1521-0111/101/2/78–86$35.00
MOLECULAR PHARMACOLOGY

dx.doi.org/10.1124/molpharm.121.000334
Mol Pharmacol 101:78–86, February 2022

Fluorizoline Blocks the Interaction between Prohibitin-2 and γ-Glutamylcyclotransferase and Induces *p21*$^{Waf1/Cip1}$ Expression in MCF7 Breast Cancer Cells

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Received June 7, 2021; accepted November 29, 2021

ABSTRACT

Prohibitin-2 (PHB2) is a scaffold protein that has pleiotropic functions, which include interacting with γ-glutamylcyclotransferase (GGCT) in the cytoplasm and repressing the transcriptional activities of the *p21*$^{Waf1/Cip}$ (*p21*) gene in the nucleus. The cytotoxic drug fluorizoline binds to PHB1/2 and exerts antiproliferative actions on cancer cells. However, the precise mechanism underlying the antiproliferative effects of fluorizoline is not fully elucidated. In the present study, we first show that fluorizoline induces p21 expression in several human cancer cell lines, including MCF7 breast cancer cells. Treatment of MCF7 cells with fluorizoline suppressed proliferation and prevented cells from entering into the DNA synthesis phase. Knockdown of p21 rescued the suppressed proliferation, indicating that fluorizoline inhibited MCF7 cell growth via the induction of p21. Overexpression of PHB2 in MCF7 cells prevented the induction of p21 expression by fluorizoline and restored the antiproliferative effects and blockade of cell cycle progression. Moreover, treatment of MCF7 cells with fluorizoline inhibited the interaction between endogenous PHB2 and GGCT proteins and reduced the level of nuclear localization of PHB2 proteins. These results indicate that targeting PHB2 with fluorizoline induces the expression of p21 and consequently blocks proliferation of cancer cells.

SIGNIFICANCE STATEMENT

This study shows that fluorizoline may be a promising novel anticancer drug candidate that induces p21 expression and blocks cell-cycle progression in human cancer cell lines. In addition, we show that fluorizoline inhibits the interaction between PHB2 and GGCT and reduces the nuclear localization of PHB2 proteins.

Introduction

Prohibitin-1 (PHB1) and -2 (PHB2) are scaffold proteins that have pleiotropic functions. These functions include the regulation of many cellular processes, such as growth factor signaling pathways, the immune response, mitochondrial dynamics, cell migration, and transcriptional activities of estrogen receptor (Ande et al., 2017; Chowdhury et al., 2017; Koushyar et al., 2015; Peng et al., 2015; Theiss et al., 2011; Wang et al., 2020). PHB1/2 interact with various factors important for determining cell fate, such as C-rapidly accelerated fibrosarcoma, protein kinase B, IkappaB kinase, mitogen-activated protein kinase kinase kinase 10, small heterodimer partner 1/2, and the 14-3-3 scaffold proteins. Due to the regulatory impact of PHB1/2 on cell proliferation, PHB1/2 are novel molecular targets for anticancer therapeutics (Wang et al., 2020).

To facilitate the development of a new class of anticancer drugs, several small molecules targeting PHB1/2 have been synthesized (Wang et al., 2020). An example of one promising molecule is fluorizoline, which directly binds to PHB1/2 and is cytotoxic to cancer cells (Pérez-Perarnau et al., 2014). Fluorizoline induces apoptosis in acute myeloid leukemia cells (Pomares et al., 2016) and chronic lymphocytic leukemia cells (Cosialls et al., 2017; Wierz et al., 2018), as well as in mouse embryonic fibroblasts and Hela cells, where this effect is mediated by upregulation of NOXA and Bcl2-like protein (Moncunill-Massaguer et al., 2015). In Hela cells, fluorizoline prevents activation of mitogen-activated protein kinase kinase 1/2 upon epidermal growth factor stimulation by disrupting the interaction between C-rapidly accelerated fibrosarcoma and active rat sarcoma virus (Yurugi et al., 2017). Moreover, fluorizoline

This work was supported by the Japan Society for the Promotion of Science [Grants 18K09192 and 20K07623] and the Takeda Science Foundation, a Scholarship for Young/Women Researchers of the PMAC.
No author has an actual or perceived conflict of interest with the contents of this article.
[1]These authors contributed equally to this work.
dx.doi.org/10.1124/molpharm.121.000334.

ABBREVIATIONS: GGCT, γ-glutamylcyclotransferase; p21, p21$^{WAF1/CIP1}$; PHB, prohibitin.

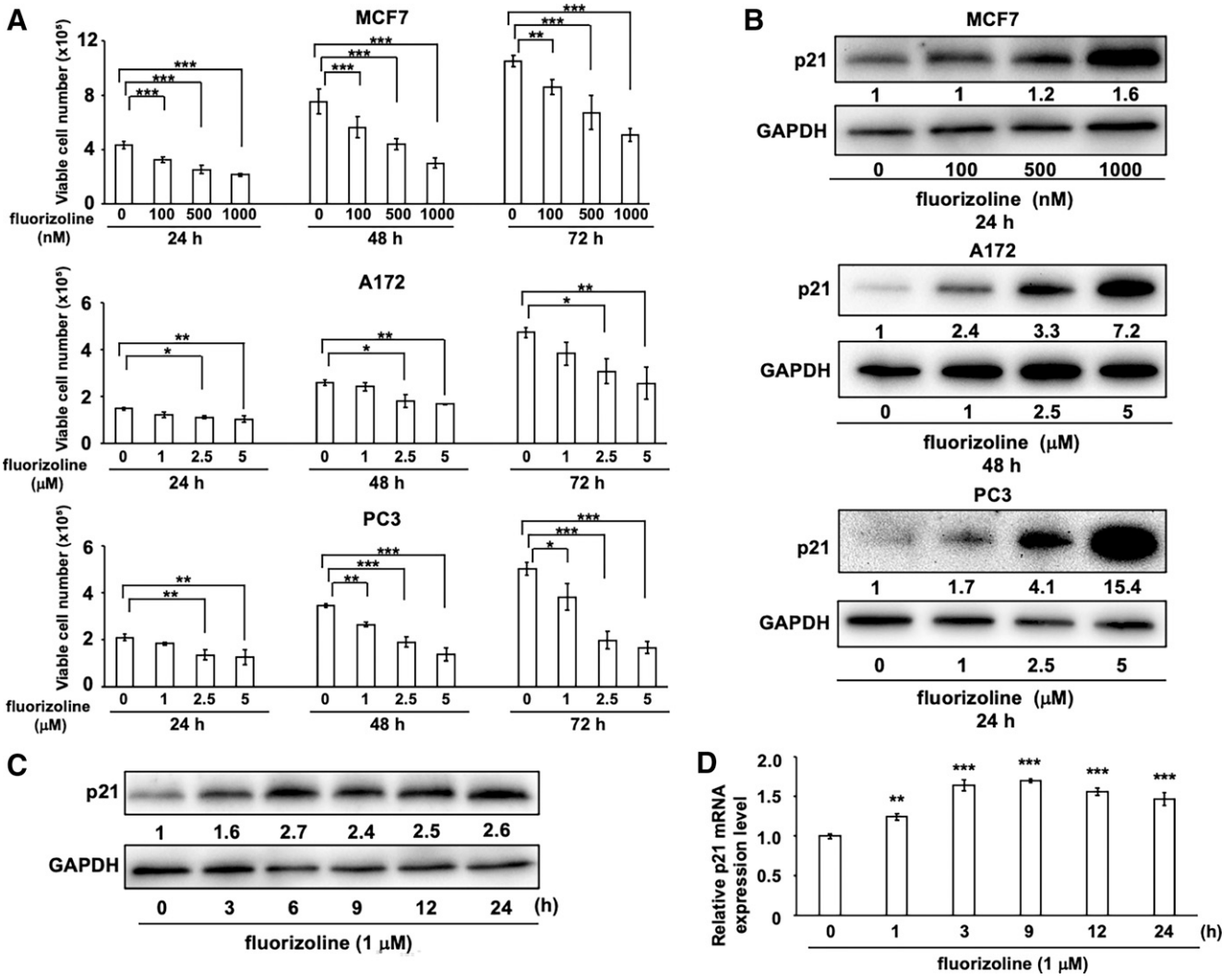

Fig. 1. Fluorizoline inhibits MCF7 proliferation and induces p21 expression. MCF7, A172, and PC3 cells were treated with the indicated concentrations of fluorizoline for the indicated durations. (A) The number of trypan blue-negative viable cells is shown. The DMSO-treated solvent control is shown as a fluorizoline concentration of 0 nM (MCF7; $n = 6$, A172; $n = 3$, PC3; $n = 3$). (B) Concentration-dependent expression of p21 in MCF7, A172, and PC3 cells analyzed by Western blot analysis is shown. glyceraldehyde-3-phosphate dehydrogenase is shown as a loading control. The time-course of p21 protein (C) and mRNA (D) expression levels in MCF7 cells were analyzed by Western blot analysis and RT-qPCR, respectively ($n = 3$). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by one-way ANOVA with Bonferroni's multiple comparison test).

inhibits protein synthesis in cancer cells due to its effects on multiple components of the translational machinery, including promoting phosphorylation of initiation factor 2 and elongation factor 2 (Jin et al., 2020). Recently, it was confirmed that the cytotoxicity of fluorizoline in nematodes and human cancer cells requires the expression of PHB1/2 (Saura-Esteller et al., 2021). Despite these studies indicating the anticancer potential of fluorizoline, the precise mechanisms that underlie the antiproliferative effects of this compound are not fully understood.

Increasing evidence shows that γ-glutamylcyclotransferase (GGCT; formerly known as chromosome 7 open reading frame 24) promotes the proliferation of cancer cells. GGCT catalyzes the production of 5-oxoproline and free amino acids from gamma-glutamyl peptide to promote glutathione biosynthesis. It is highly expressed in a wide range of cancer types, including urothelial, breast, lung, brain, and ovarian cancers (Kageyama et al., 2018). Knockdown or pharmaceutical inhibition of GGCT blocks proliferation of various human cancer cell lines. GGCT knockdown in MCF7 and MDA-MB-231 breast cancer cells upregulates the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ (p21) and/or $p16^{INK4a}$ to promote autophagy, followed by cellular senescence (Matsumura et al., 2016; Taniguchi et al., 2018a; Taniguchi et al., 2019).

Recently, we showed that PHB2 is a novel binding partner of GGCT (Taniguchi et al., 2018b). The interaction between PHB2 and GGCT promotes the nuclear translocation of PHB2, which binds to the promoter region of the *p21* gene, thereby repressing transcription of the *p21* gene in MCF7 breast cancer cells. The finding that PHB2 is a key regulator of the expression of *p21*, which is a pivotal cell cycle regulator and a downstream target of p53 (el-Deiry et al., 1993), led us to investigate the effects of fluorizoline on p21 expression in cancer cells. In the present study, we show that fluorizoline induces p21 expression in several human cancer cell lines and also demonstrate that fluorizoline suppresses cell cycle progression,

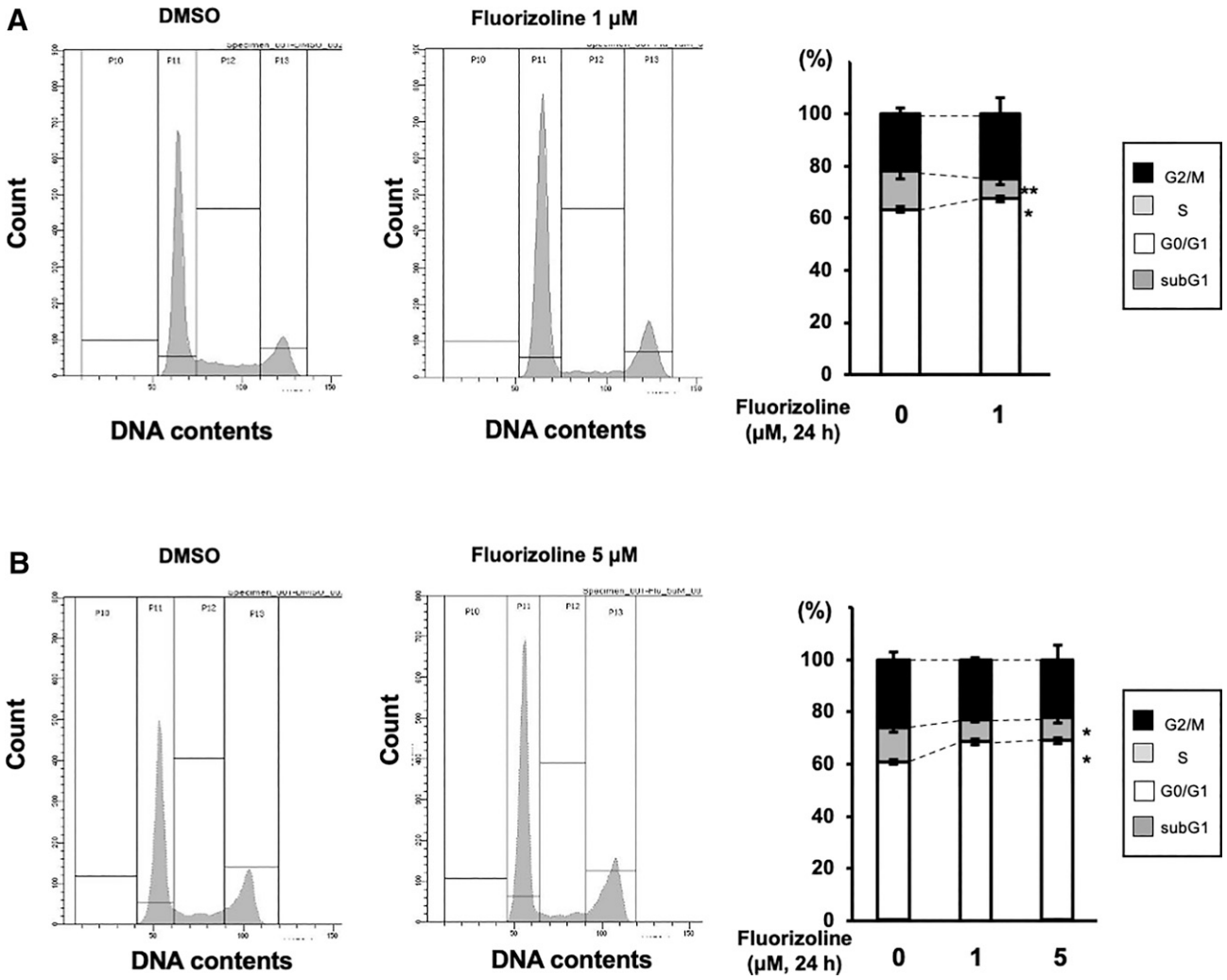

Fig. 2. Fluorizoline induces cell cycle arrest. (A) MCF7 cells were treated with 1 μM of fluorizoline (or DMSO control) for 24 h, and the distribution of cell cycle phases was analyzed by flow cytometry. Representative histograms and quantified distributions are shown. (Control; $n = 7$, 1 μM; $n = 9$). (B) A172 cells were treated with 1 μM or 5 μM of fluorizoline (or DMSO control) for 24 h, and the distribution of cell cycle phases was analyzed by flow cytometry. Representative histograms and quantified distributions are shown. (Control; $n = 6$, 1 μM; $n = 3$, 5 μM; $n = 3$). (*$P < 0.05$, **$P < 0.01$ by two-tailed Student's t test).

blocks the interaction between PHB2 and GGCT, and inhibits the nuclear localization of PHB2 in MCF7 cells.

Materials and Methods

Cell Culture. MCF7 human breast cancer, A172 glioblastoma, and PC3 prostate cancer cells were obtained from RIKEN BioResource Center (Tsukuba, Japan) and cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, South Logan, UT, USA) and 1% penicillin and streptomycin. Cells were maintained in 5% CO_2 at 37°C.

Reagent. The PHB ligand fluorizoline was synthesized following an established procedure (Pérez-Perarnau et al., 2014) and dissolved inDMSO.

Cell proliferation assay. Briefly, 1×10^5 MCF7, A172, and PC3 cells per well were seeded in 6-well plates and treated with fluorizoline at the indicated concentrations. A trypan blue dye exclusion test was performed using 0.4% trypan blue solution (Wako Pure Chemical Industries, Osaka, Japan) and a Countess II automated cell counter (Thermo Fisher Scientific, Waltham, MA, USA). Cell viability assays were performed using cell count reagent SF (WST-8 assay, Nacalai Tesque, Kyoto, Japan) and a CellTiter-Glo kit (Promega, Madison, WI, USA).

Antibodies. The following antibodies were used: mouse monoclonal antibodies specific for p21 (dilution 1:200, 556430, BD Biosciences, Franklin Lakes, NJ, USA), glyceraldehyde-3-phosphate dehydrogenase (dilution 1:1000, 016-25523, Wako), the V5 epitope tag (V5) (dilution 1:1000, R960-25, Thermo Fisher Scientific), and PHB2 (dilution 1:200, Sc-133094, Santa Cruz Biotechnology, Dallas, TX, USA); a sheep monoclonal antibody against GGCT (dilution 1:500, AF5086, R&D systems, Minneapolis, MN, USA) ; and a rabbit monoclonal antibody against Lamin B1 (dilution 1:1000, 12987-I-AP, Proteintech, Rosemont, IL, USA). Horse anti-mouse immunoglobulin G–horseradish peroxidase (IgG-HRP) conjugates were purchased from Vector Laboratories (dilution 1:2000, PI-2000, Burlingame, CA, USA). Rabbit anti-sheep IgG-HRP conjugates were purchased from Thermo Fisher Scientific (dilution 1:5000, 81-8620). The HRP-linked goat anti-rabbit IgG (dilution 1:2000, 7074) and mouse IgG1 isotype controls (5415) were purchased from Cell Signaling Technology (Danvers, MA, USA). A mouse GGCT mouse monoclonal antibody (TSS-M01) was purchased from Cosmo Bio (Tokyo, Japan).

Western Blot Analysis. Western blot analysis was performed as described previously (Taniguchi et al., 2018b). Briefly, cell lysates were prepared in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with

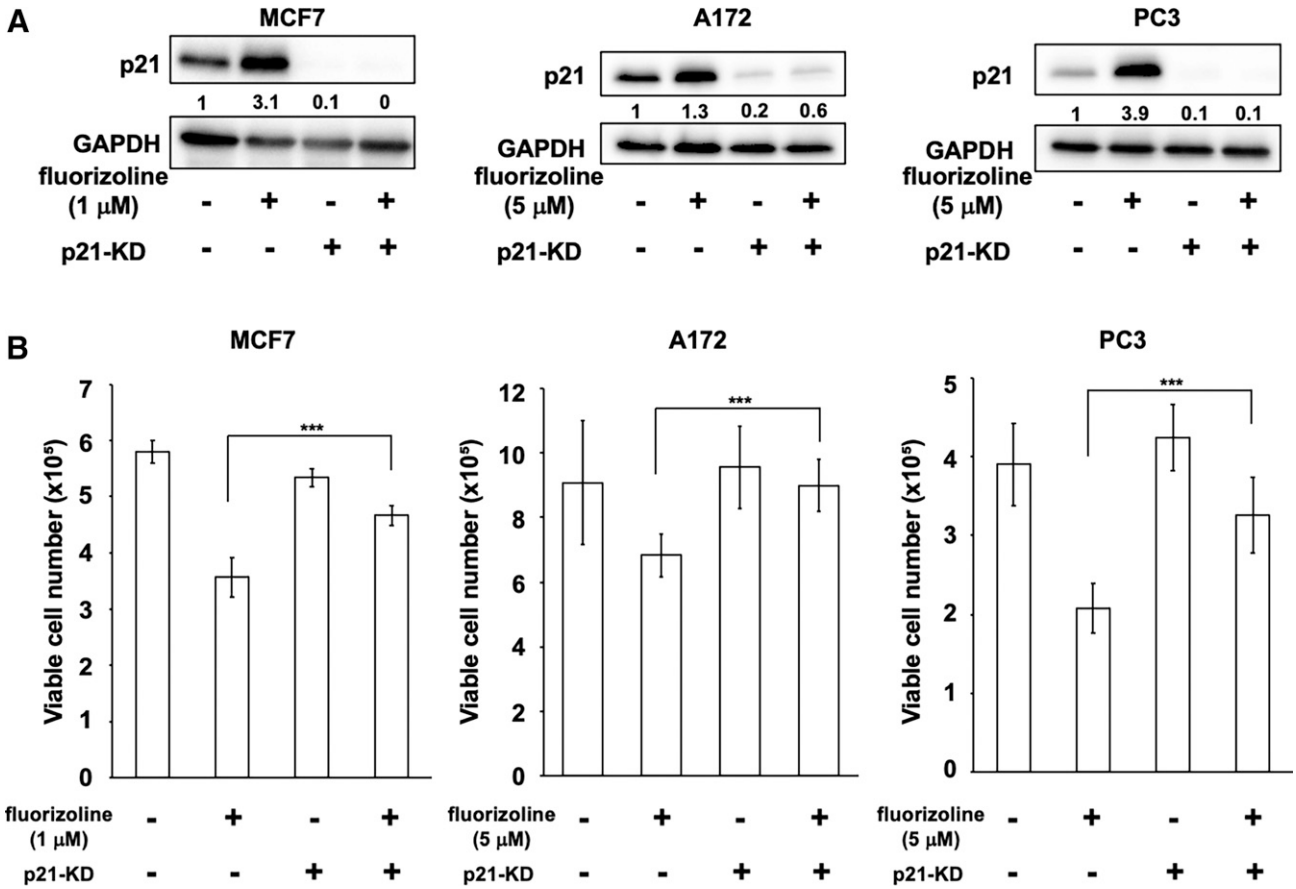

Fig. 3. p21 knockdown restores cell proliferation suppressed by fluorizoline. MCF7, A172, and PC3 cells were transfected with siRNA targeting p21 or non-target control siRNA and treated with 1 μM or 5 μM of fluorizoline for 48 h. (A) The expression levels of p21 protein were analyzed by Western blot analysis. GAPDH is shown as a loading control. (B) The number of trypan blue-negative viable cells is shown (MCF7: $n = 3$, A172: control siRNA; $n = 11$, p21 siRNA; $n = 9$, PC3: $n = 6$) (***$P < 0.001$ by 2-way repeated-measures ANOVA).

a protease inhibitor cocktail (Nacalai Tesque). The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). After blocking with 3% fat-free dried milk in Tris-buffered saline /0.05% Tween-20, or polyvinylidene difluoride blocking reagent for Can Get Signal (TOYOBO, Osaka, Japan), the membranes were incubated with primary antibodies in 3% bovine serum albumin/Tris-buffered saline /0.05% Tween-20, Can Get Signal immunoreaction enhancer solution (TOYOBO), or signal enhancer HIKARI solution (Nacalai Tesque). Proteins were visualized using the Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA) or Chemi-Lumi One Super (Nacalai Tesque) and were detected using the ChemiDoc XRS plus system (Bio-Rad).

Transfection of SiRNA Targeting p21. Synthesized siRNAs were purchased from RNAi (Tokyo, Japan) or Gene Design (Osaka, Japan). The siRNA sequences were as follows: non-targeting, 5′-GUACCGCACGUCAUUCGUAUC-3′ (sense) and 5′- UACGAAUGACGUGCGGUACGU-3′ (anti-sense); p21, 5′- CUUCGACUUUGUCACCGAG-3′ (sense) and 5′-CUCGGUGACAAAGUCGAAG-3′ (anti-sense). Transient transfection of siRNA targeting p21 was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, MCF7 and PC3 cells were seeded in 6-well plates (5×10^4 cells/well), cultured for 24 h, and transfected with siRNAs at a concentration of 10 nM. At 24 h post-transfection, the cells were treated with 1 μM or 5 μM of fluorizoline for 48 h and then collected and analyzed with Western blot and cell counting as described above.

PHB2 Overexpression. The procedure was carried out as described in (Taniguchi et al., 2018b). Briefly, the pENTR221-PHB2 vector (clone no. 100011434, DNAFORM, Yokohama, Japan) was recombined into the pEF-DEST51 vector using a Gateway LR Clonase II enzyme mix (Thermo Fisher Scientific). Next, 2×10^6 MCF7 cells were transfected with 2 μg of V5-tagged PHB2 vector or pEF-DEST51 empty vector by electroporation using an Amaxa cell line Nucleofector kit V and a Nucleofector 2b device (Lonza, Visp, Switzerland) with a P-020 program optimized for MCF7 cells. DMEM containing 10 μg/ml of blasticidin was used for clone selection.

RT-qPCR Analysis. Total RNA was extracted from MCF7 cells lysed with TRIzol (Thermo Fisher Scientific) and purified with an RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized by reverse transcription using ReverTra Ace qPCR RT master mix (TOYOBO). Synthesized cDNA was analyzed by RT-qPCR with THUNDERBIRD SYBR qPCR mix (TOYOBO) using a LightCycler 96 system (Roche Diagnostic, Indianapolis, IN, USA). Gene expression levels were normalized against the housekeeping gene h*ARF1*. Specific primers were purchased from Eurofins Genomics (Tokyo, Japan): hARF1, 5′- GACCACGATCCTCTACAAGC -3′ (forward) and 5′- TCCCACACAGTGAAGCTGATG -3′ (reverse); p21 $^{WAF1/CIP1}$, 5′-GCAGACCAGCATGACAGATTT-3′ (forward); 5′-GGATTAGGGCT TCCTCTTGGA-3′ (reverse).

Bromodeoxyuridine (BrdU) Incorporation Assay. MCF7 cells were treated with 1 μM of fluorizoline for 24 h. The proportion of MCF7 cells in the DNA synthesis phase was assessed after BrdU-pulse for 1.5 h using an APC BrdU flow kit (BD Biosciences) according to the manufacturer's instructions. At least 10,000 cells per sample were analyzed.

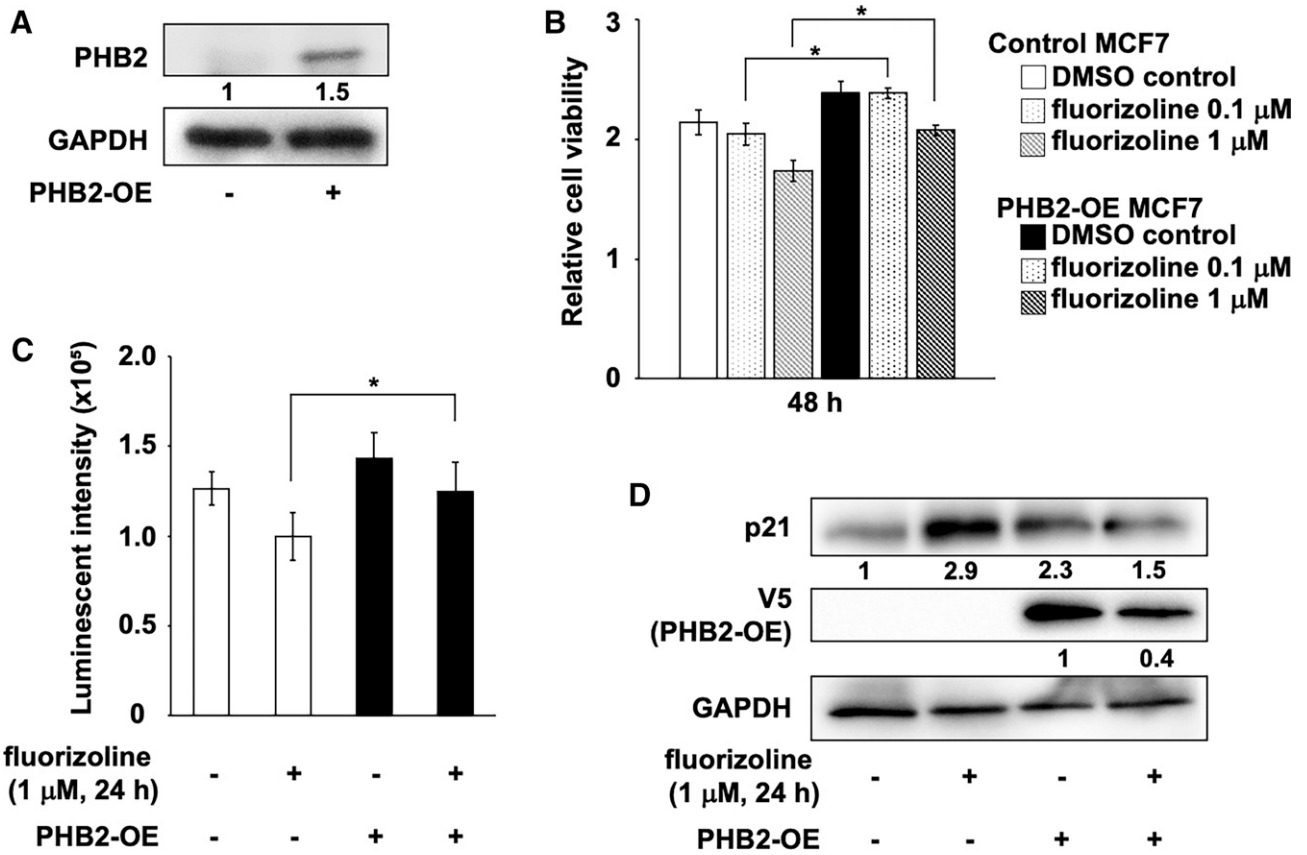

Fig. 4. PHB2 plays a crucial role in regulating changes in p21 expression and cell growth induced by fluorizoline treatment. (A) PHB2-overexpressing (PHB2-OE) or empty vector-transfected (Control) MCF7 cells were prepared, and expression levels of the introduced PHB2 proteins were analyzed by Western blotting. (B) The relative cell viability of control and PHB2-OE MCF7 cells treated with 0.1 or 1 μM of fluorizoline (or DMSO control) was analyzed by a WST-8 cell viability assay ($n = 5$). (C) The relative cell viability of control and PHB2-OE MCF7 cells treated with 1 μM of fluorizoline for 24 h was analyzed by CellTiter-Glo 2.0 luminescent cell viability assay (Control; $n = 4$, PHB2-OE; $n = 5$). (D) Western blot analysis of p21, V5 tag (linked to the C-terminus of overexpressed PHB2), and GAPDH in MCF7 cells 24 h after treatment with 1 μM of fluorizoline. GAPDH is shown as a loading control. (*$P < 0.05$ by 2-way repeated-measures ANOVA)

Cell Cycle Assay. MCF7 cells or A172 cells were seeded at 1.5×10^5 cells in 6-cm dishes. The next day, the cells were treated with 1 μM or 5 μM of fluorizoline for 24 h. The cells were harvested in PBS, and stained with a Cell Cycle Assay Solution Blue (Dojindo, Kumamoto, Japan). Cell cycle ratios of the stained cells were analyzed using FACS Fortessa (BD Biosciences).

Apoptosis Assay. Apoptosis induction was assessed using the MEBCYTO Apoptosis Kit (MBL, Nagoya, Japan). Annexin V-positive and propidium iodide (PI)-negative cells in the early phase of apoptosis, Annexin V-positive and (PI)-positive cells in the late phase of apoptosis were detected by flow cytometry using a BD LSRFortessa X-20 cell Analyzer (BD Biosciences). At least 10,000 cells were analyzed for each experiment.

Co-immunoprecipitation. The antibody-bead complexes were prepared by incubating 10 μg of anti-GGCT (6-1E, Cosmo Bio) or isotype control antibody with 1.5 mg of Dynabeads protein G (Thermo Fisher Scientific). MCF7, A172, and PC3 cells were cultured in 10-cm dishes, treated with fluorizoline at the indicated concentrations for the indicated durations, and then lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate-Na, and 0.1% SDS) supplemented with a protease inhibitor cocktail (Nacalai Tesque). Mixtures comprising cell lysate and each antibody-bead complex were rotated overnight at 4°C. After washing with PBS, the complexes were eluted in SDS-sample buffer, denatured at 95°C for 5 minutes, and analyzed by Western blotting.

Fractionation of Nuclear/Cytoplasmic Proteins. MCF7, A172, and PC3 cells were treated with fluorizoline at the indicated concentrations for the indicated durations, and then cellular proteins were fractionated into cytoplasmic and nuclear fractions using a LysoPure nuclear and cytoplasmic extractor kit (Wako), according to the manufacturer's protocol.

Subcellular Localization Analysis of PHB2 Proteins by Immunofluorescent Staining. MCF7 cells were treated with fluorizoline for 24 h. The cells were fixed with 10%(v/v) formaldehyde in PBS for 30 minutes at room temperature. Then, permeabilization was performed using 0.5% triton-X 100 containing PBS for 15 minutes at 4°C. After blocking with 0.1% triton-X 100, 1% bovine serum albumin containing PBS for 45 minutes at room temperature, the cells were incubated in primary antibodies, anti-PHB2 antibody (dilution, 1:50) in Can Get Signal immunostain solution A (TOYOBO) for 1 h at room temperature. Then, cells were incubated in secondary antibodies, polyclonal rabbit anti-mouse Immunoglobulins FITC (Dako, Carpinteria, CA, USA) (dilution, 1:800) in Can Get Signal Immunostain Solution A for 1 h at room temperature. Nuclei were counterstained with 4, 6-Diamidino-2-phenylindole (DAPI) (Lonza Basel, Switzerland) in PBS for 5 minutes at room temperature. Images were obtained with an Eclipse Ti confocal microscope (Nikon, Tokyo, Japan). Quantitative analysis of fluorescence of PHB2 in nuclei were performed by ImageJ 1.53k software (NIH, Bethesda, MD, USA).

Statistical Analysis. All data were obtained from at least three independent experiments and expressed as the mean ± S.D. Statistical analysis was performed using a two-tailed Student's t test, one-way ANOVA with Bonferroni's multiple comparison test, or 2-way repeated-measures ANOVA with BellCurve for Excel (Social Survey

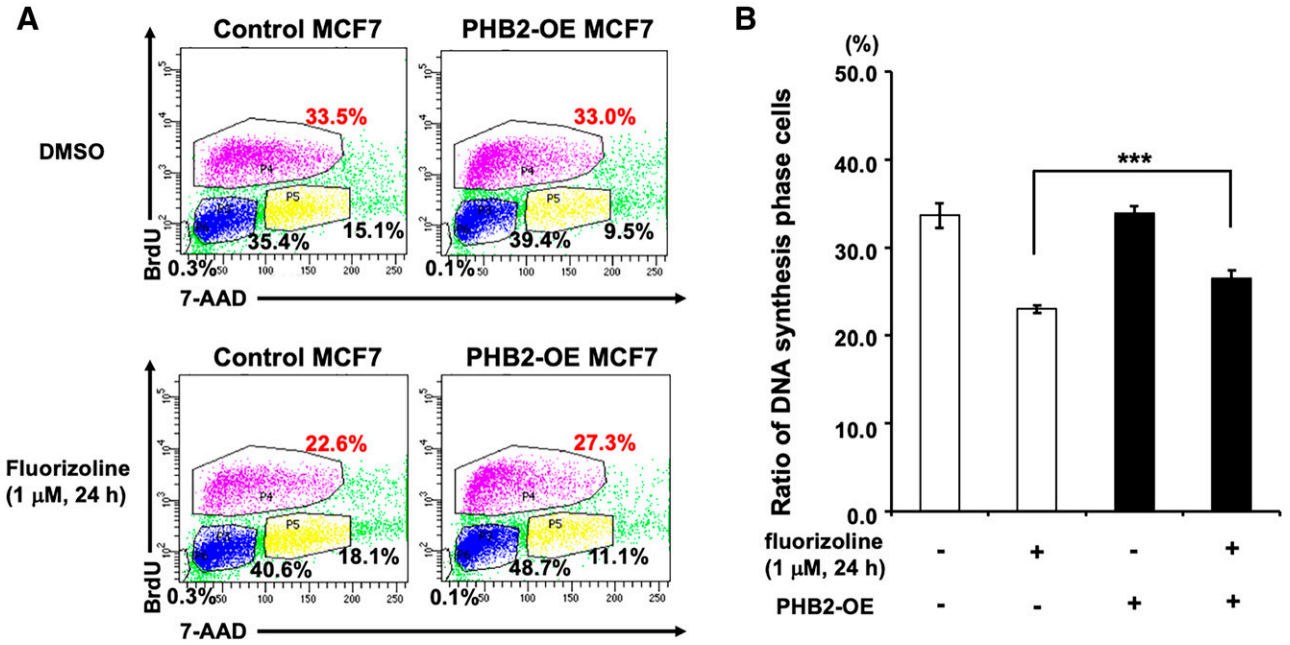

Fig. 5. Fluorizoline-induced blockade of cell cycle progression is mediated by PHB2. (A) The fraction of control or PHB2-overexpressing (PHB2-OE) MCF7 cells in S-phase was measured by bromodeoxyuridine incorporation. PHB2-OE MCF7 cells treated with 1 μM of fluorizoline for 24 h were measured by flow cytometry. (B) Quantitative analyses of the proportion of cells in S-phase ($n = 4$, $^{***}P < 0.001$ by 2-way repeated-measures ANOVA).

Research Information Co., Ltd. Tokyo, Japan). A P value < 0.05 was deemed significant.

Results

Fluorizoline Inhibits MCF7 Proliferation and Induces p21 Expression. First, we examined the effects of fluorizoline treatment on the growth of MCF7 breast cancer cells. A trypan blue dye exclusion test demonstrated that treatment of MCF7 cells with fluorizoline for 24, 48, and 72 h suppressed cell growth in a concentration-dependent manner (Fig. 1A). We next examined whether fluorizoline treatment induces p21 expression. Western blot analyses demonstrated that treatment of MCF7 cells with fluorizoline for 24 h significantly increased p21 protein expression in a concentration-dependent manner (Fig. 1B). Fluorizoline also suppressed proliferation (Fig. 1A) and induced p21 expression (Fig. 1B) in A172 human glioblastoma and PC3 prostate cancer cells. A time-course analysis of 1 μM of fluorizoline treatment in MCF7 cells showed that expression levels of p21 protein

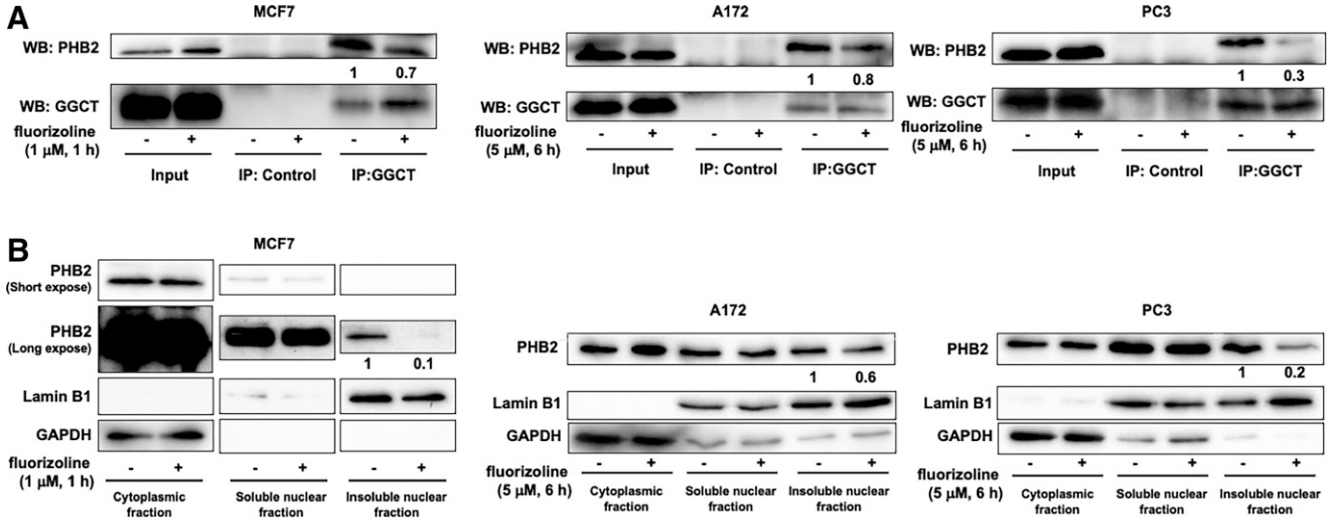

Fig. 6. Fluorizoline blocks the interaction between PHB2 and GGCT and decreases PHB2 protein levels in the nuclear fraction. (A) MCF7, A172, and PC3 cells were treated with fluorizoline at the indicated concentrations for the indicated durations. Endogenous GGCT proteins were immunoprecipitated from cell extracts using mouse anti-GGCT monoclonal or isotype control antibodies. prohibitin-2and GGCT proteins in the precipitated samples (and lysate input) were analyzed by Western blotting. (B) The proteins were fractionated into cytoplasmic, soluble nuclear, and insoluble nuclear components and were analyzed by Western blotting. Lamin B1 and GAPDH are shown as loading controls.

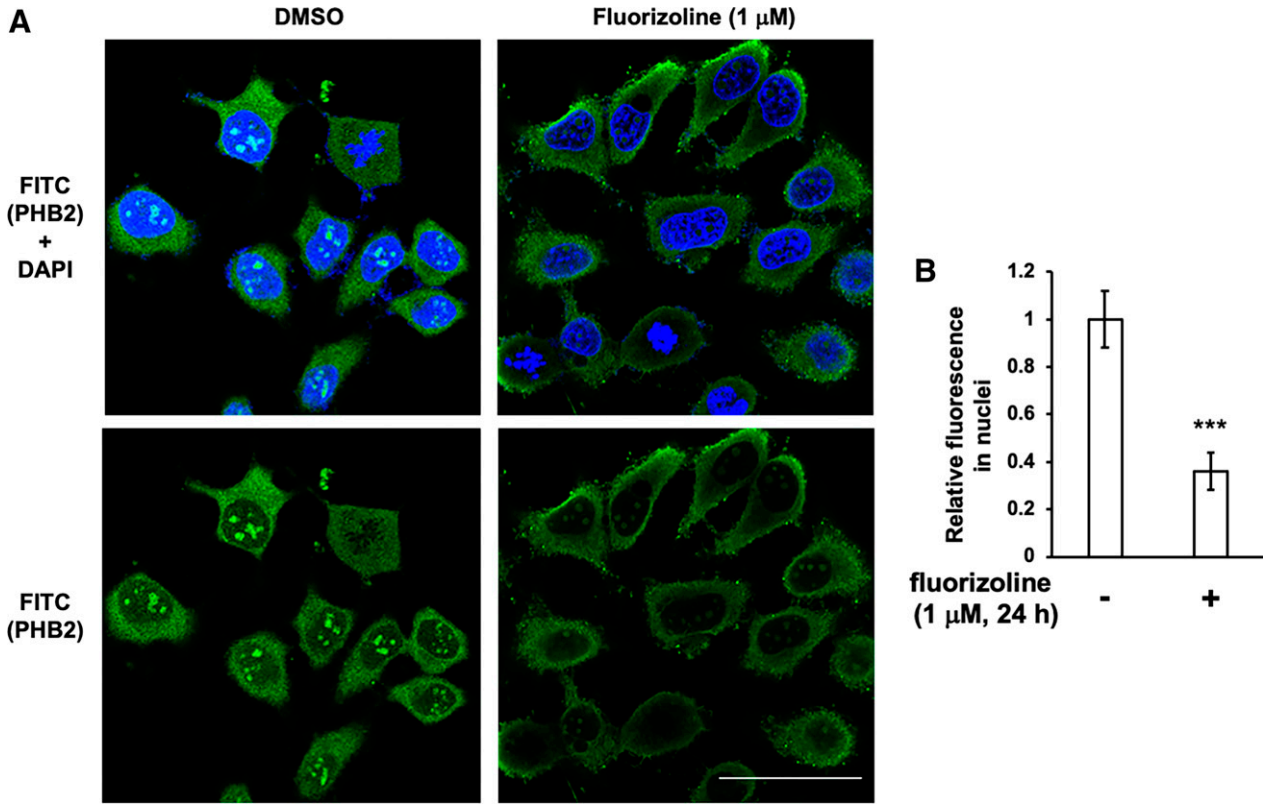

Fig. 7. Fluorizoline reduces the nuclear localization of PHB2. (A) MCF7 cells were treated with 1 μM of fluorizoline for 24 h. Immunofluorescent analysis was performed using anti-prohibitin-2 (green) antibody and 4′,6-diamidino-2-phenylindole (blue). Scale bar, 50 μm. (B) Quantified analysis of fluorescent signal of prohibitin-2 in nuclei are shown. ($n = 9$, $^{***}P < 0.001$ by two-tailed Student's t test).

increased 3 h after treatment (Fig. 1C). An RT-qPCR analysis demonstrated that 1 μM of fluorizoline significantly induced mRNA expression levels of p21 from 1 h treatment (Fig. 1D). These results indicate that fluorizoline induces p21 expression and inhibits proliferation of MCF7 breast cancer cells.

Fluorizoline Induces Cell Cycle Arrest but not Apoptosis. Cell cycle analysis revealed significant increases of the G0/G1 phase population and decreases of the S phase population with fluorizoline in MCF7 cells or A172 cells. However, no increase of sub G1 population in the cell-cycle analyses was observed (Fig. 2A-B). Furthermore, Annexin V-positive cells were not observed in the fluoriozoline-treated MCF7 and A172 cells (data not shown). These results suggest that fluorizoline does not induce apoptosis in MCF7 cells and A172 cells but mainly induces cell-cycle arrest.

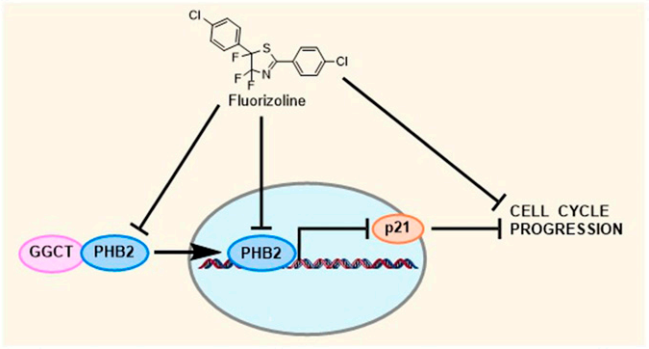

Fig. 8. Fluorizoline disrupts the interaction of prohibitin-2 with γ-glutamylcyclotransferase and its translocation into the nucleus, resulting in an upregulation of p21 and a blockade of cell cycle progression in MCF7 cells.

Induction of p21 Mediates Proliferative Suppression of MCF7 Cells by Fluorizoline. Next, we performed knockdown of p21 in MCF7, A172, and PC3 cells to test whether inhibition of cell proliferation by fluorizoline would be restored by suppression of p21. We confirmed the efficient knockdown of p21 by Western blot analysis (Fig. 3A). The p21 knockdown significantly rescued the suppressed proliferation of MCF7, A172, and PC3 cells by treatment with 1 μM or 5μM of fluorizoline for 48 h (Fig. 3B). These results indicate that fluorizoline inhibits proliferation of MCF7 cells in a p21-induction dependent manner.

Overexpression of PHB2 Restores Growth in Fluorizoline-Treated MCF7 Cells. To confirm that fluorizoline suppresses MCF7 cell proliferation by inhibiting PHB2 activity we prepared PHB2-overexpressing MCF7 cells and examined the effects of fluorizoline. We confirmed the overexpressed PHB2 proteins by Western blot analysis (Fig. 4A). Assessments of proliferation in empty vector-transfected (Control) and PHB2-overexpressing (PHB2-OE) MCF7 cells treated with 0.1 μM or 1 μM of fluorizoline for 48 h, the WST-8 cell viability assay showed that PHB2 overexpression restored cell growth at 48 h (Fig. 4B). The ATP-based cell viability assay showed partially yet significantly restored cell growth in the PHB2-overexpressing MCF7 cells treated with 1 μM of fluorizoline for 24 h (Fig. 4C). Consistent with these findings, Western blot analyses demonstrated that overexpression of PHB2 prevented the fluorizoline-mediated increase in p21 expression (Fig. 4D). These results suggest that fluorizoline increases p21 expression and

suppresses cellular proliferation by modulating the activity PHB2.

Overexpression of PHB2 Restores Cell Cycle Progression in Fluorizoline-Treated MCF7 Cells. Since p21 is an important regulator of the cell cycle, we next investigated the effects of fluorizoline on cell cycle progression in parental and PHB2-overexpressing MCF7 cells. A BrdU incorporation assay demonstrated that fluorizoline significantly reduced the number of MCF7 cells in S-phase, and that PHB2 overexpression partially but significantly restored cell cycle progression (Fig. 5A-B). These results indicate that fluorizoline suppresses the growth of MCF7 cells by inhibiting cell cycle progression, and this effect is associated with increases in p21 expression.

Fluorizoline Blocks the Interaction between PHB2 and GGCT Proteins and Reduces the Nuclear Localization of PHB2. PHB2 interacts with GGCT, and the interaction promotes the nuclear localization of PHB2 proteins, which then bind to the promoter of the *p21* gene and repress transcription (Taniguchi et al., 2018b). We finally investigated the effects of fluorizoline treatment on the interaction between PHB2 and GGCT proteins. Results of a co-immunoprecipitation assay using monoclonal antibodies against GGCT revealed that fluorizoline treatment blocked the interaction between endogenous PHB2 and GGCT proteins (Fig. 6A). Consistent with the results, PHB2 protein expression in the insoluble nuclear fraction (Fig. 6B) and nuclear localization of PHB2 (Fig. 7A-B) were reduced after fluorizoline treatment. These results suggest that fluorizoline prevents PHB2 from interacting with GGCT, which then inhibits the nuclear localization of PHB2 proteins, resulting in the induction of p21 (Fig. 8).

Discussion

Here, we first show that fluorizoline induces p21 expression in human cancer cell lines. Our time-course analysis demonstrated that fluorizoline treatment as short as 1 h significantly increased the mRNA levels of p21, suggesting that fluorizoline directly influences p21 expression. Moreover, knockdown of p21, which was prevented from its induction by fluorizoline treatment, significantly cancelled the proliferative inhibition of MCF7 cells by fluorizoline. We provide evidence from a co-immunoprecipitation assay showing that fluorizoline blocked the interaction between PHB2 and GGCT. A previous study demonstrated that the interaction between GGCT and PHB2 promotes the nuclear translocation of the PHB2 protein, which plays an important role in repressing p21 expression (Taniguchi et al., 2018b). Consistent with those findings, we show that fluorizoline treatment markedly reduces the amount of PHB2 protein in a chromatin-bound insoluble nuclear fraction; indeed, PHB2 protein could not be detected in this fraction even after long chemiluminescence exposure times in Western blot analysis. This meant it was not feasible to perform chromatin immunoprecipitation analysis following fluorizoline treatment using PHB2 antibodies because of the absence of the target PHB2 proteins.

Our findings showing that overexpression of PHB2 restored the cellular proliferation that was inhibited by fluorizoline indicate that fluorizoline targets PHB2. In particular, PHB2 overexpression prevented fluorizoline-mediated increases in p21 expression. The fluorizoline-mediated blockade of cell cycle progression was prevented by PHB2 overexpression, as shown by the BrdU incorporation assay. These results also indicate that the effects of fluorizoline were mediated by p21 induction. However, recovery of proliferation and cell cycle arrest by PHB2 overexpression were partial, suggesting existence of other mechanisms for fluorizoline or potential involvement of PHB1 instead of PHB2. It has been reported that PHB2 exerts pleiotropic functions in the nucleus, where it acts as a transcriptional regulator binding to MyoD, MEF2, HDAC1/5, EZH2, RNF2, or estrogen receptors (Thuaud et al., 2013). Further clarification of the binding partner for PHB2 on the *p21* promoter would provide useful information on the mechanisms that underly the complicated functions of PHB2.

Our findings show that fluorizoline induces p21 expression in several human cancer cell lines. The increase in p21 expression, antiproliferative effects, and blockade of cell cycle progression mediated by fluorizoline treatment were reversed by PHB2 overexpression, indicating that fluorizoline specifically targets PHB2. Fluorizoline inhibited the interaction between GGCT and PHB2, which decreased the nuclear localization of PHB2. Taken together, these results shed new light on the mechanism of action of fluorizoline and illustrate how this small molecule can be used to explore new aspects of PHB signaling.

Acknowledgments

The authors thank Momoka Endo for experimental support.

Authorship Contributions

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