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HES1-mediated inhibition of Notch1 signaling by a Gemini vitamin D analog leads to decreased CD44⁺/CD24^{-low} tumor-initiating subpopulation in basal-like breast cancer

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Abstract

Tumor-initiating cells (also known as cancer stem cells) are the subpopulation of cells shown to be responsible for tumor initiation, maintenance and recurrence. In breast cancer, CD44⁺/CD24^{-low} cells were identified as tumor-initiating cells. We previously reported that a Gemini vitamin D analog, 1 α ,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol (BXL0124), reduced CD44⁺/CD24^{-low} cells in MCF10DCIS basal-like breast cancer cells. Since Notch has been identified as one of the key signaling pathways involved in breast cancer stem cells, the effect of BXL0124 on the Notch signaling pathway was investigated in breast cancer. The CD44⁺/CD24^{-low} subpopulation of MCF10DCIS cells showed elevated Notch1 signaling and increased cell proliferation compared to the CD44⁺/CD24^{high} subpopulation. Treatment with the Gemini vitamin D analog BXL0124 decreased the level of activated Notch1 receptor. In addition, mRNA and protein levels of the Notch ligands, Jagged-1, Jagged-2 and DLL1, were significantly reduced by treatment with BXL0124, which was followed by repression of c-Myc, a key downstream target of Notch signaling. Interestingly, HES1, a known downstream target of Notch signaling, was rapidly induced by treatment with BXL0124. The inhibitory effect of BXL0124 on Notch signaling was reversed by knockdown of HES1. Overexpression of HES1 inhibited Notch1 signaling and reduced the CD44⁺/CD24^{-low} subpopulation, confirming a role of HES1 in Notch1 signaling. In conclusion, the Gemini vitamin D analog, BXL0124, represses the tumor-initiating subpopulation

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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by HES1-mediated inhibition of Notch1 signaling. The present study demonstrates BXL0124 as a potent inhibitor of Notch signaling to target tumor-initiating cells in basal-like breast cancer.

Keywords

Notch signaling; Tumor-initiating cells; Gemini vitamin D analog; CD44⁺/CD24^{-/low} cells; HES1; Breast Cancer

1. Introduction

Breast cancer is a heterogeneous disease which can be divided into three major subtypes, estrogen receptor (ER)-positive, HER2-overexpressing and basal-like subtype [1]. Each subtype has distinct gene signatures and is associated with selective targeted therapies with different clinical outcomes [1]. Endocrine therapy and HER2-targeting antibodies have been effective targeted therapies for ER-positive and HER2-overexpressing breast cancer patients, respectively [2]. However, basal-like breast cancer, which has the worst prognosis among those subtypes, still lacks the targeted therapy with proven clinical benefit [2]. Many of ER-positive and HER2-overexpressing tumors eventually develop resistance against targeted therapies, urging additional therapeutic strategies to overcome the resistance [2].

Tumor-initiating cells (also known as cancer stem cells) are a subpopulation of cancer cells which are considered to be responsible for the initiation, maintenance and recurrence of tumors [3]. Various cell surface markers, such as CD44, CD49, CD24 and CD133, have been used to isolate the tumor-initiating subpopulation of cells from different types of tumors, including breast, lung, colon and pancreas [3]. In breast cancer, tumor-initiating cells were initially identified by sorting for cells expressing high levels of CD44 but little or very low levels of CD24 (CD44⁺/CD24^{-/low} cells), and as few as 100 of these cells were shown to form tumors in animals [4]. The CD44⁺/CD24^{-/low} cells were chemoresistant [5, 6] and enriched in residual breast tumors after chemotherapy [7]. Moreover, the CD44⁺/CD24^{-/low} cells were most frequently found in basal-like subtype, which is associated with high risk of early relapse within the first 2–5 years after treatment [8]. Therefore, targeting these tumor-initiating cells may have beneficial implications, eliminating recurrence and metastasis of breast cancer [9, 10].

Recent studies have shown that signaling pathways involved in maintaining the stem cell population, such as Hedgehog, Wnt and Notch, are activated in tumor-initiating cells [11–13]. Among them, Notch signaling is known to be one of the key pathways regulating tumor-initiating cells [14]. Notch receptors are single transmembrane proteins activated by interaction with ligands from adjacent cells, mediating cell-cell communication [15]. Upon activation by ligands, Notch receptors undergo two sequential proteolytic cleavages first by a disintegrin and metalloproteinase family of metalloproteinases and second by the gamma-secretase complex, producing cleaved Notch (c-Notch) [15]. The cleaved Notch then translocates to the nucleus where it regulates expression of its target genes, such as HES1, HEY2 and c-Myc [15]. Notch signaling is known to play key roles in cell-fate determination of embryonic stem cells and maintenance of adult stem cells [15]. In addition to its roles in stem cells, aberrant Notch signaling has been reported in different types of human cancers,

such as T-cell acute lymphoblastic leukemia, chronic lymphocytic leukemia, glioblastoma and breast cancer [16–18].

In breast cancer, several studies reported that the expression of Notch receptors is higher in breast cancer tissues than the normal breast epithelium [19, 20]. In addition, activation of Notch signaling is correlated with pathologic features of poor prognosis such as high tumor grade [21] and basal-like subtype [22]. In animal studies, overexpression of constitutively active Notch receptors was shown to promote development of mammary tumors [23, 24], supporting the tumorigenic potential of Notch signaling in breast cancer. Moreover, recent studies reported that the activation of Notch signaling is necessary for maintenance of CD44⁺/CD24^{-low} tumor-initiating cells in breast cancer [14, 25].

Gemini vitamin D analogs are a group of novel synthetic analogs of 1 α ,25(OH)₂D₃, a hormonally active form of vitamin D, which were developed to achieve better anti-cancer activity with lower toxicity [26]. Gemini vitamin D analogs have two identical side chains at carbon 20 (Fig. 1), inducing the structural rearrangement of the ligand binding domain of vitamin D receptor (VDR) and acting as a more potent agonist of VDR than the natural vitamin D [27, 28]. We have previously demonstrated the inhibitory activities of novel synthetic Gemini analogs of 1 α ,25(OH)₂D₃ on breast cancer *in vitro* and *in vivo* [5, 29–32]. Recently, we have shown that a Gemini vitamin D analog, BXL0124, repressed the expression of a tumor-initiating cell marker CD44 and reduced the CD44⁺/CD24^{-low} subpopulation in MCF10DCIS cells, a basal-like human breast cancer cell line derived from the MCF10A cell line with the ability to form ductal carcinoma *in situ* (DCIS)-like lesions in animals [31]. The mechanism by which BXL0124 reduces the CD44⁺/CD24^{-low} subpopulation, however, is not understood. Based on the crucial role of Notch signaling in tumor-initiating cells, we hypothesized that Notch might be a key signaling pathway targeted by BXL0124 to suppress the CD44⁺/CD24^{-low} subpopulation in breast cancer. In the present study, we report that BXL0124 inhibits Notch signaling via the transcriptional repressor HES1, leading to the reduction of the CD44⁺/CD24^{-low} subpopulation in basal-like breast cancer.

2. Materials and Methods

2.1. Reagents and cell culture

1 α ,25(OH)₂D₃ and a Gemini vitamin D analog BXL0124 (1 α ,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol) provided by BioXell, Inc. (Nutley, NJ) (Fig. 1) [33] were dissolved in dimethyl sulfoxide. The MCF10DCIS.com cell line (MCF10DCIS) was provided by Dr. Fred Miller at the Barbara Ann Karmanos Cancer Institute (Detroit, MI) [34]. The MCF10DCIS cell line was authenticated by short tandem repeat profiling at American Type Culture Collection (Manassas, VA). HES1 overexpressing MCF10DCIS cells were generated by transducing the MCF10DCIS cells with lentivirus containing HES1 expression vector (Plasmid 17624: EF.hHES1.Ubc.GFP) (Addgene, Cambridge, MA) [35]. The transduced cells were sorted by FACS using MoFlo XDP Cell Sorter (Beckman Coulter, Brea, CA) to obtain GFP-labeled HES1 overexpressing MCF10DCIS cells (DCIS-HES1) and GFP-unlabeled control MCF10DCIS cells (DCIS). Cells were maintained in DMEM/F-12 medium supplemented

with 5% horse serum, 1% penicillin/streptomycin, and 1% HEPES solution at 37°C and 5% CO₂.

2.2. Cell sorting and flow cytometry with CD44 and CD24 staining

The detailed procedure was described previously [31]. MCF10DCIS cells were stained with antibodies against CD44-APC (Cat. 559942) and CD24-PE-CyTM7 (Cat. 561646) from BD bioscience (San Jose, CA). The stained MCF10DCIS cells were sorted by MoFlo XDP Cell Sorter (Beckman Coulter) into three subpopulations (CD44⁺/CD24⁻, CD44⁺/CD24^{low} and CD44⁺/CD24^{high}), and the sorted cells were utilized for further analysis. DCIS and DCIS-HES1 cells were stained with the antibodies against CD44-APC and CD24-PE-CyTM7 and analyzed by flow cytometry using FC500 Analyzer (Beckman Coulter).

2.3. [³H] thymidine incorporation assay

The procedure was described previously [29]. In brief, the three subpopulations (CD44⁺/CD24⁻, CD44⁺/CD24^{low} and CD44⁺/CD24^{high} cells) of MCF10DCIS cells were seeded into each well of 24-well plate (8,000 cells/well), and grown overnight. On the next day, the cells were incubated for 72 h with or without BXL0124 treatment for the thymidine incorporation assay. The amount of [³H] thymidine uptake was analyzed by a Beckman liquid scintillation counter (Fullerton, CA) to determine cell proliferation rate.

2.4. MTT assay

We previously reported the details of the MTT assay [36]. DCIS and DCIS-HES1 cells were seeded into each well of 96-well plate (1,000 cells/well), and incubated for 24, 48 and 72 h. The absorbance was measured with a spectrophotometer (Tecan US, Durham, NC) to determine cell proliferation rate.

2.5. Western blot analysis

The detailed procedure was described previously [37]. The primary antibody against CD44 (sc-7298), which recognizes both the standard form of CD44 (CD44s, 82 kDa) and variant forms of CD44 (CD44v, 100~250 kDa), was from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies recognizing c-Notch1 (4147), Notch1 (4380), Notch2 (5732), Notch3 (5276), Jagged-1 (2155), Jagged-2 (2210), DLL1 (2588), c-Myc (5605) and HES1 (11988) were from Cell Signaling Technology (Beverly, MA); vitamin D receptor (VDR) (GR37) was from Millipore (Billerica, MA); β -actin (A1978) was from Sigma-Aldrich (St. Louis, MO). Secondary antibodies were from Santa Cruz Biotechnology. Densitometric analysis of western blots was conducted by using image J (NIH, Bethesda, MD) to quantify protein levels.

2.6. Knockdown of VDR or HES1 by siRNA

The detailed procedure was described previously [31]. MCF10DCIS cells were incubated with 1 μ M of VDR siRNA (A-003448-13-0010, Thermo Fisher Scientific Inc., Waltham, MA) or 1 μ M of HES1 siRNA (A-007770-20-0010, Thermo Fisher Scientific Inc.) in Accell siRNA delivery medium (Thermo Fisher Scientific Inc.) for 72 h to knockdown VDR or

HES1, respectively. Non-targeting siRNA (D-001910-02-20, Thermo Fisher Scientific Inc.) was utilized as a negative control with same experimental procedure.

2.7. Fluorescence microscopy

The detailed procedure was described previously [36]. Cells were incubated overnight at 4°C with a primary antibody to c-Notch1 (1:100 dilution, Cell Signaling Technology). Then, the cells were incubated with fluorophore-conjugated secondary antibody (1:200 dilution, Alexa Fluor 488, Invitrogen) and TO-PRO-3 iodide nuclear antibody (1 µM, Invitrogen) at room temperature for 60 and 15 minutes, respectively. The images were taken using confocal microscope with laser at 488 nm (c-Notch1) and 633 nm (TO-PRO-3).

2.8. Nuclear and cytoplasmic fractionation

The nuclear and cytoplasmic fractionation was performed with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Cat. 78833, Thermo Fisher Scientific Inc.). In brief, MCF10DCIS cells were incubated with vehicle control or BXL0124 for 24 h and harvested with EDTA-Trypsin for fractionation. The nuclear and cytoplasmic fractions were isolated separately by following the manufacturer's instructions. The amount of a given protein in each fraction was determined by Western blot analysis.

2.9. Quantitative polymerase chain reaction (qPCR)

The procedure was described previously [29]; the labeled primers for Notch1, Notch2, Notch3, Notch4, Jagged-1, Jagged-2, DLL1, DLL2, c-Myc, HES1, VDR and glyceraldehyde 3-phosphate dehydrogenase were obtained from Life Technologies (Carlsbad, CA).

2.10. Statistical Analysis

Statistical significance was evaluated using Student's *t* test for two sets of data or one-way ANOVA with Tukey's test for more than two sets of data.

3. Results

3.1. CD44⁺/CD24^{-low} subpopulation shows elevated Notch signaling and increased cell proliferation

Recent studies have demonstrated that active Notch signaling is required to maintain the tumor-initiating subpopulation (CD44⁺/CD24^{-low}) in breast cancer cells [12, 38]. We previously reported that the novel Gemini vitamin D analog, BXL0124, reduced the CD44⁺/CD24^{-low} tumor-initiating subpopulation and inhibited cell proliferation in MCF10DCIS basal-like breast cancer cells [31]. To study whether BXL0124 decreases tumor-initiating subpopulation by targeting Notch signaling, we first investigated the correlation between Notch signaling and the tumor-initiating subpopulation of MCF10DCIS cells. While most studies demonstrated CD44⁺/CD24⁻ (CD44⁺/CD24^{neg}) and CD44⁺/CD24^{low} combined cells as the tumor-initiating subpopulation, some studies specified only CD44⁺/CD24⁻ cells as the tumor-initiating subpopulation [4, 39, 40]. To clarify the role of each subpopulation, we sorted MCF10DCIS cells into three subpopulations (CD44⁺/CD24⁻, CD44⁺/CD24^{low} and CD44⁺/CD24^{high}) (Fig. 2A). All three subpopulations expressed high levels of CD44 [a standard form of CD44 (CD44s, 82 kDa) and variant forms of CD44 (CD44v, 100~250

kDa)] (Fig. 2B). The activation of Notch signaling was determined by measuring protein levels of activated Notch1 receptor (cleaved form of Notch1, c-Notch1), Notch ligand (Jagged-2) and a downstream target of Notch signaling (c-Myc). The levels of c-Notch1, Jagged-2 and c-Myc were markedly higher in CD44⁺/CD24⁻ and CD44⁺/CD24^{low} subpopulations compared to the CD44⁺/CD24^{high} subpopulation (Fig. 2B). CD44⁺/CD24⁻ and CD44⁺/CD24^{low} subpopulations showed significantly higher proliferation rate than the CD44⁺/CD24^{high} subpopulation (Fig. 2C). Treatment with BXL0124 lowered the levels of c-Notch1, Jagged-2 and c-Myc (Fig. 2D) as well as cell proliferation rate in all three subpopulations (Fig. 2E). The activity of Notch1 signaling and its regulation by BXL0124 were similar in CD44⁺/CD24⁻ and CD44⁺/CD24^{low} cells (Fig. 2B and 2D), confirming the combined CD44⁺/CD24^{-/low} subpopulation as tumor-initiating cells.

3.2. 1 α ,25(OH) $_2$ D $_3$ and BXL0124 inhibit Notch1 signaling in a vitamin D receptor (VDR)-dependent manner

In the previous study, the synthetic analog BXL0124 demonstrated much more potent activity than naturally occurring 1 α ,25(OH) $_2$ D $_3$ to inhibit proliferation of MCF10DCIS cells [36]. We compared 1 α ,25(OH) $_2$ D $_3$ with BXL0124 for their inhibitory effects on Notch signaling. Both 1 α ,25(OH) $_2$ D $_3$ and BXL0124 decreased protein levels of c-Notch1, total Notch3, Jagged-1, Jagged-2, DLL1 and c-Myc in a dose-dependent manner with the greatest effects on c-Notch1 and Jagged-2. BXL0124 showed much stronger activity than 1 α ,25(OH) $_2$ D $_3$ (Fig. 3A). Total Notch1 and total Notch2 were not affected by either 1 α ,25(OH) $_2$ D $_3$ or BXL0124 (Fig. 3A). The induction of CYP24A1 mRNA, a direct target gene of VDR, by 1 α ,25(OH) $_2$ D $_3$ and BXL0124 was also compared, and BXL0124 was much more potent than 1 α ,25(OH) $_2$ D $_3$ (Fig. S1). However, the difference of potency between BXL0124 and 1 α ,25(OH) $_2$ D $_3$ for the induction of CYP24A1 mRNA was lesser than those for the inhibition of c-Notch1 and Jagged-2, suggesting an additional VDR-independent activity of BXL0124 to regulate those molecules. In a time course study, the protein level of c-Notch1 was markedly reduced by BXL0124 at 12 and 24 h, while the protein level of total Notch1 was not affected (Fig. 3B). BXL0124 treatment decreased Jagged-1 at 4, 12 and 24 h, and Jagged-2 at 12 and 24 h, respectively (Fig. 3B). Because repression of c-Notch1 and Jagged-2 by BXL0124 was most significant at 24 h, further analysis was carried out with 24 h treatment with BXL0124. Activated Notch receptor translocates to the nucleus [15]. Thus, we examined c-Notch1 levels in the nucleus by confocal microscopy and immunoblotting of subcellular fractions. MCF10DCIS cells treated with the vehicle control showed strong c-Notch1 staining in nuclei, which was decreased with BXL0124 treatment (Fig. 3C). A subcellular fractionation study showed that the majority of c-Notch1 protein was localized in the nucleus, while most of total Notch1 and Jagged-2 were present in the cytoplasm (Fig. 3D). BXL0124 treatment decreased the level of c-Notch1 in the nucleus. VDR level in the nucleus was markedly increased by BXL0124 (Fig. 3D). Since most of the biological actions of vitamin D occur through the VDR, involvement of VDR in the repression of Notch signaling by BXL0124 was investigated by using VDR siRNA. The repression of c-Notch1 and Jagged-2 by BXL0124 was reduced by knockdown of VDR, indicating that the inhibition of Notch1 activation by BXL0124 is a VDR-dependent event (Fig. 3E).

3.3. Regulation of mRNA expression levels of Notch signaling molecules by BXL0124

The effects of BXL0124 on mRNA levels of Notch signaling components, such as receptors, ligands and downstream targets, were examined in a time course study. The mRNA levels of Notch receptors, Notch1, Notch2 and Notch3 were not affected by BXL0124 (Fig. 4A). The mRNA expression of Notch4 was undetectable (PCR cycle numbers were over 32) (data not shown). The mRNA levels of Jagged-2 and DLL1 were increased over the 24 h time periods possibly due to the increased cell-cell contact, while Jagged-1 level remained constant (Fig. 4B). The mRNA levels of Notch ligands, Jagged-1, Jagged-2 and DLL1, were significantly decreased by BXL0124 at 8, 16 and 24 h (Fig. 4B). The mRNA expression of DLL2 was too low for reliable detection (PCR cycle numbers were over 32) (data not shown). The mRNA level of c-Myc, a key downstream target of Notch signaling, was significantly decreased by BXL0124 at 16 and 24 h (Fig. 4C), which occurred later than the repression of Notch ligands. Interestingly, HES1, a known downstream target of Notch signaling, was rapidly induced by BXL0124 at 1, 4, 8, 16 and 24 h (Fig. 4C).

3.4. HES1 is required for the inhibition of Notch1 signaling by BXL0124

The rapid induction of HES1 mRNA by BXL0124 was confirmed with additional earlier time point studies (10 min, 30 min and 2 h) (Fig. 5A). The expression of HES1 mRNA was significantly increased by BXL0124 as early as 30 min, and the induction of HES1 was maintained up to 24 h (Fig. 5A). The protein level of HES1 was also markedly increased by BXL0124 at 1 and 4 h (Fig. 5B). Although HES1 is a well-known Notch effector regulating cell proliferation and differentiation during embryogenesis [41], HES1 can also antagonize Notch signaling by directly repressing the expression of Notch ligands in a context dependent manner [42]. Therefore, the involvement of HES1 in Notch signaling was investigated by using HES1 siRNA. Although HES1-targeting siRNA substantially decreased HES1, there was a considerable amount of HES1 remaining. Because of the partial depletion of HES1, BXL0124 still increased HES1 and caused down-regulation of c-Notch1 and Jagged 2 (Fig. 5C). However, the extent of c-Notch1 and Jagged-2 reduction by BXL0124 was decreased by the knockdown of HES1, indicating that the inhibition of Notch1 signaling by BXL0124 is a HES1-dependent event (Fig. 5C). The induction of VDR by BXL0124 was not affected by HES1 knockdown (Fig. 5C), suggesting that VDR is an upstream effector of HES1 for the regulation of Notch1 signaling by BXL0124.

3.5. Overexpression of HES1 decreases the expression of Notch ligands and inhibits Notch1 signaling

To further investigate a specific role of HES1 in Notch signaling, HES1 was overexpressed in MCF10DCIS cells, and the effects of HES1 overexpression on Notch1 signaling were examined. MCF10DCIS cells were transduced with lentiviral HES1-expression vector labeled with green fluorescent protein (GFP), and then sorted into GFP-negative control cells (DCIS) and GFP-positive HES1-overexpressing cells (DCIS-HES1) (Fig. 6A, left panel). Stable maintenance of HES1 expression in DCIS-HES1 cells was confirmed by a 2nd cell sorting of DCIS-HES1 cells 4 passages after the 1st cell sorting (Fig. 6A, right panel). DCIS-HES1 cells (2.6 ± 0.5) showed a significantly higher level of HES1 mRNA than DCIS cells (1.0 ± 0.1) ($p < 0.01$) (Fig. 6B). The protein level of HES1 was also increased in DCIS-

HES1 cells compared to DCIS cells (Fig. 6C). The protein levels of Jagged-2, c-Notch1 and c-Myc were markedly decreased by the overexpression of HES1, while those of total Notch1 and VDR were not affected by the overexpression of HES1 (Fig. 6C). The mRNA levels of Jagged-2, DLL1 and c-Myc were also significantly decreased by HES1 overexpression, but the mRNA levels of Notch1 and Jagged-1 were not affected (Fig. 6D).

3.6. HES1 overexpression reduces the CD44⁺/CD24^{-low} subpopulation and decreases proliferation of MCF10DCIS cells

The effect of HES1 overexpression on the CD44⁺/CD24^{-low} subpopulation was investigated by analyzing DCIS and DCIS-HES1 cells after staining with CD44 and CD24. Both DCIS (GFP-negative) and DCIS-HES1 (GFP-positive) cells expressed high levels of CD44 (Fig. 7A). Single staining of CD24 showed that DCIS-HES1 cells had a markedly increased fraction of CD24^{high} cells compared to DCIS cells (Fig. 7A). Double staining with CD44 and CD24 showed that CD44⁺/CD24^{-low} cells were significantly lower in DCIS-HES1 cells ($25.6 \pm 3.8\%$) than DCIS cells ($50.6 \pm 3.7\%$) ($p < 0.01$), while CD44⁺/CD24^{high} cells were significantly higher in DCIS-HES1 cells ($58.7 \pm 4.5\%$) than DCIS cells ($29.9 \pm 3.4\%$) ($p < 0.01$) (Fig. 7B). The proliferation rates of DCIS-HES1 cells at 48 h (0.267 ± 0.025) and 72 h (0.556 ± 0.084) were significantly lower than those of DCIS cells at 48 h (0.378 ± 0.015 , $p < 0.01$) and 72 h (0.849 ± 0.111 , $p < 0.01$), respectively (Fig. 7C).

4. Discussion

Aberrant activation of Notch signaling has been reported in DCIS as well as in invasive breast cancer [43, 44]. Interestingly, increased activation of Notch1 signaling is shown in CD44⁺/CD24^{-low} tumor-initiating cells of breast cancer [12, 25]. Recent studies have demonstrated that inhibition of Notch1 signaling by knockdown of Notch1 receptor or Notch1 receptor-targeting antibody reduced the number of tumor-initiating cells [12, 38], indicating the important role of Notch1 signaling in the maintenance of these cells. Here, we showed that Notch1 signaling was highly activated in the CD44⁺/CD24^{-low} subpopulation of MCF10DCIS cells (Fig. 2). In addition, the inhibition of Notch1 signaling by either treatment with a Gemini vitamin D analog BXL0124 or HES1 overexpression resulted in the reduction of the CD44⁺/CD24^{-low} subpopulation of MCF10DCIS cells (Figs. 2 and 7), supporting the critical role of the Notch1 signaling pathway in the maintenance of the CD44⁺/CD24^{-low} subpopulation of breast cancer.

With the accumulating evidence for the important role of Notch signaling in human cancer, various approaches such as receptor-targeting antibodies and inhibitors of co-activators have been developed to inhibit Notch signaling [14]. In particular, gamma-secretase, which mediates the second cleavage of the intracellular domain of Notch, has been a key target to inhibit Notch signaling [45]. However, gamma-secretase inhibitors are not exclusively specific to Notch signaling since this protease has many other substrates such as ErbB4, syndecan-3 and CD44 [45]. In addition, gamma-secretase inhibitors were shown to inhibit the proteasome which might have adverse effects in humans [46]. In the present study, BXL0124 markedly reduced Notch ligands but not Notch receptors, and the decrease of Notch ligands was positively correlated with the reduced activation of Notch receptor (determined by protein level of c-Notch1) (Fig. 3A and 3B). These results demonstrate that

BXL0124 inhibits Notch signaling by targeting Notch ligands, emphasizing the potential of BXL0124 as an inhibitor of Notch signaling with a different mechanism of action from gamma-secretase inhibitors.

HES1, a basic helix-loop-helix transcriptional repressor, plays a critical role in the development of many tissues, such as brain, eye and pancreas [47]. Since HES1 is a well-established downstream effector of Notch signaling, it has been used as an indicator of Notch signaling activity in many cancers [48, 49]. However, the biological functions of HES1 have been shown to vary in a context-dependent manner, even exhibiting inverse correlation with Notch signaling in some cases [50, 51]. In ER-positive breast cancer, estrogen promoted Jagged1-induced Notch signaling [52], while it inhibited HES1 expression leading to increased cell proliferation [53]. Furthermore, constitutively high expression of HES1 inhibited Notch signaling by directly repressing the expression of Notch ligands, such as Jagged-1 and DLL1, in embryonic stem cells [42]. These results indicate that HES1 could be involved in Notch signaling as an inhibitor of Notch ligands, suggesting possible a negative feedback regulation of Notch signaling. Here, we demonstrated that BXL0124 repressed the expression of Notch ligands in a HES1 dependent manner (Fig. 5C). In addition, overexpression of HES1 decreased the expression levels of Notch ligands (Fig. 6C and 6D), supporting the role of HES1 as an inhibitor of Notch signaling. Moreover, the mRNA level of HES1 was rapidly induced by BXL0124 as early as 30 min (Fig. 5A), and was followed by the repression of mRNA of Notch ligands (Fig. 4), suggesting that HES1 might be a key effector of BXL0124 to inhibit Notch signaling in breast cancer. Figure 7D shows a proposed schematic diagram for the action of the Gemini vitamin D analog BXL0124 on Notch signaling and CD44⁺/CD24^{-low} tumor-initiating cells.

c-Myc, a critical transcriptional regulator of cellular proliferation, differentiation and apoptosis, is one of the well-known targets of the Notch signaling pathway [24]. The direct up-regulation of c-Myc by activated Notch1 was shown to induce growth of T-cell acute lymphoblastic leukemia and lymphoma cells [54, 55]. In a transgenic mouse model overexpressing activated Notch1, c-Myc was a direct transcriptional target of Notch1 and was required for Notch1-induced mammary tumorigenesis [24]. In our study, the increased levels of c-Myc and c-Notch1 in the CD44⁺/CD24^{-low} subpopulation were correlated with high cell proliferation (Fig. 2B and 2C), and BXL0124 repressed cell proliferation as well as the levels of c-Myc and c-Notch1 (Fig. 2D and 2E). These results suggest that the Notch1/c-Myc signaling axis might be a key target of BXL0124 to inhibit tumor-initiating cells in breast cancer.

5. Conclusion

The present study demonstrates that BXL0124 inhibits Notch signaling by rapid induction of HES1, resulting in the reduction of the CD44⁺/CD24^{-low} subpopulation and proliferation of MCF10DCIS cells. Overexpression of HES1 also represses Notch signaling to decrease CD44⁺/CD24^{-low} subpopulation and proliferation of MCF10DCIS cells, confirming a role of HES1 in Notch signaling. Our study suggests the Gemini vitamin D analog, BXL0124, may be useful as an inhibitor of Notch signaling to target tumor-initiating cells in breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ER	estrogen receptor
GFP	green fluorescent protein
CD44s	standard form of CD44
CD44v	variant form of CD44
MCF10DCIS	MCF10DCIS.com
DCIS	GFP-unlabeled control MCF10DCIS
DCIS-HES1	GFP-labeled HES1 overexpressing MCF10DCIS
qPCR	quantitative polymerase chain reaction
VDR	vitamin D receptor

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Highlights

- Notch1 signaling is activated in tumor-initiating cells of breast cancer.
- The Gemini vitamin D analog BXL0124 inhibits Notch1 signaling in breast cancer.
- Vitamin D and the Gemini vitamin D analog BXL0124 repress Notch ligands.
- The inhibition of Notch1 signaling by BXL0124 is mediated by induction of HES1.
- BXL0124 is a Notch inhibitor targeting tumor-initiating cells in breast cancer.

Figure 1.

The structures of 1α,25(OH)₂D₃ and the Gemini vitamin D analog BXL0124 (1α,25-dihydroxy-20R-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol).

Figure 2. Increased cell proliferation and Notch1 activation in CD44⁺/CD24⁻ and CD44⁺/CD24^{low} subpopulations of MCF10DCIS cells

(A) MCF10DCIS cells were labeled with antibodies against cell surface markers, CD44 and CD24, and sorted into three subpopulations (CD44⁺/CD24⁻, CD44⁺/CD24^{low} and CD44⁺/CD24^{high}). (B) After cell sorting, the protein levels of indicated molecules in the three subpopulations were examined by Western blot analysis, with β-actin used as a loading control. (C) The cell proliferation rates of three subpopulations were measured by [³H] thymidine incorporation. Three separate experiments, each with triplicates, were conducted (**p < 0.01). (D) Three subpopulations of MCF10DCIS cells after sorting were incubated with vehicle control or BXL0124 (10 nM) for 24 h. The protein levels of indicated molecules were examined by Western blot analysis, with β-actin used as a loading control. (E) Three subpopulations of MCF10DCIS cells after sorting were incubated with vehicle control or BXL0124 (10 nM) for 72 h. The cell proliferation rates were determined by [³H] thymidine incorporation assay. Two separate experiments, each with triplicates, were conducted (**p < 0.01).

Figure 3. Inhibition of Notch signaling by BXL0124 in MCF10DCIS cells

(A) MCF10DCIS cells were treated with vehicle control or given doses of 1α,25(OH)₂D₃ or BXL0124 for 24 h. The protein levels of indicated molecules were examined by Western blot analysis, with β-actin used as a loading control. (B) MCF10DCIS cells were treated with vehicle control or BXL0124 (10 nM) for given time periods. (C) MCF10DCIS cells were treated with vehicle control or BXL0124 (10 nM) for 24 h. Nuclear localization of c-Notch1 was determined by staining MCF10DCIS cells with antibody against c-Notch1 (Green). Nuclei were stained with TO-PRO-3 (blue). (D) MCF10DCIS cells were treated with vehicle control or BXL0124 (10 nM) for 24 h. The protein levels of indicated molecules in cytoplasm or in nucleus were examined by Western blot analysis. β-Actin and lamin A were used as loading controls for cytoplasm and nucleus fractions, respectively. (E) MCF10DCIS cells were transfected with non-targeting siRNA (Neg siRNA) or VDR-targeting siRNA (VDR siRNA) and treated with BXL0124 (10 nM) for 24 h.

Figure 4. Regulation of mRNA levels of Notch signaling molecules by BXL0124

MCF10DCIS cells were treated with vehicle control or BXL0124 (10 nM) for given time periods. The mRNA expression levels of (A) Notch receptors, (B) Notch ligands and (C) downstream targets of Notch signaling were determined. Three separate experiments, each with duplicates, were conducted (* $p < 0.05$, ** $p < 0.01$).

Figure 5. Involvement of HES1 in the inhibition of Notch signaling by BXL0124

MCF10DCIS cells were treated with vehicle control or BXL0124 (10 nM) for given time periods. (A) The mRNA expression levels of HES1 were determined. Three separate experiments, each with duplicates, were conducted (** $p < 0.01$). (B) The protein levels of HES1 were examined by Western blot analysis, with β -actin used as a loading control. (C) MCF10DCIS cells were incubated without siRNA (w/o siRNA), with non-targeting siRNA (Neg siRNA) or with HES1-targeting siRNA (HES1 siRNA), and treated with BXL0124 (10 nM) for 24 h.

Figure 6. Inhibition of Notch signaling by HES1 overexpression

(A) MCF10DCIS cells were transduced with GFP-labeled HES1-expressing vectors and sorted to GFP-negative control cells (DCIS) and GFP-positive HES1-overexpressing cells (DCIS-HES1). DCIS-HES1 cells were checked again for stable maintenance of HES1-expressing vectors after four passages. (B) The mRNA levels of HES1 in DCIS and DCIS-HES1 cells were determined. Three separate experiments, each with duplicates, were conducted (** $p < 0.01$). (C) The protein levels of indicated molecules were examined by Western blot analysis, with β -actin used as a loading control. (D) The mRNA levels of indicated molecules in DCIS and DCIS-HES1 cells were determined. Three separate experiments, each with duplicates, were conducted (** $p < 0.01$).

Figure 7. Reduction of CD44⁺/CD24^{-low} subpopulation and cell proliferation rate by HES1 overexpression

(A) DCIS (GFP-negative) and DCIS-HES1 (GFP-positive) cells were stained with antibodies against CD44 or CD24, and the number of cells expressing CD44, CD24 or GFP was determined by flow cytometry. (B) After double staining with CD44 and CD24 antibodies, the number of cells for CD44⁺/CD24⁻, CD44⁺/CD24^{low} and CD44⁺/CD24^{high} subpopulations was determined in DCIS and DCIS-HES1 cells by flow cytometry. The average percentage of CD44⁺/CD24^{-low} combined subpopulation and CD44⁺/CD24^{high} subpopulation from three independent experiments was shown in the graph. (C) DCIS and DCIS-HES1 cells were grown for 24, 48 and 72 h. Cell proliferation rates were determined by the MTT assay. Three separate experiments, each with quadruplicates, were conducted (**p < 0.01). (D) A schematic diagram for the inhibition of Notch1 signaling and CD44⁺/CD24^{-low} subpopulation by BXL0124.