2-(4-Hydroxy-3-methoxyphenyl)-benzothiazole suppresses tumor progression and metastatic potential of breast cancer cells by inducing ubiquitin ligase CHIP

Hiromi Hiyoshi¹,², Natsuka Goto¹, Mai Tsuchiya¹, Keisuke Iida³, Yuka Nakajima¹,², Naoya Hirata⁴, Yasunari Kanda⁴, Kazuo Nagasawa³ & Junn Yanagisawa¹,²

¹Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8577, Japan, ²Center for Tsukuba Advanced Research Alliance, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8577, Japan, ³Faculty of Technology, Tokyo University of Agriculture and Technology-TUAT, 2-24-16 Naka-cho, Koganei-shi, Tokyo 185-0031, Japan, ⁴Division of Pharmacology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku 158-8501, Japan.

Breast cancer is the most common malignancy among women and has poor survival and high recurrence rates for aggressive metastatic disease. Notably, triple-negative breast cancer (TNBC) is a highly aggressive cancer and there is no preferred agent for TNBC therapy. In this study, we show that a novel agent, 2-(4-hydroxy-3-methoxyphenyl)-benzothiazole (YL-109), has ability to inhibit breast cancer cell growth and invasiveness in vitro and in vivo. In addition, YL-109 repressed the sphere-forming ability and the expression of stem cell markers in MDA-MB-231 mammosphere cultures. YL-109 increased the expression of carboxyl terminus of Hsp70-interacting protein (CHIP), which suppresses tumorigenic and metastatic potential of breast cancer cells by inhibiting the oncogenic pathway. YL-109 induced CHIP transcription because of the recruitment of the aryl hydrocarbon receptor (AhR) to upstream of CHIP gene in MDA-MB-231 cells. Consistently, the antitumor effects of YL-109 were depressed by CHIP or AhR knockdown in MDA-MB-231 cells. Taken together, our findings indicate that a novel agent YL-109 inhibits cell growth and metastatic potential by inducing CHIP expression through AhR signaling and reduces cancer stem cell properties in MDA-MB-231 cells. It suggests that YL-109 is a potential candidate for breast cancer therapy.
Aryl hydrocarbon receptor (AhR) has also recently emerged as a potential therapeutic target for breast cancer. The AhR is a basic helix-loop-helix transcription factor that was initially identified as a receptor for environmental toxins, such as dioxin\textsuperscript{16}. Ligand binding to the receptor triggers formation of a heterodimeric nuclear AhR complex, which binds to dioxin response elements in target gene promoters to induce transcriptional activation\textsuperscript{3}. Several studies have demonstrated that the AhR may be a potential drug target for several diseases, including endometrial, prostate, pancreatic, and ER-positive breast cancers\textsuperscript{2-17}. In addition, the antitumor effects of compounds belonging to the 2-(4-amino-3-methylphenyl) benzothiazole group are mediated by AhR in ER-positive breast cancer cells\textsuperscript{18-20}. Phortress, the lysine amide produg of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole, has completed Phase I clinical evaluations\textsuperscript{18,21}. In addition to 2-(4-aminophenyl) benzothiazoles, the relatively non-toxic selective AhR modulators (SAhRMs) are highly effective agents for inhibiting hormone-responsive breast cancer growth in animal models\textsuperscript{18-22}. Although 2-(4-aminophenyl) benzothiazoles and SAhRMs are less effective against ER-negative breast cancer cells, AhR is also expressed in these cells\textsuperscript{19,23,24}. Therefore, we hypothesized that ideal agents might exert the antitumor effects mediated by AhR signaling in both ER-positive and -negative breast cancer cells.

In this study, we demonstrated that the novel agent 2-(4-hydroxy-3-methoxyphenyl)-benzothiazole (YL-109) has ability to inhibit breast cancer progression in TNBC, MDA-MB-231 cells, and ER-positive breast cancer MCF-7 cells. In addition, YL-109 suppresses the proliferation and invasiveness of MDA-MB-231 cells, both in vitro and in vivo. Moreover, YL-109 suppresses the properties of breast cancer stem cells. Furthermore, we demonstrated that YL-109 increases CHIP expression by the recruitment of AhR to an upstream region of the gene. Consistent with these observations, CHIP or AhR knockdowns inhibit the suppressive effects of YL-109 on anchorage-independent growth and invasiveness. Taken together, our findings indicate that YL-109 is a novel antitumor agent that can induce CHIP expression through AhR signaling, and that it represents a promising candidate for a new therapeutic strategy against TNBC.

Results

YL-109 inhibits cell proliferation, motility, and invasiveness in breast cancer cells. It has been reported that 2-(4-aminophenyl)-benzothiazoles have anti-proliferative activity in MCF-7, ER-positive breast cancer cells\textsuperscript{18-20}. Therefore, we investigated the effects of 2-(4-hydroxy-3-methoxyphenyl)-benzothiazole, YL-109 (Figure 1a) on cell proliferation in MCF-7 cells. YL-109 possesses characteristic hydroxyl group at C4, whereas 2-(4-aminophenyl)-benzothiazoles have the amino group at this position. YL-109 strongly inhibited cell proliferation of MCF-7 cells in a dose-dependent manner (IC\textsubscript{50} = 85.8 nM) (Figure 1b and c). Surprisingly, YL-109 had an anti-proliferative effect in a dose-dependent manner (IC\textsubscript{50} = 402 μM) on MDA-MB-231 cells, known as TNBC cells, unlike 2-(4-aminophenyl)-benzothiazoles (Figure 1b and c)\textsuperscript{18}. We subsequently tested whether YL-109 inhibited anchorage-independent growth in poly-HEMA coated plates and colony formation in soft agar (Figure 1d and e). Under short-term detached conditions using poly-HEMA coated plate, YL-109 suppressed cell survival in MCF-7 cells, but not in MDA-MB-231 cells (Figure 1d). In contrast, YL-109 decreased the number of colonies in MDA-MB-231 cells under long-term detached conditions on soft agar (Figure 1e). Moreover, to examine the effects of YL-109 on the metastatic and invasive potential of MDA-MB-231 cells, we performed migration and invasion assays. In the migration assay, YL-109 reduced the ability of cells to migrate (Figure 1f). YL-109 also significantly decreased the number of cells that penetrated the Matrigel-coated membrane (Figure 1g). In addition, YL-109 inhibited cell proliferation and invasiveness of BT-20 cells, also known as TNBC cells (see Supplementary Fig. S1 online). These results suggest that YL-109 inhibits cell proliferation and suppresses the metastatic potential of breast cancer cells.

YL-109 inhibits both tumor growth and cancer metastasis of breast cancer cells in vivo. Using a nude mouse xenograft model, we investigated the effects of YL-109 in vivo. Mice treated with vehicle showed significantly enlarged tumors, whereas mice treated with YL-109 showed attenuated tumor growth using MCF-7 cells (Figure 2a). Interestingly, YL-109 also suppressed tumor growth in mice injected with MDA-MB-231 cells (Figure 2b). Next, to examine the effect of YL-109 against metastatic activity, we performed an in vivo lung metastasis assay using MDA-MB-231 cells. We discovered several metastatic tumors in the lungs of mice injected with MDA-MB-231 cells in the vehicle (Figure 2c). Compared with the vehicle control, YL-109 significantly reduced lung metastasis (Figure 2c). We also quantified lung metastasis using real-time RT-PCR, which confirmed our observations (Figure 2d). These results suggest that YL-109 suppresses tumor progression of breast cancer cells in vivo.

YL-109 suppresses breast cancer progression by inducing CHIP expression. We previously demonstrated that CHIP suppresses tumorigenesis and the metastatic cellular phenotypes of breast cancer cells both in vitro and in vivo\textsuperscript{8}. It has also been indicated that CHIP expression is significantly associated with prognostic parameters in breast cancer patients\textsuperscript{25}. Therefore, we examined whether YL-109 induced CHIP expression. We observed that YL-109 increased both CHIP mRNA and protein levels (Figure 3a and b). Next, to investigate whether the antitumor effects of YL-109 were mediated by CHIP, we performed colony formation and Matrigel invasion assay using siRNA for CHIP (Figure 3c--e). YL-109-induced inhibition of cell growth and invasive potential were decreased by CHIP knockdown (Figure 3d and e). These results demonstrate that YL-109 inhibits breast cancer progression by inducing CHIP expression.

YL-109 activates AhR signaling to induce CHIP expression. Previous studies indicated that 2-(4-aminophenyl)-benzothiazoles activate AhR and increase transcription of AhR target genes, such as Cyp1a1\textsuperscript{18-20}. We observed that YL-109 induced Cyp1a1 expression in MCF-7 and MDA-MB-231 cells (Figure 4a). To explore AhR participation in the mechanisms underlying the induction of CHIP by YL-109, we examined whether CHIP expression levels were affected by knocking down of AhR with siRNA (Figure 4b and c). We observed that YL-109-induced increases in CHIP mRNA were reduced by AhR knockdown (Figure 4c). We subsequently examined AhR recruitment to the promoter region in CHIP gene, which contains potential AhR response elements. Chromatin immunoprecipitation (ChIP) assay demonstrated that the interaction between AhR and the CHIP promoter is potentiated by YL-109 (Figure 4d). Our data suggest that YL-109 induces CHIP expression through recruitment of AhR to upstream of CHIP gene.

YL-109 exerts antitumor effects through AhR signaling. To examine whether AhR signaling mediated the inhibition of cell proliferation by YL-109, we performed MTT assay using the AhR antagonist α-naphtoflavone (α-NF) and siRNA against AhR (Figure 5a and b). Treatment with α-NF and knockdown of AhR expression abolished the inhibitory effect of YL-109 on cell proliferation of MCF-7 cells (Figure 5a and b). Moreover, YL-109-induced inhibition of colony formation and invasive potential were repressed by AhR knockdown (Figure 5c and d). Our data suggest that the antitumor effects of YL-109 are mediated by AhR signaling.

YL-109 reduces the property of cancer stem cells in breast cancer cells. Recently, we have shown that CHIP suppresses stem cell properties in breast cancer cells\textsuperscript{25}. Therefore, we evaluated whether
YL-109 affects breast cancer stem cells. One property of cancer stem cells is their ability to form tumor spheres, denoted mammospheres in the case of breast cancer. We observed that cells derived from the MDA-MB-231 cell line formed mammospheres, whereas YL-109 markedly inhibited mammosphere formation (Figure 6a). To confirm the effects of YL-109 on the population of cancer stem cells in MDA-MB-231 cells, we used flow cytometry with cancer stem cell marker ALDH. Consistent with the effects on mammosphere formation, YL-109 decreased ALDH-positive cell population (Figure 6b). In addition, YL-109 significantly decreased mRNA levels of Klf-4 and Notch target gene Hes1 in MDA-MB-231 mammosphere cultures (Figure 6c). Taken together, these results indicate that YL-109 inhibits the properties of breast cancer stem cells.

**Discussion**

In the present study, we revealed that the novel agent YL-109 has antitumor activity in TNBC cells. It was previously reported that 2-(4-aminophenyl)-benzothiazoles have anti-proliferative activity in ER-positive breast cancer cells, that is mediated by the AhR signaling pathway. AhR is expressed in breast cancer cells regardless of ER expression. However, TNBC cells exhibit a poor response to benzothiazoles and SAhRMs because they do not activate AhR signaling. Consistent with previous reports on benzothiazole antitumor activity, YL-109 inhibited cell proliferation through AhR activation in ER-positive breast cancer cells. In addition, we observed that YL-109 could activate AhR signaling in MDA-MB-231 cells, which is necessary for YL-109 to exert antitumor effects in these cells. These results suggest that YL-109 inhibits breast cancer progression by AhR signaling activation in TNBC cells.

Our results also demonstrated that the YL-109-induced AhR signaling activation resulted in increased CHIP expression. We previously reported that CHIP suppresses tumor progression in human breast cancer by inhibiting oncogenic pathways. In mice, tumor growth and metastasis were significantly inhibited by CHIP expression, whereas CHIP knockdowns in breast cancer cells resulted in rapid tumor growth and metastatic phenotypes. In this study, we observed that YL-109 treatment increased CHIP expression and CHIP knockdown inhibited the suppressive effects of YL-109 on both breast cancer cell growth and invasiveness. Moreover, YL-109 induced CHIP expression by recruiting AhR to an upstream region of the CHIP gene. We also found that the multiple AhR-responsive element sites exist in the promoter region of CHIP, which contain the core sequence 5'-GCGTG-3'. Taken together, these findings indicate...
that YL-109 inhibits breast cancer progression by inducing CHIP expression through AhR signaling.

Our data demonstrated that YL-109 increased CHIP levels in MDA-MB-231 cells. We also observed that YL-109 inhibited anchorage-independent cell growth in soft agar as well as xenograft tumor growth, but not in poly-HEMA coated plates. For assays of in vitro cell growth, cells were detached and suspended, either in poly-HEMA coated plates for 24 h, or in soft agar for 3 weeks. In short-term growth experiments such as the poly-HEMA assay, we did not observe cell growth inhibition by YL-109, because YL-109-induced inhibition of cell growth presumably requires the elevation of CHIP levels. Consistent with this, in long-term growth experiments such as soft agar colony formation and xenograft tumor growth, YL-109 inhibited cell growth of TNBC cells. Furthermore, this effect was repressed by the knockdown of CHIP or AhR in TNBC cells.

In contrast, CHIP levels are considerably higher in MCF-7 cells than in MDA-MB-231 cells, and we observed that YL-109 did not affect CHIP expression in MCF-7 cells (data not shown). However, YL-109 was able to inhibit the growth of MCF-7 cells in both short-term and long-term growth experiments. These observations suggest that CHIP does not contribute to the effects of YL-109 on cell growth in ER-positive breast cancer cells.

Previous studies have reported that the inhibition of ER signaling can result from cross talk with the ligand-activated AhR. AhR ligands strongly suppress estrogen-induced responses in the rodent uterus, mammary tumors, and human breast cancer cells. Treatment of ER-positive breast cancer cells with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces proteasome-dependent degradation of endogenous ERα. In this study, we demonstrated that YL-109 inhibits cell proliferation through the AhR signaling pathway in MCF-7.

Figure 2 | YL-109 suppresses both tumor growth and metastasis of breast cancer cells in vivo. (a and b) Effects of YL-109 on tumor growth in a mouse xenograft model. Mice were treated with DMSO (vehicle) or YL-109 (15 mg/kg) for every 2 days. Tumor growth curves showed tumor volume in nude mice inoculated with MCF-7 (a) or MDA-MB-231 cells (b). Tumor growth was monitored twice each week. Bars represent mean ± s.d. (n = 3–6). * indicates p<0.05 and ** indicates p<0.01 by two-way ANOVA with Bonferroni’s post hoc test. (c and d) Effects of YL-109 on tumor metastasis in vivo. MDA-MB-231 cells were injected into the tail veins of nude mice. Forty-two days after the injections, lungs were collected. Representative images of sections from lungs are shown (c). Left panels show the images of H&E staining (50x). Immunohistochemistry for human cytokeratins is shown in middle (50x) and right (200x) panels. Asterisks indicate metastatic tumor growth. The lung metastasis was quantified by real-time RT–PCR (d). Specific primers for human HPRT were used. * indicates p<0.05 and ** indicates p<0.01 by student’s T test vs. DMSO-treated mice.
cells. These findings suggest that YL-109 may induce ERα degradation because of AhR activation in ER-positive breast cancer cells. Taken together, our results show that YL-109 suppresses cell growth through AhR signaling in both ER-positive and triple-negative breast cancer cells. In TNBC cells, YL-109 increases CHIP levels by AhR activation. In contrast, in ER-positive cells, YL-109 induces ERα degradation by AhR activation.

The treatment options for patients with TNBC, including those with ER-negative breast cancer, have not yet been established. In this study, we have demonstrated that YL-109 inhibits tumor growth using a different mechanism from anti-estrogens in ER-positive breast cancer cells. Therefore, we predict that YL-109 will also exert an antitumor effect in tamoxifen-resistant breast cancer. Thus, YL-109 might have the ability to treat with several subtypes of breast cancer.

Drug resistance is a serious problem in breast cancer therapy, and cancer stem cells contributed to chemotherapeutic drug and radiation resistance. Breast cancer stem cells form mammospheres and express stem cell markers. We observed that YL-109 markedly inhibited mammosphere formation and decreased the ALDH-positive cell population in MDA-MB-231 cells. Moreover, YL-109 significantly decreased \( Klf-4 \) and \( Hes1 \) expression in mammospheres derived from MDA-MB-231 cells. \( KLF-4 \) is essential for the maintenance of breast cancer stem cells and cell invasiveness, and \( Hes1 \) is a target of Notch signaling, which may be important for the self-renewal of breast cancer stem cells.

Figure 3 | YL-109 inhibits breast cancer progression by inducing CHIP expression. (a and b) Effect of YL-109 on CHIP levels in MDA-MB-231 cells. MDA-MB-231 cells were cultured in the absence or presence of YL-109 (1 μM). Total RNA was prepared from the indicated cells and the expression of CHIP was analyzed using real-time RT–PCR (a). Protein levels of CHIP were determined by Western blotting (b). Full length images of blots are represented in Supplementary Fig. S2. (c) Knock-down of CHIP by treatment with siRNA targeting CHIP. The levels of CHIP were examined by western blotting. Full length images of blots are represented in Supplementary Fig. S2. (d) Effects of CHIP knockdown on YL-109-induced reduction of anchorage-independent cell growth in MDA-MB-231 cells. CHIP expression was knocked down in MDA-MB-231 cells, and cells were plated in soft agar dishes in the absence or presence of YL-109 (1 μM). After incubation for 3 weeks, colonies were examined under a microscope and colonies with a diameter of more than 50 μm were counted. (e) Effects of CHIP knockdown on YL-109-reduced invasiveness in MDA-MB-231 cells. MDA-MB-231 cells were knocked down of CHIP and pre-cultured in the absence or presence of YL-109 (1 μM) for 48 h. The cells were seeded onto filters with Matrigel matrix-coated upper chambers in the absence or presence of YL-109 (1 μM). After 24 h incubation, invaded cells were stained using crystal violet. * indicates p<0.05, ** indicates p<0.01, and n.s. indicates p>0.05 by student’s T test vs. DMSO-treated cells.
Therefore, our results suggest that YL-109 inhibits the properties of breast cancer stem cells and can be used for patients with drug-resistant cancer.

In summary, we demonstrated that YL-109 is a novel antitumor agent that suppresses tumor progression in TNBC cells and inhibits cancer stem cell properties, unlike other benzothiazoles. Furthermore, we show that YL-109 induces CHIP expression through AhR signaling. Taken together, we propose a new therapeutic strategy for breast cancer including TNBC, and prodrug of YL-109 improved water solubility and chemically stability may be helpful in development of new pharmacological treatments for TNBC.

**Methods**

**Cell culture.** MCF-7, MDA-MB-231 and BT-20 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI1640 supplemented with 10% fetal bovine serum (FBS). For the experiments, cells were seeded in phenol red-free DMEM containing 4% charcoal-stripped FBS. After 24 h, the medium was exchanged for phenol red-free DMEM containing 1% charcoal-stripped FBS with or without ligands.

**RNA interference.** For transfection of siRNAs, cells at 30–50% confluency were transfected with 20 nmol/L of siRNA using Lipofectamine RNAi max (Invitrogen) according to the manufacturer’s protocol. All siRNAs were purchased from Invitrogen. The siRNA duplexes

CHIP: 5’-CCAGCGCUCUUCGAUCGGGAAGAA-3’;
AhR: 5’-GAGAAUUCAUAUAUACAGGCGUCGAA-3’.

Figure 4 | YL-109 induces CHIP expression through AhR activation. (a) Effects of YL-109 on Cyp1a1 mRNA expression in breast cancer cells. MCF-7 or MDA-MB-231 cells were cultured in the absence or presence of YL-109 (1 μM). Cyp1a1 mRNA level was quantified using real-time RT-PCRs. (b) Knockdown of AhR by treatment with siRNA targeting AhR. Western blotting was used to examine the levels of AhR. Full length images of blots are represented in Supplementary Fig. S2. The representative blots for AhR were cropped to clarify relevant bands. (c) Effects of AhR knockdown on YL-109-increased CHIP expression. AhR expression was knocked down in MDA-MB-231 cells, and cells were cultured in the absence or presence of YL-109 (1 μM). CHIP expression was analyzed using real-time RT-PCRs. (d) AhR recruitment to CHIP promoter by YL-109. MDA-MB-231 cells were cultured in the absence or presence of YL-109 (1 μM). CHIP assay was performed with control IgG or anti-AhR antibodies. Immunoprecipitated DNA was examined using real-time RT-PCR and primers specific for the CHIP promoter. Samples were normalized to the amount of input DNA. * indicates p<0.05, ** indicates p<0.01, and n.s. indicates p>0.05 by student’s T test vs. DMSO-treated cells.

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CHIP: 5’-CCAGCGCUCUUCGAUCGGGAAGAA-3’;
AhR: 5’-GAGAAUUCAUAUAUACAGGCGUCGAA-3’.
Stealth™ RNAi reporter control was used as a negative control.

MTT assay. Cells were incubated in DMEM containing 4% charcoal-stripped FBS with DMSO or YL-109 (1 μM) for 96 h. The proliferation of cultured cells was measured by MTT assay using the MTT Cell Count Kit (Nakarai tesque).

Poly-HEMA. One gram of poly-(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma-Aldrich) was dissolved in 25 mL 99.5% ethanol and mixed overnight at 37°C. The poly-HEMA stock solution were added to 12-well plates and plates were left to dry for a few hours. After drying, the plates were washed with PBS. Cells were plated in the poly-HEMA-coated 12-well plates at a density of 1 × 10^5 cells (MCF-7) or 1.5 × 10^5 cells (MDA-MB-231) per well and incubated for 24 h. Cells were treated with 0.2% trypan blue. Viable cells were counted using Countess Automated Cell Counter (Invitrogen).

Soft agar colony-formation assay. For soft agar assays, 2 × 10^5 cells were suspended in DMEM containing 0.35% agar and layered on top of 1 mL of DMEM solidified with 0.6% agar in each well of a 6-well plate. After growing at 37°C for 3 weeks, colonies were counted under the microscope. The reported results represent the averages of three independent experiments.

Invasion and migration assay. The invasive potentials of MDA-MB-231 and BT-20 cells were tested with Matrigel invasion chambers (24-well format, 8 μm pore size; BD Biosciences). After incubation in DMEM containing 1% charcoal-stripped FBS with DMSO or YL-109 (1 μM) for 48 h, suspensions (0.5 mL) containing 1 × 10^5 cells (MDA-MB-231) or 0.5 × 10^5 cells (BT-20) were added with vehicle alone (DMSO) or YL-109 (1 μM), and transferred into insert chambers. These cells were then incubated for 24 h at 37°C with 0.75 mL of DMEM containing 4% charcoal-stripped FBS and each ligand in the bottom chambers. After incubation, the cells on the upper surface of the filter were removed, and invading cells were fixed in methanol. Fixed cells were stained with crystal violet and counted under a microscope. Migration assays were performed using the same procedure, except that the insert chambers were not coated with Matrigel and cells in chamber were incubated for 12 h.

Tumor xenograft models. BALB/cAJcl-nu/nu female mice at 4-5 weeks of age were purchased from CLEA Japan. The mice were kept in a pathogen-free environment under controlled conditions of light and humidity. MCF-7 or MDA-MB-231 cells were cultured as monolayers, trypsinized and resuspend in Matrigel (BD Biosciences) at each 1 × 10^4 or 1 × 10^5 cells/mL. Each mouse was injected subcutaneously with 100 μL of cell suspension (1 × 10^7 or 1 × 10^6 cells) in both flanks. YL-109 was subcutaneously injected in the scruff of the neck (15 mg/kg) for every 2 days. Tumor growth was suppressed by YL-109.

**Figure 5** | YL-109 suppresses breast cancer progression through AhR signaling. (a and b) Effects of AhR on YL-109-inhibited cell proliferation in MCF-7 cells. MCF-7 cells were cultured in the absence or presence of YL-109 (1 μM), AhR antagonist α-NF (1 μM) (a). MCF-7 cells knocked down of AhR were cultured in the absence or presence of YL-109 (1 μM) (b). The cell viability was measured by MTT assays. (c) Effects of AhR knockdown on YL-109-inhibited anchorage-independent cell growth in MDA-MB-231 cells. AhR expression was knocked down in MDA-MB-231 cells, and cells were plated in soft agar dishes in the absence or presence of YL-109 (1 μM). After incubation for 3 weeks, colonies were examined under a microscope and colonies with a diameter of more than 50 μm were counted. (d) Effects of AhR knockdown on YL-109-reduced invasiveness in MDA-MB-231 cells. MDA-MB-231 cells were seeded onto filters with Matrigel matrix-coated upper chambers in the absence or presence of YL-109 (1 μM). * indicates p<0.05, ** indicates p<0.01, and n.s. indicates p>0.05 by student’s T test vs. DMSO-treated cells.
monitored twice each week by measuring the tumor size using calipers; tumor volume was determined using the formula \( V = \frac{1}{2} \times \text{larger diameter} \times \text{smaller diameter}^2 \). All animal experiments were performed in accordance with institutional guidelines.

**In vivo lung metastasis analysis.** BALB/cAjl-nu/nu female mice at 4-5 weeks of age were purchased from CLEA Japan. The tail vein of each mouse was injected with MDA-MB-231 cells (5 × 10^5 cells). Forty-two days after injection, the mice were sacrificed and lung metastases were examined by hematoxylin-eosin (H&E) staining and immunohistochemistry for human cytokeratin and quantified by real-time RT-PCRs using primers specific for human HPRT that did not cross-react with the mouse homologue. Mouse \( \beta \)-actin was used for normalization.

**Immunohistochemistry.** Immunohistochemistry was performed under contract with Genostaff Co., Ltd. Tissues were fixed, dehydrated and embedded in paraffin.
were cut at 5 μm thickness. For antigen retrieval, the sections were incubated with EDTA buffer (10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid (EDTA)) (pH 9.0) at 95°C for 20 min, and then treated with 0.3% hydrogen peroxide. After blocking, the sections were incubated with anti-human-cytokeratin clone MNF-116 (Abcam) (10 μg/ml, overnight at 4°C). Immunostaining was performed with EnVision+TM system (Dako). Staining of H&E was performed by standard protocol.

Real-time RT-PCR. Tissues and cells were homogenized in 1 mL of Sepazol and total RNA was extracted according to the manufacturer’s instructions (Nacalai tesque). cDNA was synthesized from total RNA using RevaTraAce reverse transcriptase (Toyobo) and oligo dT primer. Real-time PCRs were performed to amplify fragments representing for the indicated mRNA expression using the Thermal Cycle DiceTM TP800 (Takara) and SYBR Premix Ex Taq (Takara). The primer sequences can be found in Supplementary Table S1.

Western blotting. Cells were lysed in TNE buffer [10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40 (NP-40), 0.15 M NaCl, and 1 mM EDTA] and then immunoblotted with the appropriate antibodies. A murine hybridoma monoclonal antibody against human CH50 was generated in our laboratory. The antibodies used in this study were: anti-human CH50 (1:500; Green Space Biomed, Japan), AβR (1:500; Santa Cruz), and β-actin (1:5000; Sigma) antibodies. Specific proteins were visualized using an enhanced chemiluminescence (ECL) Western blot detection system (Millipore).

Chromatin immunoprecipitation (ChIP) assay. This was done essentially as described previously19. The DNA was amplified by real-time PCR as described above. The primers for real-time PCR are as follows: forward primer: 5'-TCATGATGTCCTCCTGCTGC-3'; reverse primer: 5'-GACTGTTGAGATGGAAAG-3' for CH50 gene upstream region. Samples were normalized based on the amount of input DNA.

Mammosphere formation assay. MDA-MB-231 cells were plated onto 6-well ultra-low-attachment plates (Corning Costar) at 5 × 10^3 cells per well. Cells were maintained in serum-free with a CnT-27 medium and growth additives (CellnTEC (pH 9.0) at 95°C for 20 min, and then treated with 0.3% hydrogen peroxide. After blocking, the sections were incubated with anti-human-cytokeratin clone MNF-116 (Abcam) (10 μg/ml, overnight at 4°C). Immunostaining was performed with EnVision+TM system (Dako). Staining of H&E was performed by standard protocol.

ALDH assays. The ALDEFLUOR kit (Stem Cell Technologies, Durham, NC, USA) was used to detect cancer stem cell population with high aldehyde dehydrogenase (ALDH) enzyme activity, as previously reported35. Briefly, MDA-MB-231 cells were used to detect cancer stem cell population with high aldehyde dehydrogenase (ALDH) enzyme activity, as previously reported35. Briefly, MDA-MB-231 cells were treated with YL-109 (1 μM) for 4 days. The cells were then incubated in ALDH assay buffer containing the ALDH substrate (BAAA, 1 mM) for 30 min at 37°C. As a negative control, cells were stained under identical conditions in the presence of diethylaminothiazoledehydrogenase (DEAB, 15 μM), a specific ALDH inhibitor. FACs Aria II cell sorter (BD Biosciences) was used to measure the ALDH-positive cell population.

Statistical analysis. Data are representative of at least three different experiments. Significance of differences was determined by Student t-test analyses or by two-way ANOVA with Bonferroni’s post hoc test (tumor xenograft experiments).
K.I. and K.N. synthesized the compound. H.H., N.G., Y.N. and J.Y. wrote the manuscript. All authors reviewed the manuscript.

Additional information
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