ABCG2 Inhibitor YHO-13351 Sensitizes Cancer Stem/ Initiating-like Side Population Cells to Irinotecan

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Abstract. *Background/Aim: The aim of this study was to determine the efficacy of the combination of irinotecan and newly-synthesized ABCG2 (breast cancer-resistant protein) inhibitor YHO-13351 in cancer chemotherapy. Materials and Methods: Side population (SP) and non-SP cells from the human cervical carcinoma cell line HeLa were isolated by fluorescence-activated cell sorting. The antitumor activity of combination therapy with irinotecan and YHO-13351 was evaluated in xenograft studies in athymic BALB/c nude mice. Results: While SP cells exhibited cancer stem/initiating cell-like properties and low sensitivity to irinotecan-alone, YHO-13351 sensitized them to irinotecan in both in vitro and in vivo studies. YHO-13351 in conjunction with irinotecan reduced the increase of the SP cell ratio in the tumors compared to those observed with treatment with irinotecan-alone. Conclusion: Combination therapy with irinotecan and YHO-13351 would not only accelerate the antitumor effect of this regimen, but also play a crucial role in preventing resistance or relapse.*

Two major problems encountered in the treatment of cancer are resistance to chemotherapy and recurrence. One common approach to overcoming resistance is to try new combinations of anticancer agents with different modes of action (1, 2). The results, however, are not always satisfactory, even though benefits may be observed in some cases. Therefore, there is an urgent need to develop more effective means of sensitizing tumors to conventional chemotherapeutics and to completely eradicate cancerous cells.

One cause of resistance is an ATP-binding cassette (ABC) transporter-induced decrease in the intracellular accumulation of a drug (3). Therefore, much effort has been devoted to developing ABC transporter inhibitors such as ABCB1 (P-glycoprotein) and ABCG2 (breast cancer-resistant protein) in hope that this would bring about a breakthrough in the amelioration of drug resistance (4-6). ABCG2, in particular, is involved in the efflux of several anticancer agents, including irinotecan and its active metabolite, SN-38 (7, 8), one of the most widely prescribed drugs for many types of cancer (9). In addition, ABCG2 is a key molecule in the characterization of side population (SP) cells in the hematopoietic system, which are identified by their ability to exclude the fluorescent vital dye Hoechst 33342 (10). The ABCG2-positive subset of tumor cells is also frequently identified by its ability to exclude Hoechst, and it is often enriched with cells with cancer stem/initiating-like phenotypes (11-13). Some studies have attributed failure of chemotherapy to the survival of multi-drug chemoresistant cancer stem/initiating cells (14-16).

YHO-13177, (Z)-2-(3,4-dimethoxyphenyl)-3-[5-(4-hydroxypiperidin-1-yl) thiophen-2-yl] acrylonitrile, is a novel acrylonitrile derivative discovered by screening for ABCG2 inhibitors (17). YHO-13351, diethylaminoacetic acid 1-{5-[(Z)-2-cyano-2-(3,4-dimethoxyphenyl)vinyl]thiophen-2-yl}piperidin-4-yl ester methanesulfonate, is a water-soluble pro-drug of YHO-13177. Co-administration of irinotecan with YHO-13351 significantly increased survival time in mice inoculated with ABCG2-transduced murine leukemia P388 cells and suppressed tumor growth in a ABCG2-transduced HTC116 human colonic cancer xenograft model, whereas irinotecan-alone had little effect in these tumor models (17). Thus, the combination of YHO-13351 and irinotecan offers a potentially promising chemotherapy regimen capable of completely eradicating tumors by targeting cancer stem/initiating cells.

In this study, SP cells isolated from human cervical carcinoma HeLa cells were characterized by using hallmarks of stemness. The sensitivity of HeLa SP and non-SP cells to irinotecan (SN-38) was evaluated. The effect of YHO-13351 (YHO-13177) on this sensitivity was examined in *in vitro* cell culture and an *in vivo* xenograft mouse model. The superiority of combination cancer chemotherapy with irinotecan and YHO-13351 was also investigated.

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Key Words: YHO-13351, side population cell, irinotecan, ABCG2.

Materials and Methods

Reagents. YHO-13177 and YHO-13351 were chemically-synthesized by Yakult Honsha Co., Ltd. (Tokyo, Japan). Campto® Inj. (irinotecan hydrochloride: (+)-(4*S*)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino)carbonyloxy]-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione hydrochloride trihydrate) and its active metabolite, SN-38, were prepared by Yakult Honsha Co. Ltd. Hoechst 33342 and reserpine (Apoplon® Inj.) were purchased from Invitrogen (Carlsbad, CA, USA) and Daiichi Sankyo Co., Ltd. (Tokyo, Japan), respectively.

Animals and tumor cell lines. Male BALB/c nude mice were obtained from CLEA Japan Inc. (Tokyo, Japan) and maintained under standard laboratory conditions.

Human cervical carcinoma cell line HeLa was obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Human lung carcinoma cell line NCI-H460, human multiple myeloma cell line RPMI8226, human pancreatic adenocarcinoma cell line AsPC-1, and human colorectal carcinoma cell line HCT116 were purchased from the American Type Culture Collection (Manassas, VA, USA). The RPMI8226 cells were maintained as suspension cultures and the others as monolayer cultures. RPMI-1640 (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS)(Nichirei Biosciences Inc., Tokyo, Japan) was used for cell culture.

Staining, SP analysis, and sorting. Cells were suspended at 10^6 cells/ml in Hank's balanced salt solution (Invitrogen) containing 2% FBS and 10 mM HEPES (Invitrogen) and incubated for 60 min at 37˚C with Hoechst 33342 (2-10 μg/ml) with intermittent shaking. Reserpine (5-20 μg/ml) was applied to control cells to block exclusion of the Hoechst dye. Fluorescence-activated cell sorter analyses and sorting were performed on a MoFlo™ XDP flow cytometer cell sorter (Beckman Coulter Inc., Fullerton, CA, USA). Hoechst 33342 was excited at 350 nm by UV laser and its fluorescence detected at two wavelengths using 457/50 (Hoechst blue) and 628/32 (Hoechst red) band pass filters. Dead cells were excluded from the plots based on propidium iodide (Sigma-Aldrich Co., St. Louis, MO, USA) staining. Data were reanalyzed with the Summit® software (Beckman Coulter Inc.).

For cell sorting, $0.5\text{-}1\times10^7$ cells were collected and stained as described above. Sorted cells were collected into ice-cold RPMI-1640 medium with 50% FBS. Immediately after purification, the sorted cells were re-suspended either in medium for spheroid colony formation and cytotoxicity assays *in vitro*, or in saline for inoculation into nude mice *in vivo*.

Immunofluorescent staining and flow cytometry. For immunofluorescent staining, the cells were fixed with 4% paraformaldehyde and stained with a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody against human ABCG2 (clone 5D3; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After incubation in the dark for 30 min at room temperature, the cells were washed, resuspended in phosphate-buffered saline with 2% (v/v) FBS, and analyzed by flow cytometry.

Spheroid colony formation assay. A spheroid colony formation assay was performed by the method of Takahashi *et al*. (18). Briefly, SP and non-SP cells sorted from HeLa were inoculated separately into the wells (10 cells per well) of ultra-low attachment surface, 96-well plates (flat bottom) (Corning Inc. Life Sciences, Lowell, MA, USA) supplemented with 200 μl Cambrex serum-free epithelial basal medium (Takara, Tokyo, Japan), 4 μg/ml insulin (Sigma-Aldrich), 20 ng/ml epidermal growth factor (Invitrogen), 20 ng/ml basic fibroblast growth factor (Invitrogen), and B27 supplement (Invitrogen). After 2-3 weeks, each well was examined using a light microscope CKX41 (Olympus Co., Tokyo, Japan) and the total numbers of wells with spheroid colonies out of 96 wells were counted. Images of the spheroid colonies were recorded using a light microscope and a DP20 digital camera (Olympus Co.).

Tumorigenesis, growth rate, and asymmetrical proliferation in xenograft studies in athymic mice. Six-week-old, male, athymic BALB/c nude mice were allocated into six groups of five mice each and used for HeLa (SP or non-SP) xenografts. Sorted HeLa SP, or non-SP cells, in a 50% BD Matrigel™-saline suspension were subcutaneously inoculated into the inguen at 100, 1,000, or 10,000 cells/mouse on day 0. From days 15 to 60, tumor incidence (number of tumor-bearing animals)/number of animals injected was determined to evaluate tumorigenesis. When a tumor appeared, its long and short diameters were measured with calipers and its volume estimated according to the equation Estimated tumor volume (mm^3)=1/2 × (long diameter) × (short diameter)^2 to assess growth rate. In order to determine asymmetrical proliferation, the tumors were extirpated from two mice from each group on day 60 and HeLa cells from the tumors prepared by digestion in collagenase for Hoechst dye staining and SP analysis. Co-localizing fibroblasts derived from mouse tissues in the cell suspension were excluded from the analysis by using FITC-conjugated anti-mouse major histocompatibility complex (clone 34-1-2S; Abcam Inc., Cambridge, UK).

Cytotoxicity assay for cell survival. Cells were plated in 96-well culture plates (Corning) and different concentrations of SN-38 with or without YHO-13177 were added to the cultures. After 96 h, cell viability was measured as mitochondrial NADH-dependent dehydrogenase activity with a Cell Proliferation Assay System, TetraColor ONE (Seikagaku Co., Tokyo, Japan). The concentration of SN-38 causing 50% inhibition of cell viability (IC_{50}) was calculated by interpolation.

Antitumor activity of combination therapy with irinotecan and YHO-13351 in xenograft studies in athymic mice. A HeLa (SP or non-SP) xenograft model was used as described above. In brief, sorted HeLa SP or non-SP cells suspended in 50% BD Matrigel™-saline were inoculated subcutaneously into the inguen of BALB/c nude mice at 100 cells/mouse. When the tumor reached a size of 100-300 mm^3, the mice were allocated by tumor volume into four groups of five mice each to be treated with vehicle, irinotecan (200 mg/kg, total) alone, or irinotecan (200 mg/kg, total) in combination with YHO-13351 (600 and 1200 mg/kg, total). The administration schedules were designed based on previous studies (17, 19). Administration *via* the tail vein (irinotecan) or *per os* (YHO-13351) commenced immediately after allocation (day 1). Tumor size was measured on days 1, 5, 9, 14, and 22. Tumors were resected and weighed on day 22, and the tumor growth-inhibitory ratio (IR, %) assessed by the equation IR (%)=[1−(tumor weight of test group)/(tumor weight of control group)]×100. The total dose of irinotecan was set to 200 mg/kg to allow the effect of combined administration to be detected clearly. On the basis of a preliminary study, an approximately 30% IR was expected with single administration of irinotecan at this dose.

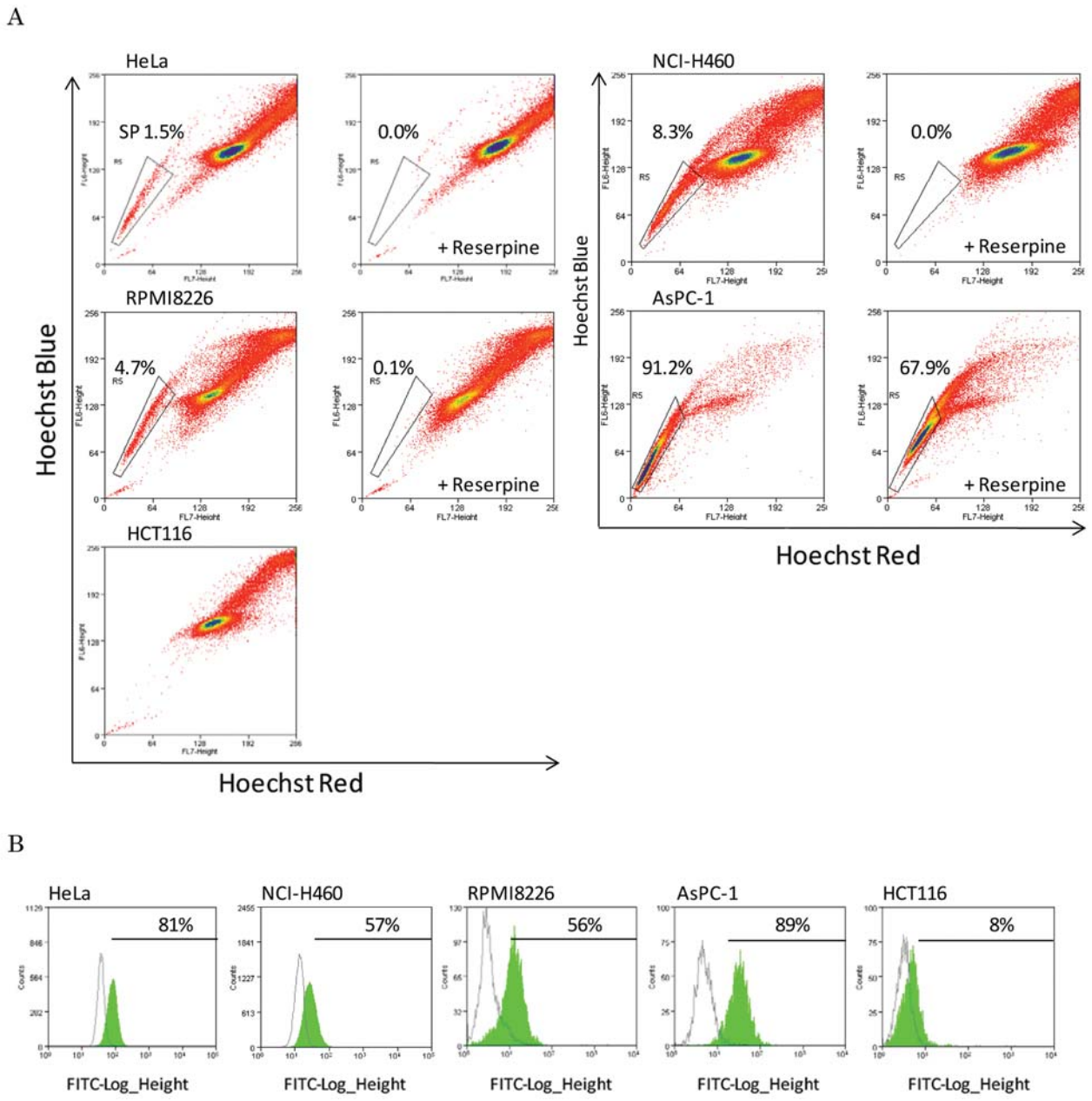

Figure 1. *Detection of side population (SP) cells and ABCG2 (breast cancer-resistant protein) in human cancer cell lines by flow cytometry. A: Presence of SP cells in human cervical carcinoma cell line HeLa, lung carcinoma cell line NCI-H460, multiple myeloma cell line RPMI8226, pancreatic adenocarcinoma cell line AsPC-1, and colorectal carcinoma cell line HCT116. Cells were stained with Hoechst 33342 in the presence or absence of reserpine and analyzed using MoFlo™ XDP. The trapezium in each panel indicates the SP area. B: ABCG2 expression in human cancer cell lines. HeLa, NCI-H460, RPMI8226, AsPC-1, and HCT116 were stained with fluorescein isotihocyanate (FITC)-conjugated mouse monoclonal antibody to human ABCG2 (filled histogram) or isotype control antibody (open histogram). FITC-positive cells were quantified by flow cytometry.*

Body weight was also measured to determine the influence of the drugs used on overall physical condition. All animal studies were conducted in accordance with the Guidelines of the Yakult Central Institute for Microbiological Research and the protocol was approved by the Institutional Animal Care and Use Committee of this institute.

Statistical analysis. The significance of differences between two groups was determined by an F-test, followed by Welch's *t*-test when the F ratio reached significance ($p<0.25$). In a multiple comparison, the significance of differences between more than three groups was determined using Dunnett's test.

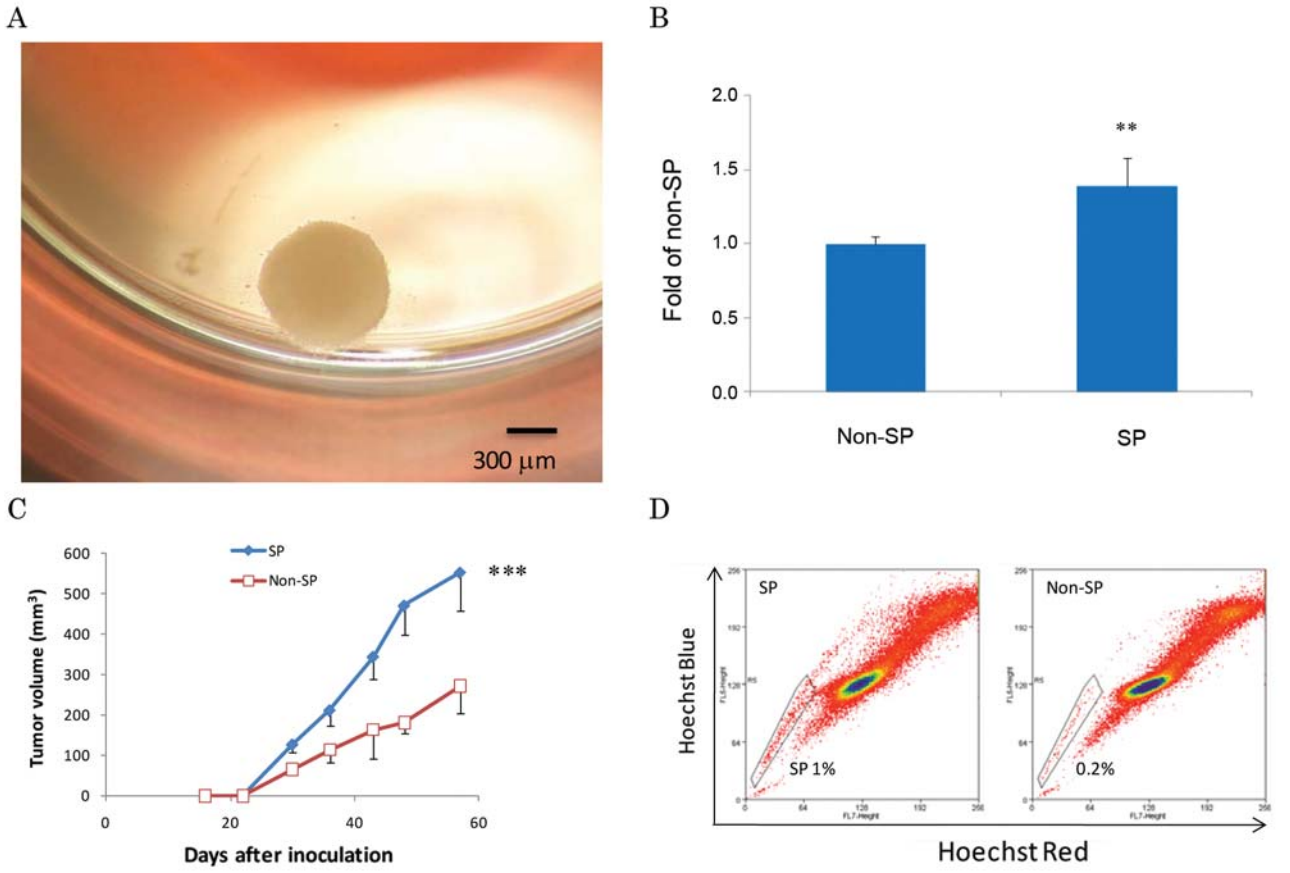

Figure 2. *Characterization of side polulation (SP) cells in HeLa by comparing spheroid colony-formation ability, tumor growth rate and asymmetrical proliferation between HeLa SP and non-SP cells. A: Spheroid colony formation of HeLa SP cells in serum-free media. HeLa SP and non-SP cells were fractionated by fluorescence-activated cell sorting for Hoechst 33342 stainability and inoculated into ultra-low attachment 96-well plates with serum-free medium (10 cells per plate) containing human epidermal growth factor (20 ng/ml) and basic fibroblast growth factor (20 ng/ml). After 2-3 weeks of culture, the number of spheroid colony-positive wells was counted. B: Relative ratio of spheroid colony-positive wells in the SP group was calculated by comparing it with that in the non-SP group (n=3). All results are given as the mean±S.D. **p<0.01 vs. non-SP cells (Welch's t-test). C: Growth of xenografts derived from injection of 100 sorted SP or non-SP cells. Values represent the means±S.D. of five mice. ***p<0.001 vs. non-SP cells (on day 57, Student's t-test). D: Asymmetrical proliferation of SP and non-SP cells in tumors at 60 days after subcutaneous injection of 100 cells into mice.*

Results

Identification of SP cells in human cancer cell lines. Firstly, the presence or absence of SP cells in selected commercially-available human cancer cell lines was determined. As shown in Figure 1A, most of them contained SP cells, although the fraction ranged from 1% to 90%. The lowest proportion of SP cells was observed in the HCT116 cell line.

Flow cytometry was used to clarify the relationship between SP cell ratio and ABCG2 expression. All the cell lines tested were clearly ABCG2-positive, except for HCT116 (Figure 1B). Notably, it was possible to detect ABCG2 by staining without fixation, that is, by live-cell staining (data not shown), in HeLa, NCI-H460 and RPMI8226 cells.

Due to its stable SP ratio and high viability after sorting, HeLa were chosen for investigation of the characteristics of SP cells and further studies.

Characterization of SP cells in HeLa. To examine the characteristics of HeLa SP and non-SP cells, spheroid colony formation was investigated. Figure 2A shows the spheroid colony that formed in a well containing SP cells. The rate of spheroid colony formation was significantly higher than that in non-SP cells (Figure 2B).

Next, the tumorigenicity of SP cells in nude mice was compared with that of non-SP cells. Even with injection of only 100 cells/mouse, the tumor incidence of non-SP cells was 5/5, which was equivalent to that of SP cells. These results showed that non-SP cells were as tumorigenic as SP

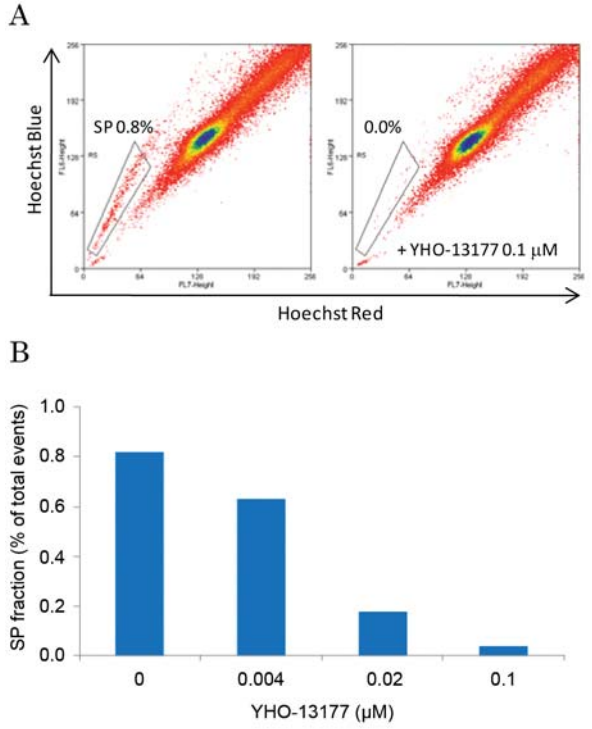

Figure 3. *Reduction in side population (SP) fraction by YHO-13177. A: SP analysis of HeLa cells. Cells were stained with 7.5 μg/ml Hoechst 33342 in the absence and presence of YHO-13177. B: HeLa cells were stained with Hoechst dye in the presence of the indicated concentration of YHO-13177 and then analyzed by a fluorescence-activated cell sorter.*

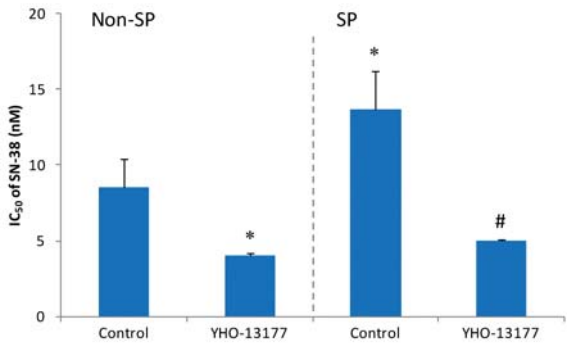

Figure 4. *Sensitization by YHO-13177 of cancer stem/initiating-like side population (SP) cells to SN-38 in vitro. HeLa SP and non-SP cells were cultured in a medium containing 0.3-100 nM SN-38 with 0.1 μg/ml YHO-13177 for 96 h. Cell viability was evaluated as described in the Materials and Methods section and the concentration of SN-38 causing 50% inhibition in cell viability (IC_{50}) was calculated. Values represent the means±S.D. of three wells. *$p<0.05$ vs. control of non-SP cells (Dunnett's test); #$p<0.05$ vs. control of SP cells (Welch's t-test).*

cells. However, tumors derived from SP cells grew faster than those derived from non-SP cells in the 100-cell inoculated mice ($p<0.001$, Figure 2C).

The SP cell ratio in the tumors derived from SP cells remained five times higher than that derived from non-SP cells at 60 days after subcutaneous injection of 100 cells (Figure 2D). The same tendency was also observed in the mice inoculated with 1,000 and 10,000 cells (data not shown).

Reduction of SP fraction by YHO-13177. SP cells may be identified by their ability to export Hoechst 33342 *via* ABCG2. Therefore, we investigated the effect of YHO-13177 and reserpine on the export of Hoechst dye by SP cells. As shown in Figure 3, YHO-13177 treatment reduced the SP cell ratio in HeLa cells in a concentration-dependent manner, with the fraction almost disappearing at 0.1 μM. This potency was 80-times stronger than that for reserpine, which was required at approximately 8 μM in order to reduce the SP cell ratio to 0% (data not shown).

Low sensitivity of SP cells to irinotecan and sensitization by YHO-13351. To determine the chemoresistance of SP cells and the ability of YHO-13177 to reduce it, the sensitivity of SP cells to SN-38 (irinotecan) in the presence and absence of YHO-13177 (YHO-13351) was investigated *in vitro* and *in vivo*. Initially, the IC_{50} of SN-38 for cell growth inhibition was compared between non-SP and SP cells. The IC_{50} for SP cells was larger than that for non-SP cells ($p<0.05$, Figure 4). YHO-13177 reduced the IC_{50} for both non-SP and SP cells ($p<0.05$), with the IC_{50} for SP cells, in particular, dropping by 64% (Figure 4).

Next, the sensitivity of tumors derived from SP cells and non-SP cells to irinotecan and the chemosensitizing effect of YHO-13351 were investigated *in vivo*. In HeLa non-SP-bearing mice, treatments with irinotecan (66.7 mg/kg/day) alone every four days for three times significantly reduced tumor volume ($p<0.001$, Figure 5A, non-SP tumor). On the other hand, in HeLa SP-bearing mice, irinotecan-alone had no significant effect on tumor growth (Figure 5A, SP tumor). However, simultaneous administration of irinotecan and YHO-13351 resulted in a marked decrease in tumor volume in the SP-bearing mice to levels similar to those seen in irinotecan-treated or irinotecan plus YHO-13351-treated non-SP-bearing mice (Figure 5A). The bodyweight of the tumor-bearing mice was also determined to assess toxicity. Change in bodyweight was very small, with the maximum reduction of 5.5% seen in the SP-bearing mice on day 12 under treatment with irinotecan and YHO-13351 (200 and 1,200 mg/kg, total, respectively) (Figure 5B). No behavioral abnormality was observed in these mice.

Inhibitory effect of YHO-13351 on irinotecan monotherapy-induced increase of SP cell ratio in SP tumors. The SP cell ratios in the tumors under each therapy were investigated

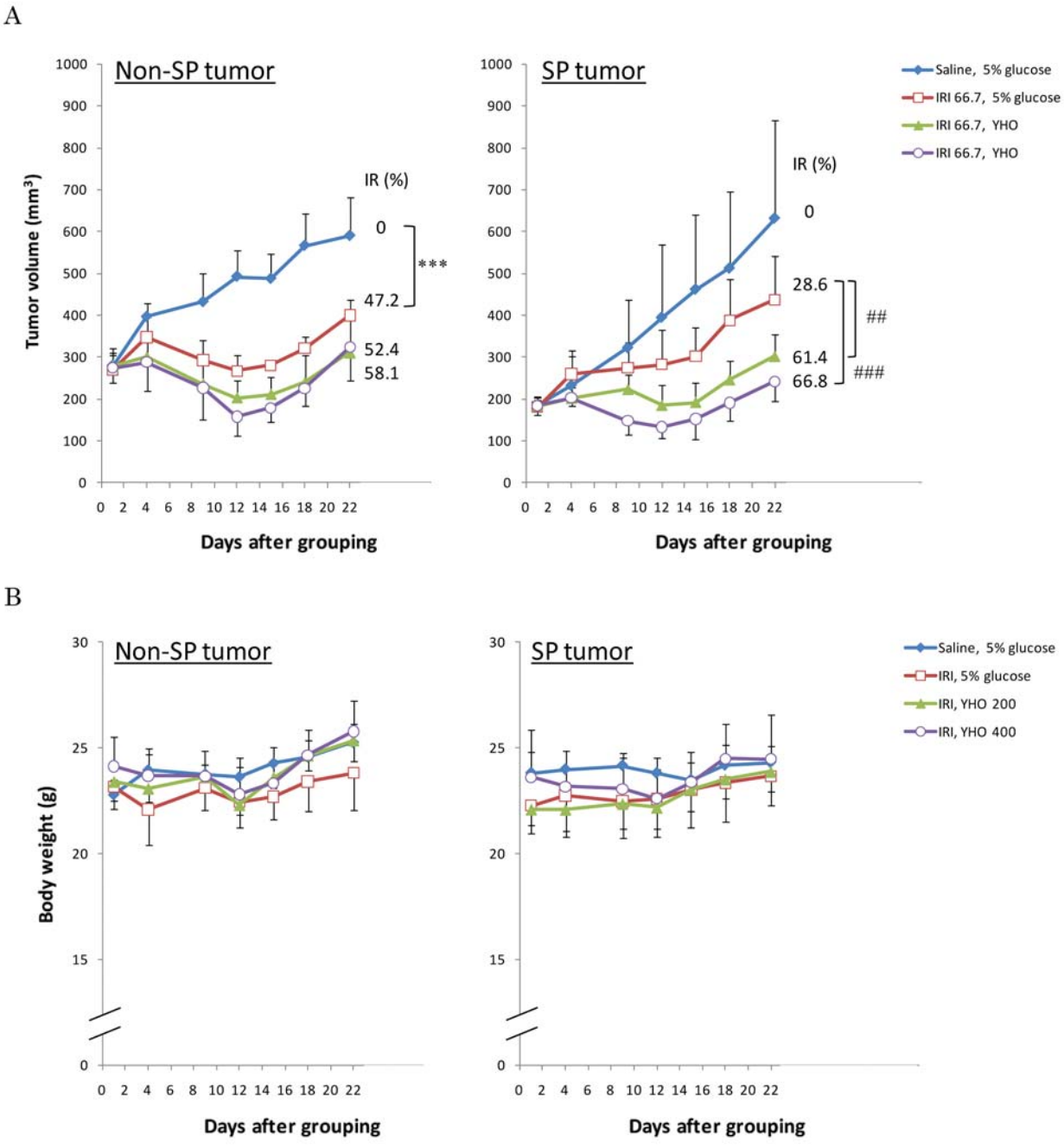

Figure 5. *Sensitization of tumors derived from side population (SP) cells to irinotecan (IRI) by YHO-13351 (YHO) in vivo. A: Mice with HeLa SP- and non-SP-cell-derived tumors were treated with 67 mg/kg irinotecan alone or 67 mg/kg irinotecan and 200 or 400 mg/kg YHO-13351 on days 1, 5, and 9 (n=5). Graphs show estimated tumor volume. All results are given as the mean±S.D. Each number besides the curve of tumor volume indicates tumor growth-inhibitory ratio (IR, %) assessed by tumor weight. ***p<0.001 vs. vehicle; ##p<0.01 and ###p<0.001 vs. irinotecan alone, respectively (Dunnett's test). B: Body weight changes in mice after administration of irinotecan and YHO-13351. Values represent the mean±S.D.*

after evaluation of the antitumor activity. In the non-SP-bearing mice groups, the ratio under each treatment was less than 0.2%, although slight differences in the SP cell ratio were observed. In the SP-bearing mice groups, on the other hand, the SP cell ratio was raised by administration of irinotecan-alone to a level at least twice as high as that in the untreated group. This increase in the SP cell ratio under irinotecan monotherapy was dose-dependently inhibited by YHO-13351 to a level below 0.2%, which was as low as that in the non-SP-bearing mice (Figure 6).

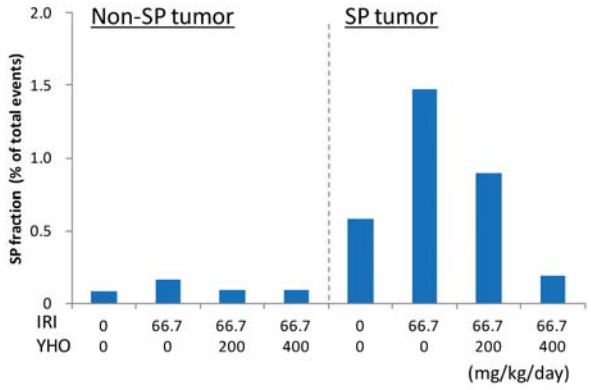

Figure 6. *Influence of irinotecan (IRI)-alone and with YHO-13351 (YHO) on side population (SP) cell ratio in tumors derived from SP cells. SP cell ratios in tumors were examined on day 22 (n=2).*

Discussion

Chemoresistance and recurrence are major problems in treating cancer, and there is an urgent need to develop more effective means of sensitizing tumors to conventional chemotherapeutics. Our primary objective was to determine the efficacy of combination treatment with irinotecan and a newly-synthesized ABCG2 inhibitor, YHO-13351, on SP tumors. SP cells exclude Hoechst 33342, and are reported to play an important role in the malignant alteration of tumors exhibiting not only low susceptibility to chemotherapeutics, but also high tumorigenicity and proliferative potential (14-16).

In this study, it was found that a selection of commercially available human cancer cell lines contained SP cells, with fractions varying from 1% to 90%, and these cell lines were ABCG2-positive. Only HCT116 cells, which contained few SP cells, were ABCG2-negative. This indicates that this ABC transporter plays an essential role in the efflux of Hoechst dye. The ratio of the ABCG2-positive population was markedly larger than that of the SP cells in all cell lines examined. This was probably because the ABCG2 molecules detected in the analysis were localized not only on the plasma membrane, but also in the cytoplasm; or because a large number of ABCG2 molecules, even if located on the cell surface, does not always contribute to efflux of Hoechst dye (20, 21).

The present results suggest that HeLa SP cells have cancer stem/initiating cell-like properties. In terms of tumorigenicity, no difference was observed between SP and non-SP cells with 100-cell inoculation in the xenograft mouse model. However, HeLa SP cells exhibited higher spheroid colony formation than non-SP cells. The spontaneous tumorigenicity of HeLa cells *in vivo* is so high that any difference between that of SP and non-SP cells might be hidden (22). Moreover, the strain of mouse may greatly influence the results. Here, tumors derived from SP cells exhibited more rapid progression and maintained a higher SP cell ratio, with asymmetrical proliferation. This suggests that HeLa SP cells, which are capable of self-renewal and re-population, possess cancer stem/initiating cell-like properties and that they form more aggressive tumors in nude mice than do HeLa non-SP cells.

Although HCT116 is frequently used in mouse xenograft models because of its high tumorigenicity, few HCT116 SP cells were detected in the present study. This suggests that HCT116 possesses a cancer stem/initiating cell-like population which does not overexpress ABCG2 and, therefore, is not associated with the efflux of Hoechst. Another possibility is the existence of secondary tumorigenic non-SP cells that are indistinguishable from cancer stem/initiating cells on the basis of assays for clonogenicity or tumorigenicity (23). It has been suggested that cancer stem/initiating cells are difficult to determine based on one property alone. Although SP cells may not always be the decisive factor in a population of cancer stem/initiating cells (24), they do seem to indicate a greater likelihood of malignancy.

YHO-13177 is a newly-discovered ABCG2 inhibitor, and YHO-13351 is a pro-drug of YHO-13177 (17). YHO-13177 inhibited the export of Hoechst dye from SP cells with a potency 80-times stronger than that of reserpine. YHO-13177, therefore, offers an excellent tool for the study of the function of ABCG2 in cell populations which include SP cells. In the present study, the non-SP cells were also mildly, but significantly, sensitized to SN-38 by YHO-13177. This suggests that SN-38 was eliminated by ABCG2 more easily than was Hoechst dye. It is, therefore, necessary to investigate the affinity of ABCG2 for Hoechst dye and SN-38.

HeLa SP cells and SP-derived tumors exhibited clear chemoresistance to irinotecan (SN-38), which was reversed by the addition of YHO-13351 (YHO-13177). The results revealed that SP cells formed more aggressive tumors than non-SP cells and that these tumors contained a higher ratio of SP cells. Moreover, while single-treatment with irinotecan amplified the tumor SP cell ratio, YHO-13351 in conjunction with irinotecan eliminated SP cells from SP-derived tumors. This increase in the SP cell ratio by irinotecan monotherapy may explain the mechanism of adverse change in cancer with conventional chemotherapy. YHO-13351 in combination with irinotecan, however, may inhibit this effect.

The present results suggest that combining irinotecan with YHO-13351 would not only accelerate the antitumor effect of this drug, but also contribute to eradicating cancer stem/initiating cells, indicating that YHO-13351 could play a crucial role in preventing resistance or relapse after conventional cancer chemotherapy.

Acknowledgements

The Authors would like to thank Professor Jeremy Williams, Tokyo Dental College, for his assistance with the English of the manuscript.

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Received February 11, 2013
Revised March 19, 2013
Accepted March 20, 2013