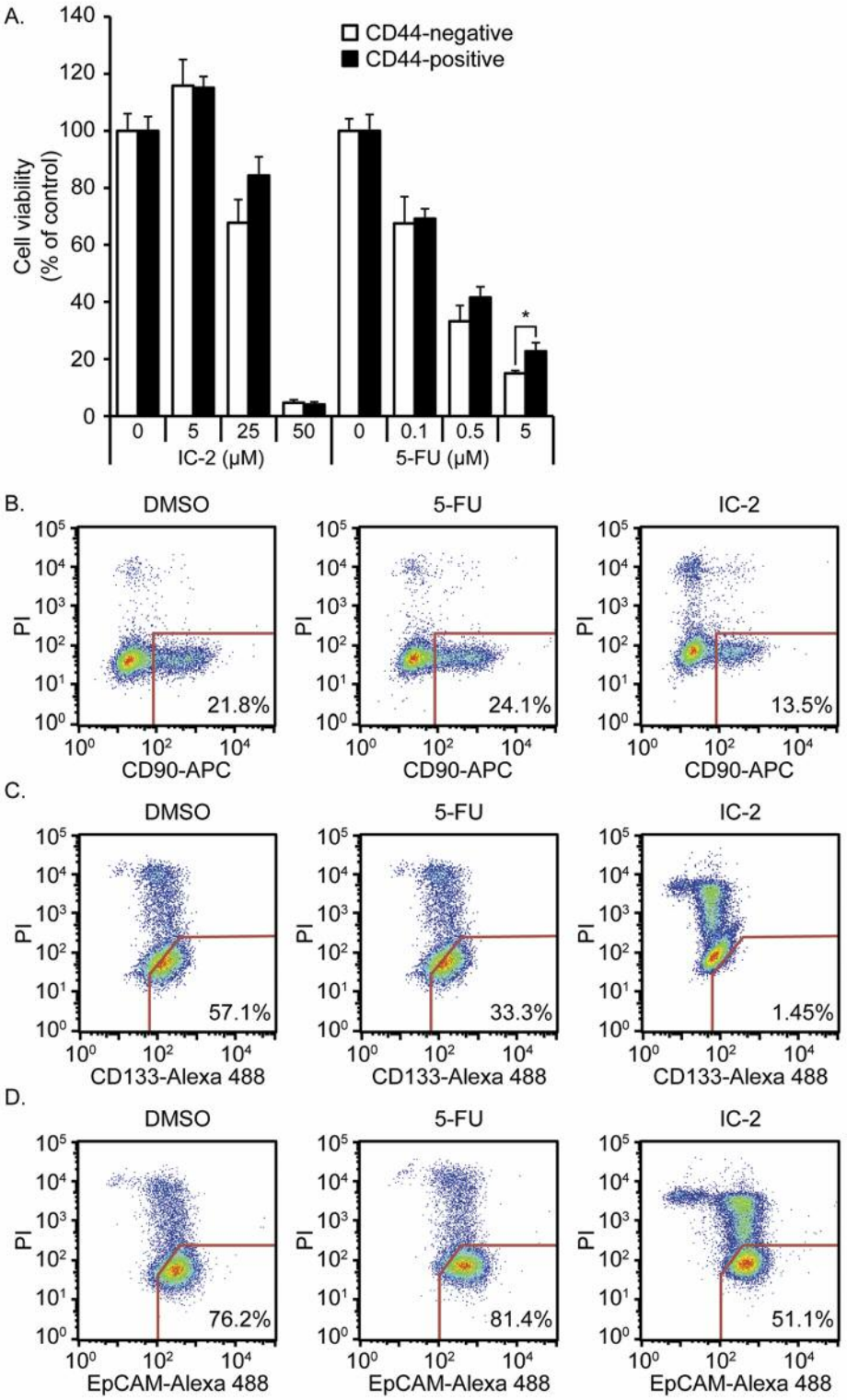

Figure 5. *IC-2 significantly suppressed the cell viability of different CSCs. A: Viability of sorted CD44-positive and CD44-negative HuH7 cells treated with IC-2 or 5-fluorouracil (5-FU) for 96 h. The value of cells treated with dimethyl sulfoxide (DMSO) was defined as 100%. Data are shown as the mean±SD (n=3). Flow cytometric analysis of HLF cells expressing CD90 (B), and of HepG2 cells expressing CD133 (C), or epithelial cell adhesion molecule (EpCAM) (D) after treatment with IC-2 or 5-FU. The percentage of positive cells are shown. Propidium Iodide (PI) was used to discriminate dead cells. Statistical significance was determined by the Student's t-test: significantly different at *p<0.05.*

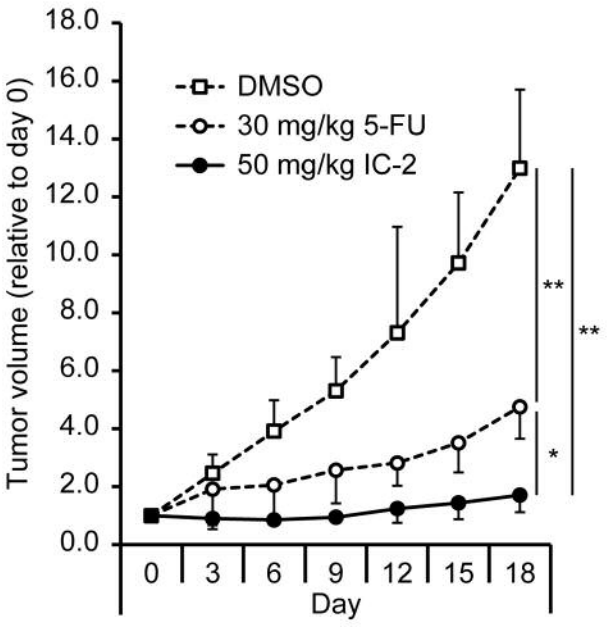

Figure 6. *IC-2 suppressed tumor growth in a mouse xenograft model transplanted with CD44-positive HuH7 cells. The mice were treated with dimethyl sulfoxide (DMSO), 30 mg/kg 5-fluorouracil (5-FU), or 50 mg/kg IC-2 for 18 days. The average tumor volumes are shown. Data are expressed as the mean±SD (n=4-5). Statistical significance at 18 days was determined by the Dunnett T3 test: significantly different at *p<0.05 and **p<0.01.*

is reportedly regulated by the following two coactivators with different functions: CBP–β-catenin complex mainly plays a role in the maintenance of stemness, pluripotency and tumorigenic phenotype, whereas p300–β-catenin complex is involved in the initiation of the differentiation program (29, 30). These reports and our findings suggest that suppression of CBP/β-catenin-mediated transcriptional activity may be more critical for the elimination of liver CSCs, rather than simply disrupting the WNT signaling pathway. Indeed, IQ-1, which promotes compensatory interaction of CBP and β-catenin by inhibiting p300–β-catenin interaction, increased CSCs in breast and bladder carcinoma cell lines (31). Furthermore, CBP–β-catenin complex inhibition with ICG-001 reduced CSC properties, including drug resistance and sphere-forming ability, in nasopharyngeal carcinoma, ovarian cancer, and leukemia cells (32-34). Although the mechanism underlying inhibition of WNT signaling pathway and suppression of liver CSCs by IC-2 treatment has yet to be fully elucidated, it is reasonable to speculate that IC-2 suppresses liver CSC properties *via* inhibition of the CBP–β-catenin complex formation as ICG-001 does. Further examinations are required to elucidate the mechanisms of IC-2 action.

Among newly synthesized derivatives, although the antitumor effect of PN-3-13 was higher than IC-2 in two-dimensional (2D) monolayer culture, it was lower than IC-2 in three-dimensional (3D) culture. It has been reported that the sensitivity to antitumor drugs usually differs between 2D and 3D culture conditions, and the 3D culture system is superior for testing clinically active antitumor drugs (35, 36). Considering this aspect, IC-2 may have potential as a clinically active agent and serve as a promising therapeutic drug targeting liver CSCs. Note that in agreement with previous reports (17, 37), we observed that the clinically available drug, 5-FU, enhanced WNT-mediated transcriptional activity and relatively increased the population of CD44- or EpCAM-positive liver CSCs. Therefore, it needs to be taken into consideration that conventional chemotherapeutic agents may contribute to the acquisition of CSC-like properties and the basis for enrichment of CSCs.

In conclusion, we found that IC-2, a novel derivative of ICG-001, has a potent antitumor effect against HCC by suppressing liver CSC properties. Therefore, IC-2 may serve as a novel therapeutic agent for targeting liver CSCs in HCC.

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