

Novel Acrylonitrile Derivatives, YHO-13177 and YHO-13351, Reverse BCRP/ABCG2-Mediated Drug Resistance *In Vitro* and *In Vivo*

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Abstract

Breast cancer resistance protein (BCRP/ABCG2) confers resistance to anticancer drugs such as 7-ethyl-10-hydroxycamptothecin (SN-38, an active metabolite of irinotecan), mitoxantrone, and topotecan. In this study, we examined the reversing effects of YHO-13177, a novel acrylonitrile derivative, and its water-soluble diethylaminoacetate prodrug YHO-13351 on the BCRP-mediated drug resistance. YHO-13177 potentiated the cytotoxicity of SN-38, mitoxantrone, and topotecan in both BCRP-transduced human colon cancer HCT116 (HCT116/BCRP) cells and SN-38-resistant human lung cancer A549 (A549/SN4) cells that express BCRP, but had little effect in the parental cells. In addition, YHO-13177 potentiated the cytotoxicity of SN-38 in human lung cancer NCI-H460 and NCI-H23, myeloma RPMI-8226, and pancreatic cancer AsPC-1 cells that intrinsically expressed BCRP. In contrast, it had no effect on P-glycoprotein-mediated paclitaxel resistance in MDR1-transduced human leukemia K562 cells and multidrug resistance-related protein 1-mediated doxorubicin resistance in MRP1-transfected human epidermoid cancer KB-3-1 cells. YHO-13177 increased the intracellular accumulation of Hoechst 33342, a substrate of BCRP, at 30 minutes and partially suppressed the expression of BCRP protein at more than 24 hours after its treatment in both HCT116/BCRP and A549/SN4 cells. In mice, YHO-13351 was rapidly converted into YHO-13177 after its oral or intravenous administration. Coadministration of irinotecan with YHO-13351 significantly increased the survival time of mice inoculated with BCRP-transduced murine leukemia P388 cells and suppressed the tumor growth in an HCT116/BCRP xenograft model, whereas irinotecan alone had little effect in these tumor models. These findings suggest that YHO-13351, a prodrug of YHO-13177, could be clinically useful for reversing BCRP-mediated drug resistance in cancer chemotherapy. *Mol Cancer Ther*; 10(7); 1252–63. ©2011 AACR.

Introduction

Drug resistance is a major problem in cancer chemotherapy. The best-characterized mechanism of drug resistance is mediated by P-glycoprotein/ABCB1, a member of the ATP-binding cassette (ABC) transporter superfamily, which pumps out a variety of structurally unrelated anticancer drugs such as the anthracyclines, vinca alkaloids, and taxanes from cells in an energy-dependent manner, resulting in a decrease in the intracellular accumulation of the drugs and resistance (1). To

overcome drug resistance, enormous efforts have been made to find inhibitors of P-glycoprotein, and a large number of clinical trials have been conducted (2). However, almost all clinical trials of P-glycoprotein inhibitors have failed to yield clinical benefit. Improvement of the clinical strategy could be necessary for successful outcomes. In addition, it has been gradually elucidated that ABC transporters other than P-glycoprotein are also associated with the drug resistance. Breast cancer resistance protein (BCRP/ABCG2) is another member of ABC transporters that mediates drug resistance (3). BCRP has been found to efflux camptothecins, including 7-ethyl-10-hydroxycamptothecin (SN-38, an active metabolite of irinotecan) and topotecan, and to confer resistance to this class of drugs (4). BCRP has also been shown to confer resistance to mitoxantrone, methotrexate, flavopiridol, gefitinib, and imatinib (5–9) by reducing the intracellular accumulation of these drugs. In addition, some evidence has emerged that BCRP in the intestine is a major determinant of the bioavailability and efficacy of orally administered BCRP substrates such as topotecan (10). Furthermore, BCRP has been regarded as a determinant

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of side population cells in tumors, a fraction highly enriched in cancer stem cells (11, 12).

It is possible that the expression of BCRP is responsible, at least in part, for clinical drug resistance. In fact, some reports have shown an association between BCRP expression and a poor response to chemotherapy. For example, the expression of BCRP has been reported in leukemic cells (13) and solid tumors (14–16) from patients, and increased expression of BCRP has been implicated in resistance to induction therapy against childhood acute myeloid leukemia (17). Furthermore, it has been reported that levels of BCRP are higher in cases of hepatic metastasis after irinotecan-based chemotherapy than in cases of irinotecan-naïve metastasis (18), and BCRP impacts clinical outcome in platinum-based chemotherapy for advanced non-small cell lung cancer (19). Therefore, inhibition of BCRP-mediated drug export could contribute to cancer chemotherapy.

Small molecule inhibitors of BCRP may be useful in reversing BCRP-mediated drug resistance, improving the bioavailability of orally administered drugs, and eliminating cancer stem cells in cancer chemotherapy. Many compounds have been shown to reverse BCRP-mediated drug resistance *in vitro*, including fumitremorgin C, an extract from *Aspergillus fumigatus* (20), GF120918, a compound developed as a P-glycoprotein inhibitor (21), estrogens like estrone and 17 β -estradiol (22), novobiocin, a coumermycin antibiotic (23), and plant-derived hydroxyflavonoids such as genistein (24). Although BCRP inhibitors have been the subject of much investigation, relatively little research has been conducted *in vivo* on the reversing effects of BCRP inhibitors on drug resistance, and no inhibitor specifically targeting BCRP has been used in clinical trials. Accordingly, more specific BCRP inhibitors that are good candidates for studies *in vivo* and could be clinically useful drugs are awaited.

YHO-13177, (Z)-2-(3,4-dimethoxyphenyl)-3-[5-(4-hydroxypiperidin-1-yl)thiophen-2-yl]acrylonitrile, is a novel acrylonitrile derivative, which was discovered by screening of in-house chemical libraries for BCRP inhibitors. 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 prodrug of YHO-13177. In this study, we examined the reversing effects of these drugs on BCRP-mediated drug resistance in various cancer cells that express BCRP *in vitro* and *in vivo*.

Materials and Methods

Materials

YHO-13177 and YHO-13351 (Fig. 1) were chemically synthesized by Yakult Honsha. Irinotecan hydrochloride, SN-38, and topotecan were also supplied by Yakult Honsha. Mitoxantrone, Hoechst 33342, paclitaxel, and doxorubicin were purchased from Sigma-Aldrich. For experiments *in vitro*, these drugs were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in all treatments, including control culture

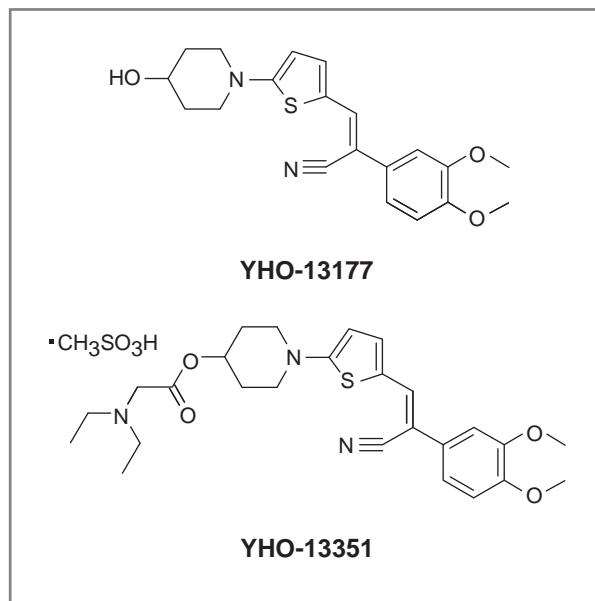


Figure 1. Chemical structures of YHO-13177 and YHO-13351.

conditions, was adjusted to 0.1%. For experiments *in vivo*, YHO-13351 and irinotecan were dissolved in 5% glucose and saline, respectively.

Cells and cell cultures

Human lung cancer A549 cells were cultured in F-12 HAM with 10% (v/v) FBS (Sigma-Aldrich) at 37°C in 5% CO₂. Human colon cancer HCT116 and epidermoid cancer KB-3-1 cells were cultured in Dulbecco's modified Eagle's medium with 10% (v/v) FBS at 37°C in 5% CO₂. Human lung cancer NCI-H23 and NCI-H460, human myeloma RPMI-8226, human pancreatic cancer AsPC-1, and murine leukemia P388 cells were grown in RPMI 1640 medium with 10% (v/v) FBS at 37°C in 5% CO₂. These cell lines were obtained from American Type Culture Collection, DS Pharma Biomedical, or Japanese Foundation for Cancer Research between 1999 and 2010. A549/SN4 cells, SN-38-resistant A549 cells, were obtained through the selection of A549 cells using increasingly high concentrations of SN-38, from 4 up to 10 ng/mL. SN-38-resistant cells were cloned using the limiting dilution technique, and one well-growing clone, A549/SN4, was selected for further experiments. HCT116/BCRP and P388/BCRP cells were established by the transduction of HCT116 and P388 cells, respectively, with the HaBCRP retrovirus, a Ha retrovirus carrying Myc-tagged human BCRP cDNA (25). K562/MDR cells were established by the transduction of K562 cells with the HaMDR retrovirus, a Ha retrovirus containing human MDR1 cDNA (26). KB-3-1/MRP1 cells were established by the transfection of KB-3-1 cells with the pCAL-MRP1 construct bearing the human MRP1 cDNA (27). All the cell lines were aliquoted, immediately frozen, and stored after acquisition. The thawed cell lines were

tested for morphology, growth, and absence of mycoplasma by microscope, TetraColor ONE (Seikagaku Corporation), and Hoechst staining, respectively, and used for experiments within 3 months. The phenotype of established cell lines was also proven by Western blotting with antibodies specific for corresponding ABC transporters or check for drug-sensitivity before or during the experiments.

Experimental animals

Six-week-old male ICR mice, female CDF1 mice, and male BALB/c nude mice purchased from Japan SLC, Inc, Charles River Japan, Inc, and CLEA Japan, Inc, respectively, were housed in polycarbonate cages (5 animals/cage) with water and food provided *ad libitum*. All experiments were carried out in accordance with the animal experimentation guidelines, and approved by the ethics committee for animal experiments, of our institute.

Western blotting

Cancer cells were lysed in solubilization buffer [10 mmol/L Tris-HCl, pH 7.4, 0.1% (w/v) NP-40, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15 mol/L NaCl, 1 mmol/L EDTA, and 10 µg/mL aprotinin]. The lysate was subjected to SDS-PAGE using 10% (w/v) gels under reducing conditions. The separated proteins were electrotransferred to Immobilon transfer membranes. Then, each membrane was reacted with anti-human BCRP monoclonal antibody (clone BXP-21, MONOSAN) and anti-porcine α -tubulin monoclonal antibody (clone TU-02, Santa Cruz Biotechnology), which were subsequently complexed with an alkaline phosphatase-conjugated or horseradish peroxidase-conjugated secondary antibody. Immunoreactive BCRP and α -tubulin were visualized indirectly using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates or ECL Plus Western Blotting Detection Reagents (GE Healthcare). In some experiments, protein expression was quantified with a LAS-3000 Luminescent Image Analyzer (Fuji Photo Film).

Cytotoxicity assay for cell survival

Cells were plated into 96-well culture plates and various concentrations of anticancer drugs with or without YHO-13177 were added to the cultures. After 72 to 120 hours, cell viability was measured as mitochondrial NADH-dependent dehydrogenase activity with a Cell Proliferation Assay System, TetraColor ONE (Seikagaku Corporation). The concentration of antitumor drugs causing 50% inhibition of cell viability (IC_{50}) was calculated by interpolation.

Assay of intracellular accumulation of Hoechst 33342

The intracellular accumulation of Hoechst 33342 was measured by a fluorometric assay. Cells (4×10^4 /well) were plated into 96-well culture plates, and allowed to attach for 18 hours at 37°C in 5% CO₂. Then, 0.5 µg/mL

Hoechst 33342 and various concentrations of YHO-13177 were added to the cultures. After 30 minutes, the intracellular amount of Hoechst 33342 was determined using a microplate spectrofluorometer (SpectraMax M5e; Molecular Devices) with excitation and emission wavelengths of 346 and 460 nm, respectively. YHO-13177 did not affect the fluorescence of Hoechst 33342. The intracellular accumulation of Hoechst 33342 was also observed with a fluorescence microscope (Biozero BZ-8100, KEYENCE).

Flow cytometry

For immunofluorescent staining, the cells were fixed with paraformaldehyde and stained with fluorescein isothiocyanate-conjugated anti-human BCRP monoclonal antibody (clone 5D3, Santa Cruz Biotechnology). After incubation in the dark for 30 minutes at room temperature, the cells were washed, resuspended in PBS with 2% (v/v) FBS, and analyzed by flow cytometry using GUAVA EasyCyte Plus System (Guava Technologies).

Pharmacokinetic studies

Blood was collected by cardiac puncture under anesthesia, using heparinized syringes after the administration of YHO-13351 to ICR mice, and centrifuged to separate the plasma. The samples in the plasma were extracted by methanol using Sirocco protein precipitation plates (Waters). Riboflavin butyrate used as an internal standard was added to plasma samples during the extraction process. The analysis of YHO-13177 and YHO-13351 in the extracts was carried out by ultra performance liquid chromatography using an Acquity UPLC system (Waters) and Acquity UPLC HSS T3 column (1.8 µm, 2.1 × 50 mm; Waters) with solvent A (10 mmol/L ammonium acetate) and solvent B (methanol). The gradient used was as follows: $t = 0$ minutes, 80% A; $t = 4$ minutes, 80% A; $t = 12$ minutes, 25% A; $t = 12.1$ minutes, 80% A; $t = 14$ minutes, 80% A. The flow rate was 0.3 mL/min and the eluate was monitored at 435 nm. The limits of quantification for YHO-13177 and YHO-13351 in plasma were 125 and 50 ng/mL, respectively.

The amount of irinotecan and SN-38 in plasma was determined by previously reported high-performance liquid chromatography with an automated online solid-phase extraction system, PROSPECT (Spark Holland; ref. 28).

The area under the plasma concentration–time curve (AUC) was calculated by the trapezoidal method. Bioavailability of administered drug was determined as the ratio of the AUC after oral and i.v. administrations.

Evaluation of antitumor activity *in vivo*

Two *in vivo* models, the P388/BCRP ascites tumor model and HCT116/BCRP xenograft model, were used to characterize the chemosensitizing properties of YHO-13351.

For the former model, P388/BCRP cells (1×10^6 /mouse) were inoculated intraperitoneally (i.p.) into CDF1 mice (5 mice/group) on day 0, and irinotecan

and/or YHO-13351 were administered i.p. once a day at the same time on days 1, 5, and 9. To evaluate the antitumor effects of irinotecan and/or YHO-13351, the survival of mice in each group was examined daily, and the survival rate, T/C (%), was calculated from the survival period using the following equation:

$$T/C (\%) = \frac{\text{(mean survival days of mice in treated group)}}{\text{(mean survival days of mice in control group)}} \times 100.$$

For the latter model, HCT116/BCRP cells (2×10^6 /mouse) were inoculated s.c. into the inguinal region of BALB/c nude mice. The long and short diameters of the tumor (mm) were measured using calipers, and tumor volume was estimated at various times with the following equation:

$$\text{Estimated tumor volume (mm}^3\text{)} = 1/2 \times (\text{long diameter}) \times (\text{short diameter})^2.$$

When the tumor volume reached about 100 mm^3 (day 0), the inoculated mice were divided into test groups (5 mice/group). Irinotecan was administered i.v. once a day on days 1, 5, and 9. YHO-13351 was administered orally at the same time as irinotecan or administered i.v. at 0 and 4 hours after irinotecan. On day 22 from the start of administration, a tumor was extirpated from each mouse and weighed, and the tumor growth inhibition rate, IR (%), was derived from the following equation:

$$IR (\%) = [1 - \frac{\text{(mean tumor weight of each treated group)}}{\text{(mean tumor weight of control group)}}] \times 100.$$

The long and short diameters of the tumor and body weight were measured once to twice weekly during the experimental period.

Statistical analysis

The statistical analysis was carried out using Dunnett's test. *P* values less than 0.05 were considered significant.

Results

Expression levels of BCRP in cell lines

To evaluate the effects of YHO-13177 and 13351 on BCRP-mediated drug resistance *in vitro* and *in vivo*, we first characterized the expression of BCRP in the cells used in this study using Western blotting. A549/SN4 cells have acquired resistance to SN-38, through exposure to increasingly high concentrations of SN-38. HCT116/BCRP and P388/BCRP cells are *BCRP*-transduced HCT116 and P388 cells, respectively. As shown in Fig. 2A, BCRP expression was detected in A549/SN4, HCT116/BCRP, and P388/BCRP cells. In contrast, the expression of BCRP was nearly or completely undetectable in the parental A549, HCT116, and P388 cells. As shown in Fig. 2B, RPMI8226, NCI-H23, NCI-H460, and AsPC-1 cells intrinsically expressed BCRP.

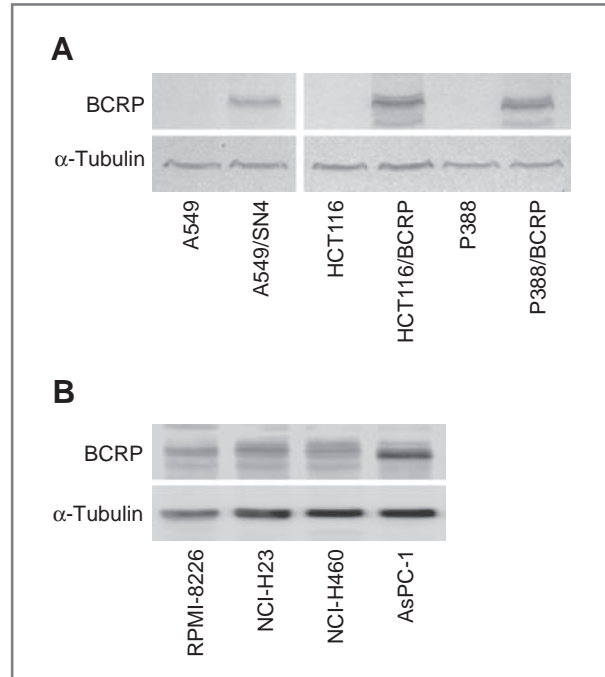


Figure 2. Analysis of BCRP expression in BCRP-mediated drug-resistant cells (A) and intrinsically BCRP-expressing cells (B). Protein extracts prepared from the cells were subjected to Western blotting as described in Materials and Methods.

Reversing effect of YHO-13177 on BCRP-mediated drug resistance *in vitro*

Initially, we examined the effect of YHO-13177 on BCRP-mediated drug resistance in *BCRP*-transduced HCT116 cells *in vitro*. As shown in Fig. 3A, HCT116/BCRP cells displayed clear resistance to SN-38, topotecan, and mitoxantrone, which are substrates of BCRP (4, 5), compared with parental HCT116 cells. When YHO-13177 was added to the HCT116/BCRP cells at final concentrations of 0.01 to $1 \mu\text{mol/L}$, it apparently reversed all of the drug resistance in a concentration-dependent manner, as shown by a shift in the cytotoxicity curves to the left. In contrast, YHO-13177 had no effect on drug sensitivity in the parental HCT116 cells that did not express BCRP. Under these experimental conditions, YHO-13177 alone had no cytotoxic effects on HCT116 or HCT116/BCRP cells. The IC_{50} value of YHO-13177 for a cytotoxic effect on HCT116 cells was higher than $10 \mu\text{mol/L}$.

The reversing effect of YHO-13177 was also evaluated in A549 cells with BCRP-mediated acquired resistance to SN-38. As shown in Fig. 3B, A549/SN4 cells showed about 10-fold resistance to SN-38, and cross-resistance to topotecan and mitoxantrone, compared with parental A549 cells. YHO-13177 reversed the resistance to all three drugs in a concentration-dependent manner at 0.01 to $1 \mu\text{mol/L}$. However, YHO-13177 had little or no effect on drug sensitivity in the parental A549 cells. Under these experimental conditions, YHO-13177 itself

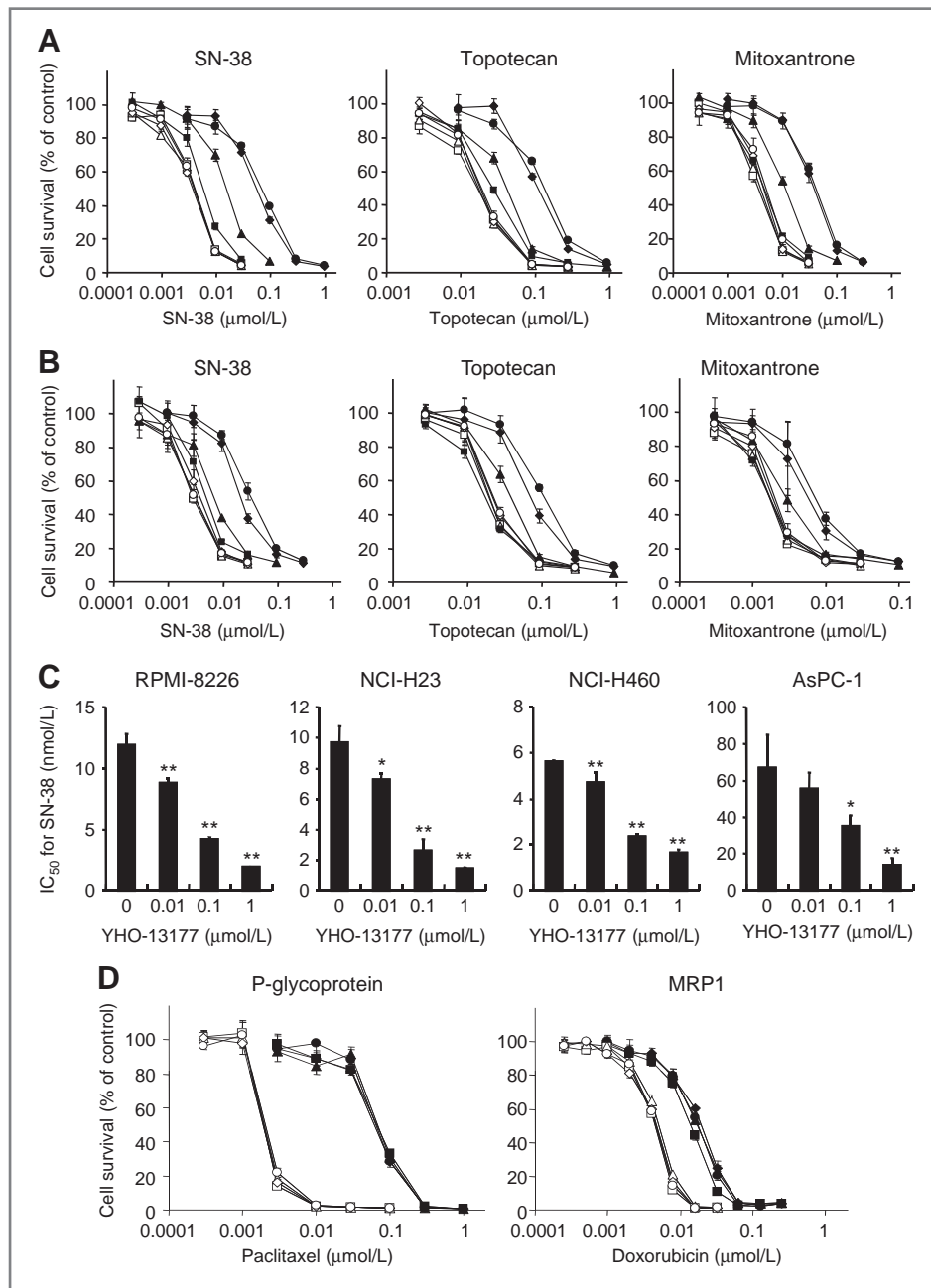


Figure 3. Effect of YHO-13177 on drug resistance and sensitivity in various cancer cells. **A**, reversing effect of YHO-13177 on BCRP-mediated drug resistance in *BCRP*-transduced cells. HCT116 (open symbols) and HCT116/*BCRP* (closed symbols) cells were cultured with SN-38, topotecan, or mitoxantrone in the absence or presence of YHO-13177 for 96 hours. The survival of the cells was presented as a percentage of the value for the control culture. **B**, reversing effect of YHO-13177 on BCRP-mediated drug resistance in SN-38-acquired resistant cells. A549 (open symbols) and A549/SN4 (closed symbols) cells were cultured with SN-38, topotecan, or mitoxantrone in the absence or presence of YHO-13177 for 96 hours. The survival of the cells was presented as a percentage of the value for the control culture. **C**, enhancing effect of YHO-13177 on the cytotoxicity of SN-38 in intrinsically *BCRP*-expressing cells. RPMI-8226, NCI-H23, NCI-H460, and AsPC-1 cells were cultured with SN-38 in the absence or presence of YHO-13177 for 96 hours. The cytotoxicity of SN-38 was represented by the IC_{50} value for SN-38. *, $P < 0.05$; **, $P < 0.01$ versus SN-38 alone. **D**, effect of YHO-13177 on P-glycoprotein or MRP1-mediated drug resistance. For P-glycoprotein, K562 (open symbols) and K562/*MDR* (closed symbols) cells were cultured with paclitaxel in the absence or presence of YHO-13177 for 72 hours. For MRP1, KB-3-1 (open symbols) and KB-3-1/*MRP1* (closed symbols) cells were cultured with doxorubicin in the absence or presence of YHO-13177 for 120 hours. The survival of the cells was presented as a percentage of the value for the control culture. \circ and \bullet , without YHO-13177; \diamond and \blacklozenge , 0.01 $\mu\text{mol/L}$ YHO-13177; \triangle and \blacktriangle , 0.1 $\mu\text{mol/L}$ YHO-13177; \square and \blacksquare , 1 $\mu\text{mol/L}$ YHO-13177. Data are means \pm SD of triplicate determinations.

was not cytotoxic in A549 or A549/SN4 cells. The IC_{50} value of YHO-13177 alone for a cytotoxic effect on A549 cells was higher than 10 $\mu\text{mol/L}$.

In addition, we examined the effect of YHO-13177 on the cytotoxicity of SN-38 in RPMI-8226, NCI-H23, NCI-H460, and AsPC-1 cells that intrinsically express BCRP. As shown in Fig. 3C, YHO-13177 significantly enhanced the cytotoxicity of SN-38 in all of the cancer cells in a concentration-dependent manner, as indicated by a reduction in IC_{50} values for SN-38. Under these conditions, YHO-13177 itself did not have cytotoxic effects on these cancer cells.

To determine the specificity of YHO-13177 for BCRP, we examined the effects of YHO-13177 on the other ABC transporters, P-glycoprotein and multidrug resistance-related protein 1 (MRP1)-mediated drug resistance in *MDR1*-transduced K562 cells and *MRP1*-transfected KB-3-1 cells, respectively. As shown in Fig. 3D, K562/*MDR* and KB-3-1/*MRP1* cells displayed resistance to paclitaxel and doxorubicin, respectively, compared with the parental cells. YHO-13177 had no effect on the P-glycoprotein and MRP1-mediated resistance at 0.01 to 1 $\mu\text{mol/L}$, the effective concentration range of YHO-13177 for the reversal of BCRP-mediated drug resistance.

Effect of YHO-13177 on intracellular drug accumulation

To determine whether the reversal of BCRP-mediated drug resistance by YHO-13177 might be associated with the inhibition of BCRP-mediated drug transport, we studied the effects of YHO-13177 on the intracellular accumulation of Hoechst 33342, a fluorescent substrate of BCRP (29), in HCT116/BCRP and A549/SN4 cells. As shown in Fig. 4, the intracellular accumulation of Hoechst 33342 was remarkably decreased in both HCT116/BCRP and A549/SN4 cells compared with their parental cells, when the cells were incubated with Hoechst 33342 for 30 minutes. YHO-13177 at 0.1 to 10 $\mu\text{mol/L}$ significantly increased the intracellular accumulation of Hoechst 33342 in both HCT116/BCRP and A549/SN4 cells in a concentration-dependent manner, and raised the intracellular Hoechst 33342 concentration to the level found in their parental cells. In contrast, YHO-13177 had no or a marginal effect on Hoechst 33342 levels in the parental HCT116 and A549 cells.

Effect of YHO-13177 on expression of BCRP protein

To further explore the mechanism by which YHO-13177 reverses the BCRP-mediated drug resistance, we examined the effect of YHO-13177 on BCRP protein levels in HCT116/BCRP and A549/SN4 cells. The cells were incubated with YHO-13177 at 0.01 to 1 $\mu\text{mol/L}$, the effective concentration range of YHO-13177 for the reversal of BCRP-mediated drug resistance *in vitro* (Fig. 3), for 96 hours. The results of Western blotting are shown in Fig. 5A, indicating that YHO-13177 partially but appreciably suppressed the expression of BCRP protein in both

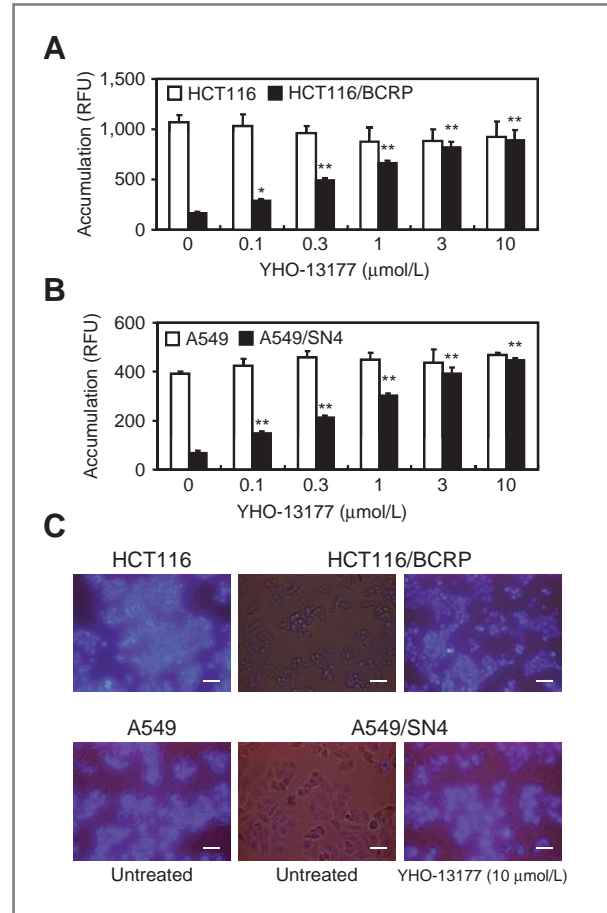


Figure 4. Effect of YHO-13177 on intracellular accumulation of Hoechst 33342. HCT116 and HCT116/BCRP cells (A), or A549 and A549/SN4 cells (B) were incubated with 0.5 $\mu\text{g/mL}$ Hoechst 33342 for 30 minutes in the absence or presence of YHO-13177. Intracellular Hoechst 33342 accumulation was quantified by measuring fluorescence with a spectrofluorometer (A and B) and observed with a fluorescence microscope (C) as described in Materials and Methods. Data are means \pm SD of triplicate determinations. *, $P < 0.05$; **, $P < 0.01$ versus Hoechst33342 alone. Scale bar, 50 μm .

HCT116/BCRP and A549/SN4 cells. In contrast, a real-time quantitative reverse transcription PCR reaction-based analysis showed that YHO-13177 did not suppress the expression *BCRP* mRNA in HCT116/BCRP and A549/SN4 cells at all (data not shown). Furthermore, we examined the time course of the effect of YHO-13177 on the expression levels of BCRP protein in HCT116/BCRP cells. As shown in Fig. 5B, the level of BCRP protein apparently decreased at more than 24 hours after treatment of YHO-13177. In contrast, YHO-13177 had no effect on the expression of BCRP protein at 0.5 to 6 hours, the effective time for increase of the intracellular accumulation of Hoechst 33342 by YHO-13177 in HCT116/BCRP cells (Fig. 4A). In addition, cell surface expression levels of BCRP protein were also confirmed by flow cytometry. As shown in Fig. 5C, YHO-13177 indeed suppressed the cell surface expression of BCRP protein at

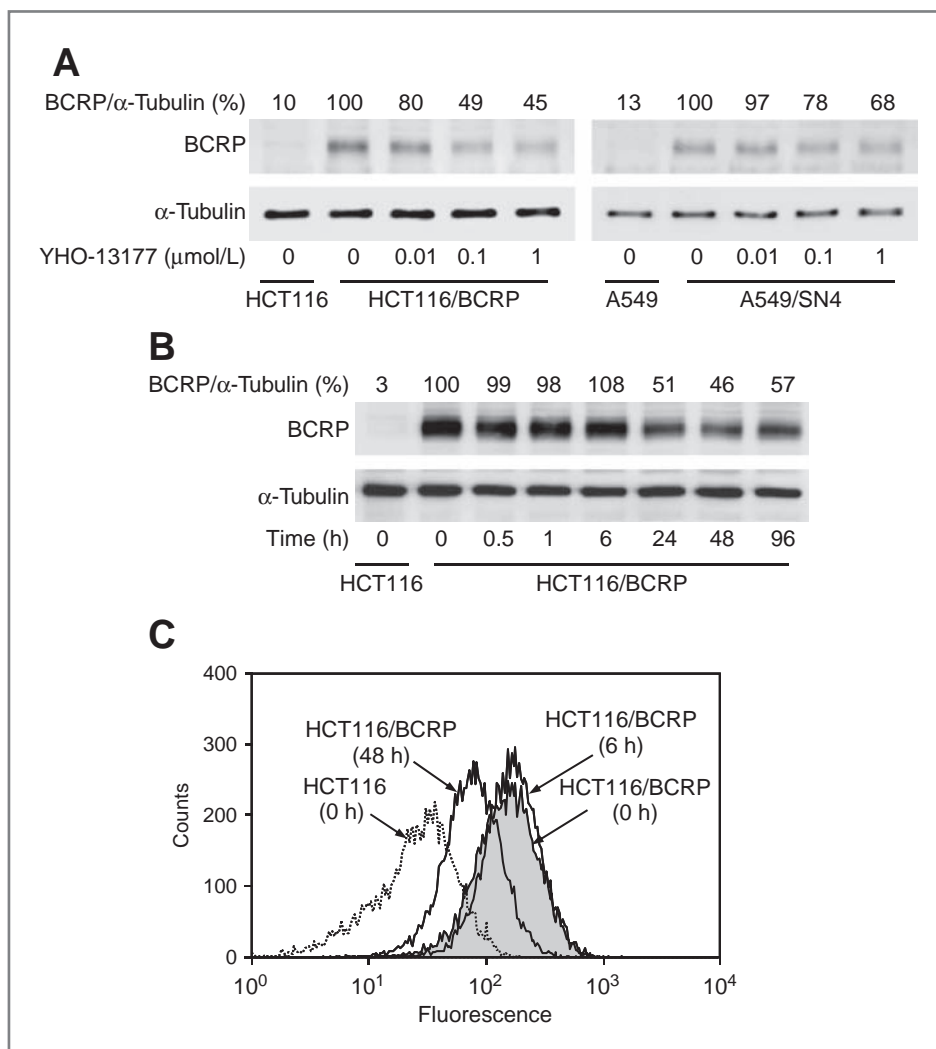


Figure 5. Effect of YHO-13177 on expression levels of BCRP protein. **A**, dose dependence of effect of YHO-13177 on expression levels of BCRP protein. HCT116 and HCT116/BCRP cells, or A549 and A549/SN4 cells were cultured in the absence or presence of YHO-13177 for 96 hours. **B**, time dependence of effect of YHO-13177 on expression levels of BCRP protein. HCT116 and HCT116/BCRP cells were cultured in the presence of 1 μ mol/L YHO-13177 for various times. Protein extracts prepared from the cells were subjected to Western blotting. The amount of BCRP was semi-quantified by densitometric analysis and calculated relative to that in control cells after normalizing for the amount of α -tubulin. **C**, effect of YHO-13177 on cell surface expression levels of BCRP protein. HCT116 and HCT116/BCRP cells were cultured in the presence of 1 μ mol/L YHO-13177 for various times, and the cell surface expression of BCRP was analyzed by flow cytometry.

48 hours but not at 6 hours after its treatment in HCT116/BCRP cells.

Conversion of YHO-13351 to YHO-13177 *in vivo*

Because YHO-13177 exhibits very low solubility in water (solubility, < 0.1 mg/mL), it is difficult to administer it to mice. Therefore, we used YHO-13351, a water-soluble diethylaminoacetate prodrug of YHO-13177 (solubility, > 100 mg/mL), *in vivo*. The plasma levels of YHO-13351 and YHO-13177 in mice after a single i.v. administration of 31 mg/kg or oral administration of 117 mg/kg YHO-13351 are shown in Fig. 6A. YHO-13351 was rapidly converted into YHO-13177 in the mice. The highest plasma concentrations of YHO-13177 after the i.v. and oral administrations were 19.7 ± 2.0 μ mol/L at 5 minutes and 27.3 ± 5.3 μ mol/L at 30 minutes. Orally administered YHO-13351 was well absorbed (bioavailability, 86.5%) and more than 1.0 μ mol/L YHO-13177 was maintained for at least 8 hours in plasma. In contrast, YHO-13351 itself was undetect-

able in plasma within 5 minutes after the i.v. and oral administrations in mice.

Enhancing effect of YHO-13351 on antitumor activity of irinotecan *in vivo*

The P388/BCRP ascites tumor model was used for the initial evaluation of the chemosensitizing effect of YHO-13351 *in vivo*. P388/BCRP cells were inoculated i.p. into mice on day 0, and irinotecan (30 mg/kg) and/or YHO-13351 (100 or 200 mg/kg) were administered i.p. once a day on days 1, 5, and 9. The survival time of P388/BCRP-inoculated mice in the control group was 13.6 ± 1.3 days. As shown in Fig. 6B, irinotecan alone was quite ineffective against P388/BCRP cells as compared with the control group (T/C, 97%). However, coadministration of irinotecan with 100 or 200 mg/kg YHO-13351 significantly increased the survival time in a dose-dependent manner. The T/C values for the combination of irinotecan with 100 and 200 mg/kg YHO-13351 were 170% ($P < 0.001$ vs. irinotecan alone) and 197% ($P < 0.001$ vs. irinotecan

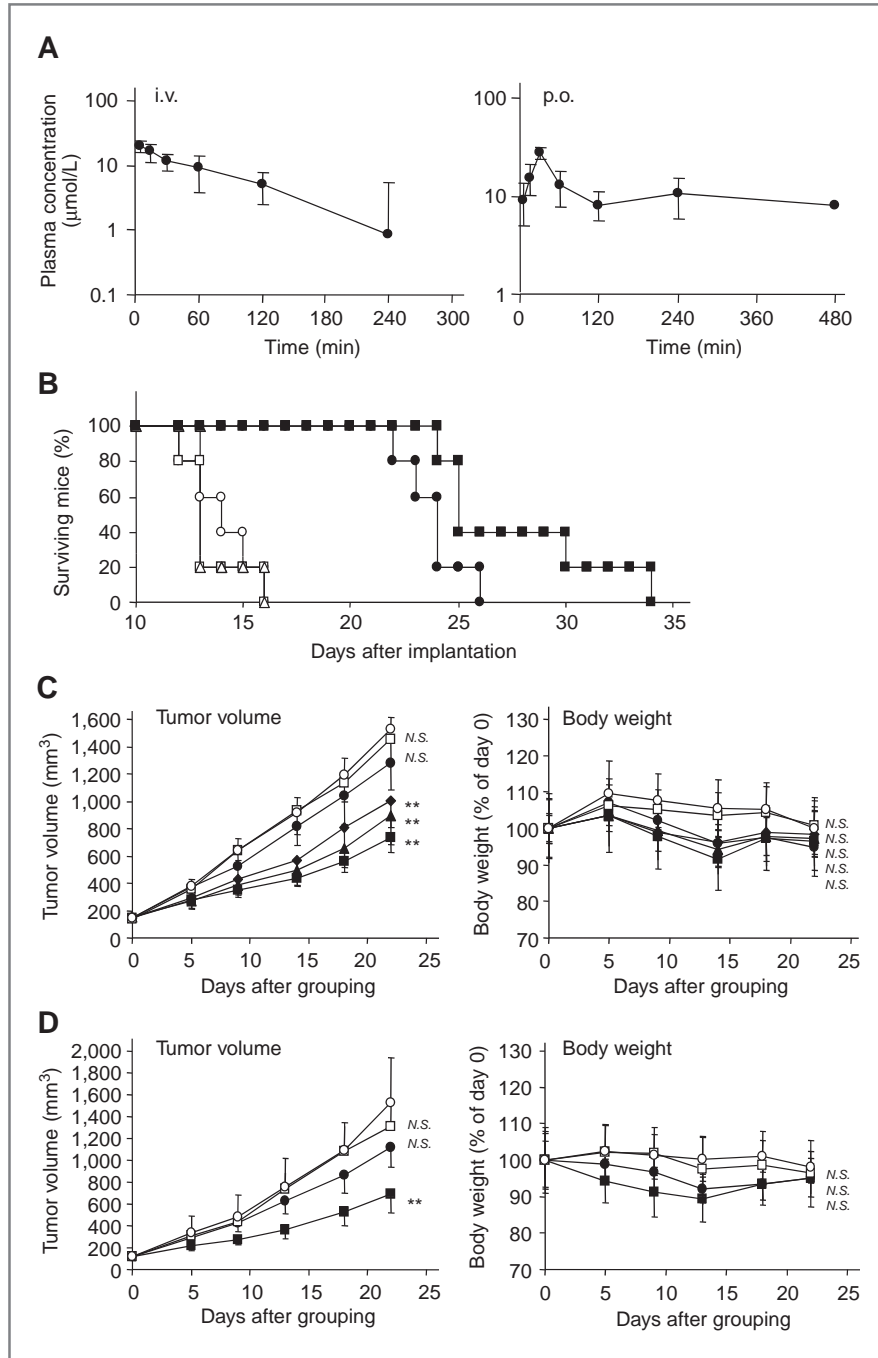


Figure 6. Enhancing effect of YHO-13351 on antitumor activity of irinotecan *in vivo*. **A**, YHO-13177 and YHO-13351 plasma levels in mice. After a single i.v. administration of 31 mg/kg or oral administration of 117 mg/kg YHO-13177 (●) and YHO-13351 (○) were determined by UPLC as described in Materials and Methods. Data are means ± SD for 5 animals. **B**, effect of YHO-13351 on antitumor activity of irinotecan in the P388/BCRP ascites tumor model. Mice were inoculated i.p. with P388/BCRP cells on day 0 and treated on days 1, 5, and 9 with 30 mg/kg irinotecan i.p. (△), 200 mg/kg YHO-13351 i.p. (□), or combinations of 30 mg/kg irinotecan i.p. with 100 mg/kg (●) or 200 mg/kg (■) YHO-13351 i.p.; ○, control. The survival of mice was examined daily. All groups consisted of 5 animals. **C**, effect of oral administration of YHO-13351 on antitumor activity of irinotecan in the HCT116/BCRP xenograft model. Mice were inoculated s.c. with HCT116/BCRP cells on day 0 and treated on days 1, 5, and 9 with 45 mg/kg irinotecan i.v. (●), 200 mg/kg YHO-13351 orally (□), or combinations 45 mg/kg of irinotecan i.v. with 50 mg/kg (◆), 100 mg/kg (▲), or 200 mg/kg (■) YHO-13351 orally, ○, control. Tumor volume and body weight were measured as described in Materials and Methods. Data are means ± SD for 5 animals. The statistical analysis was carried out on day 22. **, $P < 0.01$ versus control. NS, not significant ($P > 0.05$). **D**, effect of i.v. administration of YHO-13351 on antitumor activity of irinotecan in the HCT116/BCRP xenograft model. Mice were inoculated s.c. with HCT116/BCRP cells on day 0 and treated on days 1, 5, and 9 with 45 mg/kg irinotecan i.v. (●), 30 mg/kg YHO-13351 i.v. at 0 and 4 hours after irinotecan (■) or saline (□). ○, control. Tumor volume and body weight were measured as described in Materials and Methods. Data are means ± SD for 5 animals. The statistical analysis was carried out on day 22. **, $P < 0.01$ versus control. NS, not significant ($P > 0.05$).

alone), respectively. In contrast, YHO-13351 alone had no effect on the survival time of P388/BCRP-inoculated mice (T/C, 100%).

Furthermore, the antitumor activity of irinotecan combined with YHO-13351 was compared with that of irinotecan alone in the HCT116/BCRP xenograft model *in vivo*. When the tumor reached about 100 mm³ after the s.c. inoculation of HCT116/BCRP cells into mice, the mice were divided into test groups (day 0). Irinotecan (i.v.) and/or YHO-13351 orally were administered once a day on days 1, 5, and 9. As shown in Fig. 6C, the i.v. administration of 45 mg/kg irinotecan (half of the maximum tolerated dose) on days 1, 5, and 9 had only a small effect on tumor growth. The IR value of irinotecan alone calculated from tumor weight on day 22 was 14.6% (not significant vs. control). In contrast, the oral administration of 50, 100, or 200 mg/kg YHO-13351 at the same time as the i.v. administration of irinotecan significantly enhanced the antitumor activity of irinotecan in a dose-dependent manner. The IR values for the combination of irinotecan with 50, 100, and 200 mg/kg YHO-13351 were 39.5% ($P < 0.001$ vs. irinotecan alone), 46.4% ($P < 0.001$ vs. irinotecan alone), and 50.3% ($P < 0.001$ vs. irinotecan alone), respectively. In addition, the enhancing effect of the i.v. administration of YHO-13351 on the antitumor activity of irinotecan was also confirmed. As shown in Fig. 6D, the i.v. administration of 30 mg/kg YHO-13351 at 0 and 4 hours after the administration of 45 mg/kg irinotecan significantly enhanced the antitumor activity of irinotecan. The weight loss observed in the mice treated with irinotecan (i.v.) plus YHO-13351 (orally or i.v.) was similar to that observed in the mice treated with irinotecan alone, and body weight recovered after the treatment ceased, indicating that the combinations were well tolerated. On the other hand, YHO-13351 (orally or i.v.) alone had no effect on tumor growth or body weight in the HCT116/BCRP xenograft model.

In pharmacokinetic study, coadministration of 30 mg/kg YHO-13351 (i.v.) increased AUC_{0-inf} values of irinotecan 1.4-fold (14.5 vs. 10.4 µg/h/mL) and SN-38 1.6-fold (0.543 vs. 0.344 µg/h/mL) compared with 45 mg/kg irinotecan (i.v.) alone in mice.

Discussion

YHO-13177 was discovered by the *in vitro* screening of in-house chemical libraries for BCRP inhibitors based on restoration of the cytotoxicity of SN-38 in BCRP-expressing A549/SN4 cells. Because YHO-13177 exhibited very low solubility, we synthesized YHO-13351, a water-soluble diethylaminoacetate prodrug of YHO-13177. These compounds are novel and not structurally similar to any BCRP inhibitors described in the literature. In this study, we examined the reversing effects of YHO-13177 *in vitro* and YHO-13351 *in vivo* on BCRP-mediated drug resistance in various cancer cells that express BCRP.

YHO-13177 reversed resistance to SN-38, mitoxantrone, and topotecan, which are substrates of BCRP (4,

5), in the BCRP-transduced cells, HCT116/BCRP, and the cells that acquired BCRP-mediated resistance to SN-38, A549/SN4, but had little or no effect in the parental cells. The concentrations of YHO-13177 that produced a half-maximal reversal of the drug resistance in HCT116/BCRP and A549/SN4 cells were within the range 0.01 to 0.1 µmol/L. In addition, YHO-13177 enhanced the cytotoxicity of SN-38 in NCI-H460, NCI-H23, RPMI-8226, and AsPC-1 cells that intrinsically expressed BCRP. In contrast, it had no effect on P-glycoprotein-mediated resistance to paclitaxel in *MDR1*-transduced K562 cells and MRP1-mediated resistance to doxorubicin in *MDR1*-transfected KB-3-1 cells. Under these experimental conditions, YHO-13177 itself had no cytotoxic effect on the cancer cells. These observations indicate that YHO-13177 acts as a potent and specific inhibitor of BCRP *in vitro*.

To investigate the mechanism of the reversing effect of YHO-13177 on BCRP-mediated drug resistance, we examined the effects of YHO-13177 on the drug transport activity of BCRP and expression of the BCRP protein. YHO-13177 increased the intracellular accumulation of Hoechst 33342, a substrate of BCRP (29), in both HCT116/BCRP and A549/SN4 cells at 30 minutes after its treatment, but had no or only a marginal effect in the drug-sensitive parental cells, suggesting that BCRP-mediated drug efflux is inhibited by YHO-13177. Furthermore, YHO-13177 partially but appreciably suppressed the expression of BCRP protein in both HCT116/BCRP and A549/SN4 cells without suppression of the BCRP mRNA expression. In the time course study, more than 24 hours of incubation was necessary for YHO-13177 to suppress the expression of BCRP protein, indicating that the decrease in BCRP expression could contribute to increase the efficacy of YHO-13177 on drug resistance by its continuous treatment. Taken together, these findings suggest that the posttranscriptional downregulation of BCRP protein as well as inhibition of the drug transport function of BCRP is involved in the mechanism of action of YHO-13177. It has been reported that two distinct pathways, a lysosome-mediated pathway and an ubiquitin-mediated pathway, exist for the degradation of BCRP (30), and small compounds accelerate the degradation of the protein *in vitro* (31). In addition, estrogens have been shown to suppress the biosynthesis of BCRP at physiologic levels (32). YHO-13177 may also accelerate the turnover or decelerate the biosynthesis of BCRP protein. Further studies are needed to elucidate the exact mechanism of action of YHO-13177.

Because YHO-13177 exhibits very low solubility in water, it is difficult to administer it to mice. Therefore, we used YHO-13351, a water-soluble prodrug of YHO-13177, *in vivo*. In mice, YHO-13351 was rapidly converted into YHO-13177, and YHO-13351 itself was undetectable in plasma after i.v. and oral administration. The highest plasma concentrations of YHO-13177 were 19.7 and 27.3 µmol/L after the single i.v. administration of 31 mg/kg and oral administration of 117 mg/kg, respectively. In addition, more than 1.0 µmol/L was sustained for at least

8 hours in plasma after oral administration. These results indicate that a concentration of YHO-13177 sufficient to reverse BCRP-mediated drug resistance is achievable via the administration of YHO-13351 *in vivo*. In fact, YHO-13351 significantly increased the survival time of irinotecan-treated mice inoculated with P388/BCRP cells. Furthermore, the oral as well as i.v. administered YHO-13351 significantly enhanced the antitumor activity of irinotecan in the HCT116/BCRP xenograft model, without a remarkable decrease in body weight. In contrast, YHO-13351 alone had no effect on survival time, tumor growth, or body weight in these models. These findings suggest that YHO-13351 is well tolerated *in vivo* and may be a good candidate for a BCRP inhibitor. Additional studies are required to determine the most effective schedules of the combination therapy using YHO-13351.

It has been reported that coadministration of topotecan with BCRP inhibitor GF120918 increases AUC and decreases the clearance of topotecan in P-glycoprotein-deficient mice (21). In our study, coadministration of 30 mg/kg YHO-13351 (i.v.), the effective dose *in vivo*, also increased AUC_{0-inf} values of irinotecan 1.4-fold and SN-38 1.6-fold compared with 45 mg/kg irinotecan (i.v.) alone in mice. Therefore, although it is unclear why the enhanced AUC does not result in a remarkable decrease in body weight, the enhancing effect of YHO-13351 on antitumor activity of irinotecan *in vivo* is, at least in part, due to the higher irinotecan and SN-38 levels. On the other hand, the antitumor effect of maximum-tolerated dose (90 mg/kg) of irinotecan alone (IR, 36.7%) in additional study was not superior to that of the combination of 45 mg/kg irinotecan with YHO-13351 in this study in the HCT116/BCRP xenograft model, suggesting that the inhibition of BCRP by YHO-13177 in tumor also contributes to the enhancement of antitumor activity of irinotecan *in vivo*. Additional pharmacokinetic and toxicological studies on irinotecan and YHO-13351 combination therapy are needed.

Several known P-glycoprotein inhibitors have been found to inhibit BCRP as well. For example, GF120918, a compound developed as a P-glycoprotein inhibitor (33), is also an inhibitor of BCRP. GF120918 is a good candidate for *in vivo* and clinical studies because it can be tolerated in animals and humans at concentrations sufficient to inhibit BCRP (10, 21). Because P-glycoprotein and BCRP have overlapping substrates, inhibition of both proteins at the same time is desirable for reversing drug resistance in clinical use. In contrast, specific inhibition of BCRP also has an advantage in reversing BCRP-mediated resistance to poor substrates of P-glycoprotein such as some camptothecins including SN-38 (4) in not only research use but also clinical use considering the physiological roles of P-glycoprotein, such as protection of normal tissues (1). Although a variety of BCRP inhibitors have already been identified, there is little information regarding *in vivo* effects of specific inhibitors of BCRP on drug resistance. Fumitremorgin C, an extract from *Aspergillus fumigatus*, is a relatively specific inhibitor of BCRP (20). However, the

neurotoxicity of fumitremorgin C prevents its use *in vivo* (34). Some clinically used tyrosine kinase inhibitors such as gefitinib and imatinib have also been shown to inhibit BCRP (8, 35). However, they have intrinsic anticancer activity. Therefore, more specific BCRP inhibitors that can be used *in vivo* are needed to develop reversal agents for BCRP-mediated drug resistance in cancer chemotherapy. In this study, we showed that well-tolerated doses of YHO-13351 reversed BCRP-mediated irinotecan resistance *in vivo* following its conversion to a specific BCRP inhibitor, YHO-13177. To our knowledge, YHO-13351 may be the first inhibitor specifically targeting BCRP that has reversing effects on drug resistance *in vivo* as well as *in vitro*.

Recently, BCRP has been regarded as a molecular determinant of side population cells in tumors, a fraction highly enriched in cancer stem cells that has stem cell-like properties, such as self-renewal and multilineage proliferation (36, 37). For example, BCRP transported the fluorescent dye Hoechst 33342 out of the cells, identifying an unlabeled side population (38). The side population cells have been found in a variety of human tumors and shown to have stem cell-like properties, overexpress BCRP, and exhibit drug resistance (11, 12). According to the cancer stem cell theory, incomplete elimination of cancer stem cells by chemotherapy leads to tumor relapse, suggesting that the mechanism of stem cell resistance to chemotherapy might be a source of new therapeutic targets. Therefore, YHO-13351 used in combination with other conventional anticancer drugs may contribute to eradicating the cancer stem cells in cancer chemotherapy, thereby improving clinical outcome. Further extensive study regarding this matter is in progress in our laboratory.

In summary, the present study shows that YHO-13177, a novel acrylonitrile derivative, specifically reversed the drug resistance and potentiated the cytotoxicity of anticancer drugs in exogenous, acquired, and intrinsic BCRP-expressing cancer cells through inhibition of the drug transport function and the posttranscriptional downregulation of BCRP *in vitro*. YHO-13351, a water-soluble prodrug of YHO-13177, reversed BCRP-mediated drug resistance at well-tolerated doses following its rapid conversion to YHO-13177 *in vivo*. These findings suggest that YHO-13351 is a potent and specific inhibitor of BCRP, which may be a good candidate for studies *in vivo* and could be a clinically useful drug to reverse BCRP-mediated drug resistance in cancer chemotherapy.

Disclosure of Potential Conflicts of Interest

R. Yamazaki, Y. Nishiyama, T. Furuta, H. Hatano, Y. Igarashi, N. Asakawa, H. Kodaira, H. Takahashi, R. Aiyama, and T. Matsuzaki are employees of Yakult Honsha Co, Ltd, Tokyo, Japan.

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