Wnt/β-Catenin Small-Molecule Inhibitor CWP232228 Preferentially Inhibits the Growth of Breast Cancer Stem-like Cells

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Abstract

Breast cancer stem cells (BCSC) are resistant to conventional chemotherapy and radiotherapy, which may destroy tumor masses but not all BCSC that can mediate relapses. In the present study, we showed that the level of Wnt/β-catenin signaling in BCSC is relatively higher than in bulk tumor cells, contributing to a relatively higher level of therapeutic resistance. We designed a highly potent small-molecule inhibitor, CWP232228, which antagonizes binding of β-catenin to T-cell factor (TCF) in the nucleus. Notably, although CWP232228 inhibited the growth of both BCSC and bulk tumor cells by inhibiting β-catenin-mediated transcription, BCSC exhibited greater growth inhibition than bulk tumor cells. We also documented evidence of greater insulin-like growth factor-I (IGF-I) expression by BCSC than by bulk tumor cells and that CWP232228 attenuated IGF-I-mediated BCSC functions. These results suggested that the inhibitory effect of CWP232228 on BCSC growth might be achieved through the disruption of IGF-I activity. Taken together, our findings indicate that CWP232228 offers a candidate therapeutic agent for breast cancer that preferentially targets BCSC as well as bulk tumor cells.

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small-molecule inhibitor, namely CWP232228 (U.S. Patent 8,101,751 B2), which antagonizes the binding of β-catenin to the TCF protein in the nucleus and specifically downregulates a subset of Wnt/β-catenin-responsive genes. In vitro and in vivo studies revealed that CWP232228 suppresses tumor formation and metastasis without toxicity through the inhibition of the growth of BCSCs and bulk tumor cells. The dysregulation of insulin-like growth factor-I (IGF-I) signaling in primary breast cancers has been associated with radioresistance and tumor recurrence (20). Although IGF-I is important for the development of breast cancer, the role of this protein in BCSCs remains unclear. We also demonstrated, for the first time, the attenuation of IGF-I-mediated BCSC sphere formation mediated by CWP232228. Taken together, these results suggest that targeting β-catenin-mediated transcription using CWP232228 has significant therapeutic potential for the treatment of breast cancer.

Materials and Methods

Cell culture and reagents

The breast cancer cell lines 4T1 and 67NR were kindly provided by Dr. Wakefield, National Cancer Institute (Bethesda, MD). Human breast carcinoma cell lines MCF7, MDA-MB-435, and MDA-MB-231 were obtained from the ATCC. The human breast carcinoma cell line HS578T was obtained from the Korean Cell Line Bank (Seoul, Korea). Murine mammary cancer cell lines 4T1 and 67NR (21) and human mammary carcinoma cell lines MDA-MB-435, MDA-MB-231, MCF-7, HS578T (21) were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, 435, MDA-MB-231, MCF-7, Hs578T (21) were cultured in DMEM 450, and 5% CO2). Tumorsphere formation

from LC Laboratory (Woburn, MA) and PeproTech, respectively. CW23228 is designed by JW Pharmaceutical Corporation. Doc-

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Tumorsphere formation

Single cells were resuspended in serum-free DMEM (Invitrogen) containing R27 (Invitrogen), 20 ng/mL EGF, 20 ng/mL basic fibro-

blast growth factor (PeproTech), and 4 μg/mL heparin (Sigma-

Aldrich). Primary tumorspheres were derived by plating 50,000 to 200,000 single cells per well into 6-well ultra-low attachment dishes (Gorno). Secondary tumorspheres were plated at 50,000 cells per well. Dishes were cultivated for 7 days to enumeration of spheres. Individual spheres ≥100 μm from each replicate well (n ≥ 9 wells) were counted under an inverted microscope at ×50 magnification using the Image-Pro Plus program (Media Cybernetics). The percentage of cells capable of forming spheres, termed the “tumor-

sphere formation efficiency,” was calculated as follows: [(number of sphere formed/number of single cells plated) × 100].

Cell proliferation (cytotoxicity) assay

4T1 and MDA-MB-435 cell lines were seeded in 96-well plates. Cells were treated with increasing concentrations of CWP232228. After 48 hours of incubation, cell viability was assessed by cell counting kit-8 (CCK-8) according to the manufacturer’s instruction. The numbers of viable cells were measured at a wavelength of 450 nm using Versamax microplate reader.

Real-time PCR, stem cell PCR array, and Wnt signaling pathway PCR array

Total RNA was extracted using TRizol reagent (Invitrogen). RNA purity was verified by measuring 260/280 absorbance ratio. The first strand of cDNA was synthesized with 2 μg of total RNA using SuperScript II (Invitrogen), and one tenth of the cDNA was used for each PCR mixture containing Express SYBR-Green qPCR Supermix (BioPrince). Real-time PCR was performed using a Rotor-Gene Q (Qiagen). The reaction was subjected to 40-cycle amplification at 95°C for 20 seconds, at 60°C for 20 seconds, and at 72°C for 25 seconds. Relative mRNA expression of selected genes was normalized to HPRT and quantified using the ΔΔCt method. The sequences of the PCR primers are listed in Supplementary Table S1. The stem cell PCR array (SA Biosciences) and Wnt signaling pathway PCR array (SA Biosciences) were performed in triplicate according to the manufacturer’s instructions.

Flow cytometry

FACS analysis and cell sorting were performed using FACS Calibur and FACS Aria machines (Becton Dickinson), respective-

ly. FACS data were analyzed using FlowJo software (Tree Star). Antibodies to the following proteins were used: PE-conjugated Sca-1 (dilution 1/20), CD44 (dilution 1/40), CD24 (dilution 1/40), CD61 (dilution 1/40), CD133 (dilution 1/40), and LEF1 (dilution 1/40). The FACS gates were established by staining with isotype antibody or secondary antibody. APC-conjugated rabbit IgG antibodies (dilution, 1/500; Invitrogen) was used as the secondary antibody to visualize LEF1 protein expression. The Aldefluor kit (Stem Cell Technologies) was used to isolate the population with a high ALDH enzymatic activity. Cells were stained for ALDH1 using the Aldefluor reagent according to the manufacturer’s instructions and analyzed on FACSCalibur. As negative control, for each sample of cell aliquot was treated with 50 μmol/L DEAB, a specific ALDH inhibitor. Aldefluor™ cells were quantified by calculating the percentage of total fluorescent cells compared with a control staining reaction. FACS Aria was used to sort Aldefluor-stained cells into Aldefluor™ and Aldefluor™ cell population.

Luciferase reporter assay

4T1 cells were plated at a density of 2 × 10^4 cells per well in 48-well plates, and transfected using GeneNection transfection reagent (Genetrone Biotech Co.) according to the manufac-

turer’s protocol. The TopFlas (Addgene, ref. 22), luciferase reporter (100 ng), and Renilla luciferase thymidine kinase construct (Invitrogen; 50 ng) were used to determine luciferase activity. Luciferase activity was measured by a luminometer (GloMax, Promega), using a Dual-Luciferase assay kit (Pro-

mega), according to the manufacturer’s recommendations. Total value of reporter activity in each sample was normalized to Renilla luciferase activity.

Protein isolation and Western blot analysis

Cells were lysed in RIPA buffer (20 mmol/L Tris-HCl, pH 7.5, 200 mmol/L NaCl, 1% Triton X-100, 1 mmol/L dithiothreitol) containing protease inhibitor cocktail (Roche). The concentration of protein was measured with a Protein assay kit (Bio-Rad) as described by the manufacturer’s protocol. Total protein was sub-

tracted to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blot was probed with primary antibody; anti-

β-catenin (Cell Signaling Technology). As a loading control, anti-β-actin antibody (Santa Cruz Biotechnology) was used. Subsequently, the blots were washed in TBST (10 mmol/L Tris-

HCl, 50 mmol/L NaCl, and 0.25% Tween-20) and incubated with a horseradish peroxidase–conjugated secondary antibody. The
Informed consent was obtained from all patients. Samples were obtained from the research ethic committees at the Korea National Cancer Center.

Immunofluorescent staining

The use of fresh breast tumor specimens was approved by the research ethic committees at the Korea National Cancer Center. Informed consent was obtained from all patients. Samples were fixed with 4% paraformaldehyde for fluorescent staining. Samples were permeabilized with 0.3 mol/L glycine and 0.3% Triton X-100, and nonspecific binding was blocked with 2% normal swine serum (DAKO). Staining was performed as described previously (23), using the primary anti-ALDH1 (Abcam), and anti-LEF1 (Cell Signaling Technology). Alexa Fluor 488–conjugated rabbit IgG (Molecular Probes) was used to visualize ALDH1 and LEF1. Samples were examined by fluorescence microscopy (Zeiss LSM 510 Meta). The calculation of ALDH1 and LEF1 expression was based on green fluorescence area and density divided by cell number, as determined from the number of DAPI-stained nuclei.

Statistical analysis

All the statistical data were analyzed by GraphPad Prism 5.0 (GraphPad Software) and evaluated by the two-tailed Student t test. A P value of <0.05 was considered to indicate statistical significance.

Results

The stem cell markers Oct4, Sca-1, and ALDH1 are enriched in sphere-forming subpopulations

It has been suggested that three-dimensional (3D) sphere cultures of tumor cells from various cancer types, including breast (25), colon (6), brain, and pancreatic (26) cancers, have enriched cancer stem/progenitor cell populations. Recent studies have suggested that the stem cell markers Oct4 (27), Sca-1 (28), and ALDH1 (29) play important roles in maintaining the pluripotency of BCSCs. In the present study, we established a sphere-forming culture system to culture BCSCs as an in vitro model of breast cancer as previously described (30). To confirm whether sphere-forming subpopulations are enriched for stem cell–like properties under 3D culture conditions, we examined the expression profiles of the stem cell markers Oct4, Sca-1, ALDH1, and Sox2. Consistent with previous studies, the expression levels of these markers were higher in sphere-forming cells than in cells in monolayers (Supplementary Figs. S1A–S1C and S2B–S2D). We also performed FACS analysis to quantitate the percentage of the total cell population that consisted of CD44+/CD24− cells in both the monolayer and sphere cultures. As expected, the percentage of cells with this cell surface marker phenotype was markedly higher in sphere-forming cells than in monolayer cells (Supplementary Figs. S1D and S2A). To determine whether sphere-forming subpopulations are enriched for stem cell–like properties under 3D culture conditions, we performed an ELDA. Monolayer and sphere-forming cells were harvested and transplanted at limiting dilutions (from 50,000 to 500 cells) into mice. The repopulating unit frequency of the basal population was 1 of 1,591 for monolayer cultures and 1 of 425 for sphere cultures (Supplementary Table S2). Therefore, 4T1 sphere-forming subpopulations were compromised in their ability to repopulate functional BCSCs in a xenograft model, suggesting that these cells exhibit the characteristics of BCSCs and can be used to generate BCSCs in culture as an in vitro model for BCSC culture to evaluate the efficacy of chemotherapeutic drugs.
Enhanced expression of Wnt/β-catenin signaling-associated genes in putative BCSCs

Recently, accumulating evidence has illustrated a critical role for Wnt/β-catenin signaling in various CSCs (13). Thus, we used a stem cell PCR array to identify potential therapeutic targets in BCSCs (Supplementary Fig. S7A). Consistent with previous studies showing elevated Wnt/β-catenin signaling activity in CSCs, the signaling array results revealed that Wnt/β-catenin signaling was activated to a greater extent in sphere-forming cells than in monolayer cells (Supplementary Fig. S7B). To further assess the significance of Wnt/β-catenin signaling activity in BCSCs, we examined quantitative changes in the gene expression regulated through this pathway. The expression of downstream signaling components of the Wnt/β-catenin signaling pathway, such as Wnt1, PPAR-δ, Dvl1, LEF1, Fzd1, TCF4, and β-catenin, was markedly higher in sphere-forming cells than in monolayer cells (Fig. 1A).

Previous studies have demonstrated that aldehyde dehydrogenase 1 (ALDH1) is a marker of both normal and malignant human mammary stem cells and a predictor of poor clinical outcomes (31), and lymphoid enhancer-binding factor-1 (LEF1), is a critical regulator of Wnt/β-catenin signaling (32). Therefore, to examine the regulatory role of Wnt/β-catenin signaling in BCSCs, we analyzed the LEF1 expression levels in the ALDH-positive subpopulation. The Aldefluor-positive subpopulation showed a significantly higher level of LEF1 than the Aldefluor-negative subpopulation in multiple breast cancer cell types (Fig. 1B), suggesting that ALDH1-positive breast cancer cells represent the

**Figure 1.**
Expression profiles of Wnt/β-catenin signaling–related genes in BCSCs. A, the mRNA expression of Wnt1, TCF4, PPAR-δ, Fzd1, β-catenin, LEF1, and Dvl1 in 4T1 monolayer and sphere-forming cells was measured using real-time PCR. B, the percentages of LEF1-positive cells in both the ALDH-positive and ALDH-negative subpopulations of various breast cancer cell types (murine mammary cancer cell lines 4T1 and 67NR and human mammary carcinoma cell lines MDA-MB-435 (M435), MDA-MB-231 (M231), MCF-7, and Hs578T) were evaluated by flow cytometric analysis. C–E, the breast cancer cell lines 4T1 and MDA-MB-435 (C), premalignant MMTV-PyMT mammary tissues (kindly provided by Dr. Wakefield, National Cancer Institute, Bethesda, MD; D), and breast cancer tissues (kindly provided by Dr. Lee, National Cancer Center, Korea; E) were costained with antibodies specific for ALDH1 and LEF1. The nuclear localization of LEF1 is dependent on the cytoplasmic expression of ADLH1. DAPI staining was used to label the nuclei within each field. The results are presented as the means ± SD from three independent experiments; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
LEF1-positive subpopulation, we investigated the coexpression of these markers in breast cancer cell lines (Fig. 1C), premalignant MMTV-PyMT mammary tissues (Fig. 1D and Supplementary Fig. S8A), and tissue samples from patients with breast cancer (Fig. 1E and Supplementary Fig. S8B). As shown in Fig. 1C–E, we confirmed that the ALDH1-positive populations mostly overlapped with LEF1-positive subpopulations in both human and mouse breast cancer tissues. These results suggest that Wnt/β-catenin signaling might contribute to tumorigenic potential, representing a novel therapeutic target for eliminating BCSCs.

The β-catenin inhibitor CWP232228 suppresses BCSC growth and clonogenicity

Using a cell-based reporter system, we screened a diverse library of low molecular weight compounds for the inhibition of Wnt/β-catenin–mediated transcriptional activity. The results showed that CWP232228 was the most effective Wnt/β-catenin inhibitor (Supplementary Fig. S9A). A PK/PD analysis in mice indicated that CWP232228, administered i.v. at a dose of 200 mg/kg, generated an exposure of the compound in the blood at a concentration greater than 0.8 μg/ml for 7 hours (Supplementary Fig. S9B; Supplementary Table S3). Next, we further tested the efficacy and specificity of CWP232228 to inhibit Wnt/β-catenin signaling in breast cancer cells transiently transfected with a luciferase reporter plasmid in the presence or absence of Wnt3a. In response to CWP232228 treatment, the transcriptional activity in breast cancer cells was significantly decreased in a dose-dependent manner (Fig. 2A). Using Western blot analysis (Fig. 2B) and immunohistochemistry (Fig. 2C), we further investigated whether CWP232228 was sufficient to inhibit key components of Wnt/β-catenin signaling. Consistently, CWP232228 treatment significantly inhibited LEF1 expression in a dose-dependent manner. Approximate IC50 values were determined using a dose–response curve. In mouse (4T1) and human (MDA-MB-435) breast cancer cell lines, the IC50 values were 2 and 0.8 μmol/L, respectively (Supplementary Fig. S10).

As a functional assay, we evaluated the effect of CWP232228 on primary and secondary sphere formation. Treatment with CWP232228 resulted in the disruption of primary sphere formation of both 4T1 and MDA-MB-435 cells in a dose-dependent manner (Fig. 2D). For the secondary sphere-forming assay, treated primary spheres were collected and dissociated into single cells. The cells from treated or untreated primary spheres were replated on culture dishes without additional treatment. Interestingly, we observed that in the presence of CWP232228, the cells derived from primary spheres did not form secondary spheres as efficiently as the cells from untreated spheres (Fig. 2D). Moreover, we evaluated the effect of CWP232228 on the sphere formation of primary breast cancer cells obtained from four different patient samples. Before performing in vitro experiments, the primary breast cancer cells tested positive for cytokeratins 14 expression and negative for vimentin expression (Supplementary Fig. S3A and S3B). Treatment with CWP232228 disrupted the sphere formation of primary breast cancer cells in a dose-dependent manner (Supplementary Fig. S4A–S4D). These findings suggest that CWP232228 was sufficient to block subsequent secondary sphere formation from primary spheres in the absence of additional treatment.

In breast carcinomas, cell populations with high levels of ALDH activity are enriched in tumorigenic stem/progenitor cells (33). Therefore, we hypothesized that CWP232228 might disrupt BCSC sphere formation by regulating ALDH1 activity. To test this hypothesis, we used FACS analysis to investigate the effect of CWP232228 on ALDH activity. Indeed, the treatment of 4T1 and MDA-MB-435 cells with CWP232228 for 48 hours decreased the size of the ALDH-positive subpopulation (Fig. 2E). In this context, we examined the expression of BCSC markers in the presence or absence of CWP232228. Consistent with our hypothesis, the expression levels of these markers, including the phenotypes Sca-1+, CD133+, CD61+, CD44+/CD24−, and side populations, were significantly lower after CWP232228 treatment (Supplementary Table S4).

CWP232228 targets chemoresistant BCSCs

It is important to compare CWP232228 with another well-known small-molecule inhibitor (FH535) that targets the β-catenin/Tcf protein–protein interactions. Because deregulated cell proliferation is a hallmark of cancer cells, the antiproliferative effects of these two compounds were determined using the MTT assay. Overall, these data showed that CWP232228 more effectively suppressed the proliferative potential of MDA-MB-435 cells and at much lower doses than the well-known small-molecule inhibitor FH535 (Supplementary Fig. S5A). Normal human fibroblast cell-based dose-dependent experiments showed no marked signs of toxicity at the CWP232228 dose used in this study (Supplementary Fig. S5B).

Recently, it has been suggested that BCSCs are resistant to many conventional therapeutic approaches, including chemotherapy (24) and radiotherapy (34). Thus, although traditional approaches might kill the majority of tumor cells, some of the BCSCs remain unaffected, surviving and generating new tumors. To investigate the association between chemoresistance and Wnt/β-catenin signaling, we evaluated the available breast cancer datasets using the Oncomine dataset repository (www.oncomine.org). After specifically filtering for breast cancer datasets showing a response or nonresponse to conventional docetaxel treatment, we observed significant correlations between chemoresistance and the expression of negative (GSK3β) or positive (TCF4) regulators of Wnt/β-catenin signaling (Fig. 3A and B). Importantly, we observed that both the size of ALDH-positive populations and the sphere formation in 4T1 and MDA-MB-435 cells increased in response to conventional docetaxel treatment. However, the docetaxel-enriched ALDH-positive populations (Fig. 3C and D) and sphere formation (Fig. 3E and F) were markedly reduced after CWP232228 treatment, suggesting that CWP232228 targets BCSC populations are enriched in cells resistant to conventional chemodrugs.

CWP232228 reduces tumor growth in a murine xenograft model

We further investigated the in vivo efficacy of CWP232228 on tumorigenesis using a mouse xenograft model. Importantly, CWP232228 treatment (100 mg/kg, administered i.p.) resulted in a significant reduction in tumor volume (Fig. 4A–C). No significant changes in mortality (Supplementary Fig. S6A), body weight (Supplementary Fig. S6B), hematologic values (Supplementary Fig. S6C), and hemolytic potential (Supplementary Fig. S6D) were observed, indicating that CWP232228-associated toxicity was minimal. No obvious clinical signs, including anorexia, salivation, diarrhea, vomiting, polyuria, anuria, and fecal changes, were observed. In addition, no significant differences in body weight were observed in mice inoculated with cancer cells...
To determine whether and to what extent CWP232228 treatment affects the proportion of BCSCs in vivo, we performed FACS analysis to quantitate the percentage of the total cell population with ALDH activity in primary bulk tumors with or without CWP232228 treatment. Indeed, CWP232228 treatment led to a smaller ALDH-positive subpopulation (Fig. 4D and E). The antitumor effects of CWP232228 were further confirmed using serially regenerated secondary tumor xenografts derived from primary tumor tissues without additional treatment (Fig. 4F and G). Treatment with CWP232228 significantly reduced the incidence of secondary tumors, indicating that this molecule significantly impaired the tumor initiation potential of BCSCs. We further performed an ELDA to evaluate the inhibitory effect of CWP232228 on tumorigenesis. Following the isolation of cells from freshly digested tumor tissues, we transplanted limiting dilutions (from 50,000 to 5000) into NOD-SCID mice, and the resulting tumors were harvested and used to generate serial secondary tumors. The results showed that CWP232228 treatment significantly reduced the tumorigenic potential of BCSCs, as evidenced by the reduced formation of secondary tumor xenografts.
500 cells) of the cell preparations into mice without additional CWP232228 treatment. The repopulating unit frequency of the basal population was 1 of 1,415 and 1 of 1 for controls and 1 of 7,621 and 1 of 1,510 for CWP232228 treatment in 4T1 and MDA-MB-435 cells, respectively (Table 1). Therefore, CWP232228 did not decrease the repopulation frequency of functional BCSCs in a xenograft model.

CWP232228 targets BCSCs, bulk tumors, and metastatic tumors

An ideal and completely curative breast cancer treatment targets both BCSCs and bulk tumor cells to prevent recurrence. For each xenotransplant, we observed the significant inhibition of tumor growth induced through CWP232228 treatment alone or in combination with docetaxel (Fig. 5A). Docetaxel treatment alone moderately affected tumor growth (Fig. 5A). In bulk tumors, the percentage of ALDH1-positive cells increased with conventional docetaxel treatment (Fig. 5B). However, these docetaxel-enriched ALDH-positive populations were markedly reduced after CWP232228 treatment (Fig. 5B). Moreover, we used metastatic i.v. 4T1 cell models to investigate the effects of CWP232228 on the metastasis of breast cancer. We used cells stably expressing firefly luciferase and whole-body bioluminescence to noninvasively detect i.v. injected xenografts. Lung metastasis was significantly lower in the CWP232228-treated groups than in the untreated control groups (Fig. 5C). An overall increase in the survival of animals treated with CWP232228 was also observed (Fig. 5D). These results indicated that CWP232228 treatment alone is effective against conventional docetaxel-enriched BCSC populations, bulk tumors, and metastatic tumors in vivo.

**Suppressive effects of CWP232228 on BCSCs are achieved through the disruption of IGF-1 activity**

We compared the expression of downstream components of Wnt/β-catenin signaling between sphere-forming cells and

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### Table 1

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<tr>
<th>Treatment</th>
<th>ALDH1-positive (%)</th>
<th>4T1</th>
<th>MDA-MB-435</th>
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<tbody>
<tr>
<td>Docetaxel</td>
<td>40 ± 5</td>
<td></td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>Docetaxel + CWP232228</td>
<td>10 ± 2</td>
<td></td>
<td>0.7 ± 0.2</td>
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**Figure 3.**

Effect of CWP232228 on conventional chemodrug-enriched BCSC populations. A significant correlation between chemoresistance and the expression of negative (GSK3β; A) or positive (TCF4; B) regulators of Wnt/β-catenin signaling was observed in human breast cancer datasets available through the Oncomine dataset repository (www.oncogene.org). C–F, the primary mammospheres from 4T1 and MDA-MB-435 cells treated with vehicle alone or with docetaxel (5 nmol/L) and CWP232228 (1 μmol/L for 4T1 cells; 0.2 μmol/L for MDA-MB-435 cells) either alone or in combination were evaluated to determine the proportion of cells in the ALDH-positive subpopulation (C and D) and the relative numbers of sphere-forming units (E and F). The results are presented as the means ± SD from three independent experiments; * P < 0.05; ** P < 0.01; *** P < 0.001. TSFE, tumor sphere-forming efficiency.
monolayer cells and between nontreated and drug-treated spheres to identify potential downstream targets of CWP232228 using a Wnt/β-catenin target PCR array. We screened differentially expressed genes associated with Wnt/β-catenin signaling. Two criteria for the selection of gene expression differences were used: a significant t-test and fold-change magnitude. Among the genes examined, the level of IGF-I mRNA was significantly enhanced (~21.9-fold upregulated) in BCSCs (Fig. 6A). Interestingly, in both Wnt/β-catenin–targeting PCR array (Fig. 6B) and immunocytochemical analysis (Fig. 6C), CWP232228 treatment was correlated with decreased IGF-I levels (~8-fold downregulated) under sphere-forming conditions. Previous studies demonstrated that the expression of IGF-I in breast cancer tissues (35) and serum levels of this protein in breast cancer patients (36) are significantly higher than those in healthy individuals. Therefore, it is reasonable to hypothesize that CWP232228 suppresses the growth of BCSCs and bulk tumors through the disruption of IGF-I activity. Consistently, these results revealed that compared with control cells, IGF-I knockdown (Supplementary Fig. S12) led to smaller ALDH-positive subpopulations (Fig. 6D) and decreased BCSC sphere formation (Fig. 6E). The stimulatory effects of IGF-I on BCSC sphere formation were successfully attenuated after CWP232228 treatment (Fig. 6F). To further evaluate the CWP232228-mediated inhibition of IGF-I secretion, we performed an ELISA to quantitate the levels of IGF-I in both monolayer and sphere cultures with or without CWP232228 treatment. Consistent with immunocytochemical results, CWP232228 treatment significantly decreased IGF-I secretion under sphere-forming conditions (Supplementary

Figure 4. The effects of CWP232228 on tumorigenesis in a murine xenograft model. A, schematic representation of the experimental protocol as described in Materials and Methods. The mice were implanted with 4T1 (5 × 10⁴ cells/mouse) and MDA-MB-435 cells (5 × 10⁵ cells/mouse) through orthotopic injection into the thoracic mammary fat pads. Tumor tissue was isolated from mice bearing 4T1 or MDA-MB-435 cell tumors that had been treated with CWP232228 (100 mg/kg, i.p.) or vehicle (PBS). B and C, tumor volumes were measured as described in Materials and Methods. D and E, the effects of CWP232228 on the ALDH-positive subpopulation as a proportion of the total cells in bulk tumors were assessed through flow cytometric analysis. 4T1 and MDA-MB-435 xenografts from mice treated with CWP232228 or vehicle were dissociated into single-cell suspensions and injected into the mammary fat pads of mice in limiting dilutions (50,000, 10,000, 2,000, and 500). Tumor formation was observed for 4 weeks (4T1 cells) and 8 weeks (MDA-MB-435 cells) following inoculation. F and G, the BCSC frequency was calculated using the ELDA. The results are presented as the means ± SD from three independent experiments; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
These results suggest that the suppressive effects of CWP232228 on BCSCs are achieved through the disruption of IGF-I activity.

**Discussion**

Approximately 30% to 50% of the patients diagnosed with early-stage breast cancer are likely to progress to the metastatic stage, despite treatment with surgery and/or chemotherapy (37). Thus, the CSC concept has emerged as an important milestone in the understanding of chemodrug resistance and cancer recurrence (38). On the basis of their characteristics, targeting and eradicating CSCs represent a potential strategy for significantly improving clinical outcomes. Moreover, Fillmore and colleagues (39) revealed a 30-fold increase in the BCSC population in various breast cancer cell lines after conventional chemotherapy. The available conventional therapeutic agents primarily eliminate the bulk of a tumor mass but do not affect BCSCs (40). Thus, the identification and development of BCSC-targeting therapeutic agents is urgent.

![Figure 5](image_url)

**Table 1.** ELDA to evaluate the tumor-forming potential

<table>
<thead>
<tr>
<th>Estimated cell frequency</th>
<th>4T1</th>
<th>MDA-MB-435</th>
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<tbody>
<tr>
<td>Cell number of inoculation, % (n)</td>
<td>Control</td>
<td>CWP232228</td>
</tr>
<tr>
<td>50,000</td>
<td>100% (8/8)</td>
<td>100% (8/8)</td>
</tr>
<tr>
<td>10,000</td>
<td>100% (8/8)</td>
<td>75% (6/8)</td>
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<tr>
<td>2,000</td>
<td>63% (5/8)</td>
<td>25% (2/8)</td>
</tr>
<tr>
<td>500</td>
<td>50% (4/8)</td>
<td>0% (0/8)</td>
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Estimated CSC Frequency by ELDA (95% CI) 1/1145 (1/2850-1/703) 1/7661 (1/15301-1/3796) 6.60E-04 1/1 (1/437-1/1) 1/7510 (1/3048-1/748) 3.70E-05

NOTE: 4T1 and MDA-MB-435 xenografts from mice treated with CWP232228 or vehicle were dissociated into single-cell suspensions and injected into the mammary fat pads of mice in limiting dilutions (50,000; 10,000; 2,000; 500). Tumor formation was observed for 4 weeks (4T1 cells) and 8 weeks (MDA-MB-435 cells) following inoculation. BCSC frequency was calculated using ELDA.
In recent years, a number of studies have suggested that the dysregulation of Wnt/β-catenin signaling occurs in human breast cancer (41). In this context, the high expression of β-catenin might be an important clinical and pathologic feature of breast cancers and a predictor of poor overall survival (42). Mutations in the N-terminal domain of β-catenin have been observed in 92% of patients with metaplastic breast carcinoma (43). Consistent with these findings, the results of the present study showed that the levels of Wnt/β-catenin signaling activities observed in BCSCs were significantly higher than those of bulk cancer cells, although both bulk tumor cells and BCSCs exhibit a basal level of Wnt/β-catenin signaling (Fig. 1). These results suggested that BCSCs are sensitive to therapeutic approaches targeted against Wnt/β-catenin. The β-catenin–TCF interaction is required for functional Wnt/β-catenin signaling (15); therefore, the inhibition of Wnt/β-catenin signaling through the direct targeting of β-catenin is considered an attractive therapeutic strategy. Despite academic pursuit and industrial investment, there is currently no small-molecule inhibitor approved for human use. The majority of the inhibitors developed so far are in the preclinical or early clinical phase of development: XAV939 (Novartis Pharmaceuticals) and IV55 (Tocris Bioscience) are in preclinical trials, and OMP-18R5 (OncoMed Pharmaceuticals/Bayer), OMP-54F28 (OncoMed Pharmaceuticals/Bayer), PRI-724 (Prism Pharma Co., Ltd/Eisai), and LGK974 (Novartis Pharmaceuticals) are in phase I/II trials. If these chemical inhibitors prove to be both safe and effective for treating human cancer, then these molecules will represent powerful tools to target chemotherapy-resistant CSCs that promote metastasis.

Here, to inhibit the growth and/or self-renewal capacity of BCSCs through the suppression of β-catenin–mediated signaling, we used the small-molecule inhibitor CWP232228, identified in a high-throughput screen. Follow-up analyses using this compound revealed a reduction in the expression of a Wnt/β-catenin luciferase reporter and the inhibition of the expression of the Wnt/β-catenin target gene LEF1 (Fig. 2A–C). Notably, whereas the inhibition of β-catenin–mediated transcription through CWP232228 had inhibitory effects on the growth of BCSCs and bulk tumor cells, BCSCs showed a markedly greater degree of growth inhibition (Figs. 3–5). Moreover, CWP232228 treatment was sufficient to block subsequent secondary BCSC sphere formation in vitro (Fig. 2D) and secondary tumor development in the
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xenograft model (Table 1), without additional treatment. These findings suggest that CWP232228 inhibits the initiation of tumor development and disrupts the physiologic requirements for BCSC maintenance. The observation of markedly reduced ALDH-positive BCSC populations in bulk tumors treated with CWP232228 further supports this interpretation (Fig. 4D and E).

Breast cancer is likely to metastasize to multiple organs, primarily including the lungs, liver, and the brain. Therefore, we investigated the effect of CWP232228 on metastasis using breast cancer cell xenograft models. For this purpose, the mice were i.v. injected with mouse breast cancer cells expressing firefly luciferase (4T1-Luc), and the cells were noninvasively detected on the basis of bioluminescence. CWP232228 significantly reduced tumor bioluminescence in a fast-growing metastatic model of mouse breast cancer. This decrease in 4T1-Luc cells reflects both the number of metastatic cancer cells (data not shown), and the tumor volume as the bioluminescent signal was measured across the whole body, integrating both optical parameters (Fig. 5C). Importantly, this efficacy was achieved without affecting primary toxicity parameters, such as mortality, body weight, hematologic values, and hemolytic potential (Supplementary Fig. S6).

The aberrant activation or transcriptional activity of β-catenin has been correlated with breast stem cell amplification and tumorigenesis in a number of studies (16). Moreover, studies aiming to target CSCs have primarily focused on disrupting their self-renewal capacity rather than directly causing toxic effects; these drugs could, therefore, be less toxic than conventional cytotoxic chemotherapeutic drugs, as reflected by their higher than anticipated IC50 values (44). This result suggests that the β-catenin–TCF interaction, rather than other Wnt/β-catenin signaling components could be an effective therapeutic target in BCSCs.

The IGF signaling pathway is one of the most important regulators of the growth, migration, and invasion of various types of cancer (45). Several in vitro experimental studies have provided substantial evidence of a role for IGF-I signaling in human breast cancer. The overexpression of the IGF-I receptor in the mouse mammary gland results in rapid mammary tumorigenesis (46). Clinical studies also support the importance of IGF-I in breast cancer. IGF-I is detected at high levels in tissue specimens (35) and serum samples (36) from breast cancer patients compared with those from healthy individuals. Moreover, the constitutive activities of IGF-I in breast cancer cells are highly associated with radioresistance and tumor recurrence (20). These studies might eventually reveal a role for IGF-I in breast malignancies with respect to tumor growth, metastatic progression, and resistance to therapy. Although the IGF-I system is important in breast cancer development, the mechanisms underlying the potential role of this protein in BCSCs remain largely unknown. Cross-talk between IGF-I and Wnt/β-catenin signaling was recently reported in colon cancer (47), oligodendroglial cells (48), and chondrocytes (49). Therefore, the interactions between these two signaling pathways in BCSCs are intriguing and need further investigation. In the present study, we also provided the first evidence that IGF-I is expressed at higher levels in BCSCs than in non-BCSCs. Furthermore, we observed an attenuating effect of CWP232228 on IGF-I-mediated functions in BCSC sphere formation and ALDH-positive BCSC populations (Fig. 6). The importance of IGF-I in the growth and/or self-renewal capacity of BCSCs is consistent with previous observations from breast cancer cell lines (50). These studies suggest that IGF-I signaling is critical for the tumorigenicity and maintenance of BCSCs, and these cells could be selectively targeted to improve clinical outcomes through the inhibition of BCSCs.

In summary, these results provide the first demonstration that the small-molecule inhibitor CWP232228 inhibits Wnt/β-catenin signaling and depletes BCSCs from bulk tumors. BCSC-depleted tumor cell populations showed diminished self-renewal capacity and decreased tumorigenicity. These findings suggest that the inhibition of Wnt/β-catenin signaling suppresses the growth and functionality of BCSCs, which might be key drivers of breast cancer metastasis and recurrence. To the best of our knowledge, the effect of CWP232228 on breast tumorigenesis has not previously been assessed. Taken together, these results suggest that by antagonizing the binding of β-catenin to the TCF protein in the nucleus, β-catenin inhibitors, specifically CWP232228, might be novel BCSC-targeting agents for the treatment of breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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