The Histone Deacetylase Inhibitor Abexinostat Induces Cancer Stem Cells Differentiation in Breast Cancer with Low Xist Expression

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Abstract

Purpose: Cancer stem cells (CSC) are the tumorogenic cell population that has been shown to sustain tumor growth and to resist conventional therapies. The purpose of this study was to evaluate the potential of histone deacetylase inhibitors (HDACi) as anti-CSC therapies.

Experimental Design: We evaluated the effect of the HDACi compound abexinostat on CSCs from 16 breast cancer cell lines (BCL) using ALDEFLUOR assay and tumoursphere formation. We performed gene expression profiling to identify biomarkers predicting drug response to abexinostat. Then, we used patient-derived xenograft (PDX) to confirm, in vivo, abexinostat treatment effect on breast CSCs according to the identified biomarkers.

Results: We identified two drug-response profiles to abexinostat in BCLs. Abexinostat induced CSC differentiation in low-dose sensitive BCLs, whereas it did not have any effect on the CSC population from high-dose sensitive BCLs. Using gene expression profiling, we identified the long noncoding RNA Xist (X-inactive specific transcript) as a biomarker predicting BCL response to HDACi. We validated that low Xist expression predicts drug response in PDXs associated with a significant reduction of the breast CSC population.

Conclusions: Our study opens promising perspectives for the use of HDACi as a differentiation therapy targeting the breast CSCs and identified a biomarker to select patients with breast cancer susceptible to responding to this treatment. Clin Cancer Res; 19(23); 6520–31. ©2013 AACR.

Introduction

Acetylation of histone proteins controls transcription and regulation of genes involved in cell-cycle control, proliferation, DNA repair and differentiation (1, 2). Unsurprisingly, the expression of histone deacetylases (HDAC) is frequently altered in several malignancies (3), including breast cancer, and pharmacologic inhibitors (histone deacetylase inhibitor; HDACi) have been proposed as an alternate therapy to conventional therapeutics in solid malignancies.

Resistance to conventional therapeutic agents in cancer may be sustained by a fraction of cancer cells within the tumor, the cancer stem cells (CSC), which are able to self-renew and differentiate, giving rise to the bulk of the tumor (4). In breast cancer, in particular, this population has been shown to resist to conventional chemotherapy and radiation, suggesting that it will be imperative to target all CSC subsets within the tumor to prevent relapse and metastasis (5). Different features of CSCs have been explored in recent targeting strategies including quiescence, self-renewal, or radioresistance pathways (6). It has been demonstrated that inhibition of key signaling pathways involved in breast CSCs self-renewal reduces breast tumorigenesis and metastasis (6). Among the different anti-CSC therapeutic strategies recently developed, differentiation therapy using “epidrugs” remains poorly explored in solid tumors. Differentiation therapy aims at favoring differentiation over self-renewal programs in CSCs, inducing a depletion of the CSC population (7). Whether HDACis could influence CSCs fate remains unknown. However, during normal differentiation, the chromatin structure of stem cells undergoes major epigenetic modifications (8) with histone acetylation, which has been proposed to play a fundamental role in the control of cell-fate choice (9). The balance...
Translational Relevance

Although the overall mortality for breast cancer has recently been declining, the survival of patients with recurrent or metastatic disease has not changed significantly over the past decades. Targeting the tumorigenic cancer stem cell (CSC) population is a prerequisite to improve breast cancer treatment. Among the different anti-CSC therapeutic strategies recently developed, differentiation therapy using “epidrugs” remains poorly explored in solid tumors. In this study, we demonstrate that the histone deacetylase inhibitor (HDACi) abexinostat may be used to induce differentiation of breast CSCs. Moreover, we identify a biomarker (Xist expression) that predicts tumor response to abexinostat treatment. Thus, the use of epidrugs such as HDACi may be an effective therapeutic approach to treat breast tumor with low Xist expression.

Results

Treatment with histone deacetylase inhibitor defines two drug-response profiles in breast cancer cell lines

A series of 16 BCLs representing the molecular diversity of breast cancers were exposed for 72 hours to increasing concentrations (150 nmol/L–2.5 μM) of the HDACi abexinostat (Supplementary Table S1). According to the Gaussian Mixture Model (GMM) analysis, nine BCLs were classified as sensitive to low dose of abexinostat with an IC50 comprised between 170 and 460 nmol/L, whereas seven were classified as sensitive to high dose with an IC50 superior to 700 nmol/L (IC50 range: 715–1,650 nmol/L; Fig. 1 and Supplementary Fig. S1). These two drug-response profiles were similar when using other HDACi compounds (SAHA and valproic acid; Supplementary Fig. S2). To explain this differential response to HDACi treatment, we grouped the BCLs according to their molecular features. None of the molecular parameters tested could predict response to HDACi (Supplementary Fig. S3). To exclude an indirect cytotoxic effect of HDACi treatment, we compared our abexinostat response profile with the one of docetaxel, a conventional chemotherapeutic agent used to treat breast cancer. Abexinostat and docetaxel drug-response profiles were totally distinct, suggesting that HDACi treatment had a specific effect (Supplementary Fig. S3B). We measured histone deacetylase activity in our BCL series before and after the abexinostat treatment (Supplementary Fig. S4A and S4B). Intrinsic histone deacetylase activity was not correlated to BCL’s drug-response profile, (X-inactive specific transcript) as a potential biomarker predicting BCL response to HDACi. These results open promising perspectives for the use of HDACi as differentiation therapy targeting the CSC population of breast cancer with low Xist expression.
and both low-dose sensitive and high-dose sensitive BCLs presented a similar extinction of HDAC activity after treatment ($P < 0.01$; $t$ test). Moreover, abexinostat treatment induced a significant increase of acetylated proteins (histone H3 and $\alpha$-tubulin) after 24 hours of drug exposure (Supplementary Fig. S4C and S4D). Altogether, these results indicate that abexinostat treatment inhibits specifically HDAC activity in all BCLs tested, independently of their drug-response profile.

**Treated cells exhibit differential cell-cycle progression according to their drug-response profiles**

To determine whether drug-response profiles were dependent on apoptosis induction, we measured caspase-3/7 activation after abexinostat treatment. Surprisingly, low-dose sensitive BCLs did not present apoptosis induction, whereas high-dose sensitive BCLs did present an activation of caspase-3/7 after 48 hours of abexinostat treatment ($P < 0.01$; $t$ test; Fig. 2A). Because the abexinostat inhibitory effect observed in low-dose sensitive BCLs could not be explained by a massive cell death, we measured cell growth kinetic. As expected, the proliferation rate of high-dose sensitive BCLs decreased after 48 hours of drug exposition. Low-dose sensitive BCLs showed a transient stop in cell growth kinetic after 24 hours of treatment followed by a recovery of cell proliferation (Fig. 2B). These results suggest that abexinostat effect on low-dose sensitive BCLs may be due to a perturbation of cell-cycle progression, whereas it induced apoptosis in high-dose sensitive BCLs. To test this hypothesis, we analyzed the cell-cycle status of four BCLs (two low-dose sensitive: SK-BR-7, MDA-MB-231; two high-dose sensitive: MDA-MB-436, HCC1954) for different time points after abexinostat treatment at IC$_{50}$. Low-dose sensitive BCLs were transiently blocked in G$_1$–S phase after 24 hours ($P < 0.01$; $t$ test), whereas high-dose sensitive BCLs presented a G$_2$–M cell-cycle arrest ($P < 0.01$; $t$ test; Fig. 2C and D and Supplementary Fig. S5A and S5B). To confirm this result, we measured P21 and P27 protein expression.
using Western blot analysis (Supplementary Fig. S5C). Both proteins are checkpoint regulators of cell-cycle progression whose expression prevents G₁ to S phase transition. Western blot analysis showed that abexinostat induced P21 and P27 expression only in low-dose sensitive BCLs and in a transient fashion.

**Histone deacetylase inhibitors modulate breast cancer stem cells**

To explore whether drug-response profiles were related to an effect on the breast CSC population, we evaluated the CSC population on BCLs treated with HDACis with two different techniques, the ALDEFLUOR assay and the tumorsphere formation assay. We have previously demonstrated that BCLs contain populations with stem cell properties that can be isolated upon their aldehyde dehydrogenase activity as assessed by the ALDEFLUOR assay (18). Moreover, the capacity to generate a colony in nonadherent culture conditions (tumorsphere) has been shown to be an intrinsic property of CSCs (19). Seven BCLs (three low-dose sensitive: SUM149, SUM159, SK-BR-7; four high-dose sensitive: BrCa-MZ-01, S68, MDA-MB-436, HCC1954) were treated for 72 hours with abexinostat (IC₅₀). For each low-dose sensitive BCL tested, we observed a decrease of the CSC population with twice less ALDEFLUOR-positive cells and tumorspheres formed after treatment ($P < 0.05$; t test; Fig. 3). Conversely, high-dose sensitive BCLs treated with abexinostat presented a moderate increase of the ALDEFLUOR-positive population and no effect on tumorsphere formation. Similar results were observed using other HDACi compounds (Supplementary Fig. S6; $P < 0.05$; t test).

Abexinostat treatment induces CSC differentiation in low-dose sensitive BCLs

HDACi treatment may affect the breast CSC population through the induction of maturation process (10, 15). Therefore, we studied the protein expression of different differentiation markers by immunofluorescence, including CK5/6 and CK14 (basal markers), vimentin and E-cadherin (mesenchymal markers), and CK8/18 (luminal marker). Observed by optical microscopy, low-dose sensitive BCLs treated with abexinostat exhibited important morphologic changes with cells increased in size and with a decreased nuclear/cytoplasmic ratio. Also, treated cells flattened and generated intercellular digitations and bridges. Figure 4A shows newly formed cell clusters after abexinostat treatment. These morphologic changes were accompanied by a modification of phenotypic profiles. All BCLs analyzed presented a strong overexpression of the luminal marker

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**Figure 3.** The CSC population is differentially modulated according to abexinostat-response profile. A–D, the effect of abexinostat on the CSC population was assessed using ALDEFLUOR assay (A and B) and tumorsphere formation (C and D). Representative flow charts for ALDEFLUOR assay (A) and pictures of tumorspheres (C) are presented. ($n = 6$; *, $P < 0.05$) Error bars represent mean ± SD.
CK8/18 after HDACi treatment (Fig. 4B and Supplementary Table S2). The mesenchymal marker vimentin was lost in BCLs from the mesenchymal molecular subtype, and E-cadherin expression was induced in SK-BR-7 BCL, suggesting a reverse epithelial-to-mesenchymal transition. In the luminal BCL T47D, the small CK5/6-positive cell population, previously identified as containing the tumor-initiating cell population (23), was totally eradicated in treated cells. We also noted an induction of CK14 expression in MDA-MB-231 and SUM159 mesenchymal BCLs. In summary, our data suggest that HDACi treatment induces differentiation in low-dose sensitive BCLs, consistent with the decrease of the CSC population observed in these cell lines.

Expression of Xist IncRNA predicts drug response to abexinostat

Targeting breast CSCs is presented as a promising strategy to improve breast cancer treatment. Our findings suggest that abexinostat could be used as a novel therapeutic strategy for breast cancer through the induction of CSC differentiation. However, a biomarker is needed to predict drug response of patients with breast cancer. None of the classical molecular parameters tested could predict BCL drug response (Supplementary Fig. S2). We established and compared the gene expression profiles of low-dose and high-dose sensitive BCLs. We identified the overexpression of Xist IncRNA up to 139-fold ($P < 0.00001$, t test; FDR $q$-val: 0.03) in high-dose sensitive BCLs compared with low-dose sensitive BCLs (Fig. 5A). We validated the cDNA microarrays results by quantifying Xist expression for each BCL using quantitative real-time PCR (qRT-PCR). Xist expression level was significantly correlated between both techniques ($r = 0.84$ (0.58–0.95), $P = 8.7E-05$; Fig. 5B). We next confirmed that high-dose BCLs tended to be enriched in Xist$^{high}$ BCLs compared with low-dose sensitive BCLs ($P = 0.055$, Kruskal–Wallis rank-sum test; Fig. 5C). During the early steps of embryonic development, Xist randomly coats one X chromosome of females, allows the recruitment of chromatin modifiers, and reduces to silence an X chromosome over cell divisions. Consequently, the newly differentiated cell has one active (Xa) and one inactive (Xi) X
Several studies have reported genomic instability of X chromosomes (loss of Xi, duplications of Xa) and dysregulation of \textit{Xist} in breast, ovarian, cervical, prostate cancers, testicular germ cell tumors, and lymphoma (25, 26). To evaluate whether a variation in X chromosomes number was related to \textit{Xist} expression and drug response to abexinostat, we collected karyotype information for each BCL analyzed (Supplementary Table S3). We observed a strong correlation between X chromosomes number and drug response to abexinostat. Low-dose sensitive BCLs presented essentially X chromosome monosomy, whereas high-dose sensitive BCLs presented X chromosome normo- or polysomy ($P < 0.01$; $t$ test; Fig. 5D). Altogether these results suggest that \textit{Xist} IncRNA expression may be used as a biomarker to predict HDACi treatment effect on the breast CSC population.

\textbf{Abexinostat treatment reduces the CSC population in patient-derived xenografts with low \textit{Xist} expression}

To confirm the impact of abexinostat treatment on the CSC population from breast cancers with low \textit{Xist} expression, we utilized four different patient-derived xenografts (PDX) with distinct \textit{Xist} expression level (CRCM226X, CRCM392X, \textit{Xist}\textsuperscript{low}; CRCM389X, \textit{Xist}\textsuperscript{high}; Supplementary Fig. S8). Cells from these PDXs were transplanted orthotopically into fat pads of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Using these models, we previously demonstrated that the CSCs were contained in the ALDEFLUOR-positive population (27). We injected single cancer cells into fat pads of NOD/SCID mice and monitored tumor growth. When the tumor size was approximately 150 mm$^3$, we started treatment with abexinostat or docetaxel. Tumor growth was compared with that of placebo-treated controls. Docetaxel and abexinostat treatment had no or limited effect on PDXs growth (Fig. 6A). After 3 weeks of treatment, the animals were sacrificed and the proportion of ALDEFLUOR-positive CSCs was measured in each residual tumor (Fig. 6B). All PDX models presented an increase in the ALDEFLUOR-positive population isolated from docetaxel-treated tumors compared with the untreated control, in agreement with previous reports that described enrichment in the CSC population in residual tumors treated with conventional chemotherapy (28). In contrast, only PDXs with a low or medium \textit{Xist} expression treated with abexinostat presented a two-fold decrease of the ALDEFLUOR-positive population.
whereas abexinostat treatment induced an increase of the ALDEFLUOR-positive population of CRCM389X (Xisthigh). To functionally prove the reduction of the CSC population in the abexinostat-treated tumors with low Xist expression, we determined the ability of treated cells to form tumors in vivo by reimplanting cells from treated PDXs into secondary mice. Tumorigenicity is directly related to the presence of CSCs and this assay gives an estimate of the proportion of residual tumorigenic CSCs. For each treatment condition (placebo, abexinostat, docetaxel), 1,000 cells isolated from treated tumors were reimplanted. Cells isolated from abexinostat-treated PDXs showed an incapacity to regenerate a tumor for CRCM311X (Xistlow) and CRCM226X (Xistlow), and CRCM389X (Xisthigh; n = 4). In sharp contrast, cells isolated from docetaxel-treated tumors showed a tumor regrowth comparable with placebo-treated tumors. Interestingly, for CRCM389X (Xisthigh), cells isolated from abexinostat-treated tumors presented a higher regrowth kinetic compared with cells isolated from docetaxel- and placebo-treated tumors (Fig. 6C). These results suggest that abexinostat treatment targets the CSC population in vivo and this effect is inversely correlated to Xist expression.

Discussion

Targeting CSCs within a tumor might be critical to prevent relapse and metastasis (5). CSC biology, such as expression of self-renewal and differentiation programs, is governed by epigenetic regulation (9). Thus, epigenetic
modulation using chromatin modifiers appears as an encouraging means to control CSC fate. The rationale for differentiation therapy is to disturb the balance between self-renewal and differentiation programs. Both inhibiting self-renewal and promoting differentiation would deplete the CSC pool and allow more differentiated tumor cells to be targeted by conventional treatments.

We observed two different response profiles to HDACi in BCLs. These profiles were associated with opposite effects on the breast CSC population. On one hand, the CSC population was decreased in low-dose sensitive BCLs in association with a cellular differentiation, suggesting that CSC decrease was mediated through the induction of a mesenchymal-to-epithelial transition. In addition, cell-cycle progression was transiently stopped with an accumulation in G1 cell-cycle phase. This checkpoint before entering S phase, also called R point, has been defined as an important cell-cycle stage controlling stem cell fate allowing equilibrium between self-renewal and committed cell fate decision (29, 30). On the other hand, high-dose sensitive BCLs presented apoptosis induction with an accumulation of cells in G2–M cell-cycle phase explaining cytotoxicity. As observed, when cancer cells are treated with cytotoxic agents, the CSC population in high-dose sensitive BCLs was not depleted after HDACi treatment. Altogether, these results suggested that modulation of histone acetylation of breast CSCs is able to alter their proportion. Interestingly, it has been shown that HDACi may promote either self-renewal or differentiation of embryonic stem cells depending on the "stem cell status" and dose used (31). Moreover, this opposite effect of HDACi treatment was previously observed in different malignant diseases where HDACi was described either as a differentiating agent (11–15, 32–34) or as an oncogenic factor promoting tumor growth and metastasis formation (35–37). Recently, a study screened 30 human epithelial cancer cell lines (comprising breast, liver, gastric, and lung cancer) for their HDACi sensitivity and reported two drug-response profiles with dramatic opposite effects: 13 out of the 30 cell lines presented increased cell migration and metastasis formation, whereas cell migration was inhibited in a dose-dependent manner in the other 17 cell lines (37). This dose-dependent dual effect of HDACi may be extended to other epipodugs and particularly to demethylating agents. Indeed, a low dose of decitabine on epithelial (breast and colon) and leukemic cancer cells had no immediate toxicity, induced memory response with cell differentiation and CSC depletion in serially transplanted mice, but a high dose triggered rapid DNA damages and cytotoxicity (38). If the molecular reason explaining the dual effect of epipodug treatment is unclear, we can postulate that the abexinostat effect is mediated at the cellular level through the modulation of the CSC pool.

A direct consequence of these observations is the need for reliable biomarkers predicting response to HDACi treatment to identify patients likely to benefit from these drugs. Because none of the conventional parameters tested were able to predict HDACi response in BCLs, we performed a gene expression analysis between our two BCL groups. This analysis revealed a differential expression of the IncRNA Xist with an overexpression of Xist in high-dose sensitive BCLs. Xist is responsible for X dosage compensation of X genes between males and females (24). Normally, X inactivation is initiated in early embryogenesis but recent reports identified instances where Xist is expressed and can initiate gene repression. A wider link between X chromosome inactivation and oncogenesis has been made in a number of studies observing a gain or loss of X chromosomes in tumor cells (25, 26). In our series of BCLs, Xist expression was correlated with X chromosome number ($P < 0.01$). We observed low Xist expression in BCLs with X mono- or disomy, whereas Xist overexpression was associated with X polysomy. Our results suggest that Xist expression may be used as a predictive biomarker for effectiveness of HDACi treatment through CSC differentiation. We confirmed, in vivo, this hypothesis by using PDX with distinct Xist expression. Only the PDX with low Xist expression displayed a significant decrease of its CSC population after abexinostat treatment, whereas HDACi treatment induced an increase of the CSC population in PDX with high Xist expression.

Why tumors with a low expression of Xist are hypersensitive to HDACis is not clear and need further investigation! Interestingly, Xist has recently been described as a predictive biomarker of response to cisplatin treatment in BRCA1-defective breast cancers (39). The authors proposed that low Xist expression may be a flag for genomic instability. Indeed, loss of X is the main cause explaining low Xist transcript level. Moreover, BRCA1-defective cells present chromosome segregation errors due to compromised spindle checkpoint (40). Consequently, BRCA1-deficient cancer cells are sensitive to treatment inducing DNA damage, and Xist expression would be a surrogate marker of DNA repair defect. Interestingly, it has been shown that HDAC enzymes are critically important to enable functional homologous recombination (HR) by controlling the expression of the RAD51 gene and promoting the proper assembly of HR-directed subnuclear foci (41). Thus, HDACi may favor DNA damage in cancer cells with an important genomic instability such as cells presenting a low Xist gene expression. If this hypothesis can explain the low-dose sensitivity of BCLs with low Xist expression, it cannot explain the effect of HDACi treatment on the breast CSC population of these cell lines. One molecular mechanism debated for the role of Xist in tumorigenesis is its interaction with BRCA1 protein (42–46). Because BRCA1 has showed important role in the regulation of breast stem cell biology (47, 48), we can hypothesize that BRCA1 pathway is differentially regulated under HDACi treatment between low-dose and high-dose sensitive BCLs. Further studies are needed to decipher the precise underlying mechanism.

In conclusion, our study identifies for the first time a biomarker predicting breast cancer response to HDACi. It points out a lasting benefit of Xist low-expressing breast tumors treated with low-dose HDACi and the importance of epigenetic partners such as IncRNAs. IncRNAs said to be "dark matter" are more and more characterized (49) and increasing evidence imply them as critical in controlling...
stem cell fate (50) and oncogenesis (51). Recently it was demonstrated, using Xist-deficient mice, that Xist loss results in X reactivation and consequent genome-wide changes that lead to hematologic cancer through hematopoietic stem cell aberrant maturation (52).

Ultimately, deciphering the role of lncRNAs in cancer biology will help improve cancer understanding and treatment.

Materials and Methods

Ethics statement

Use of anonymous human tissue samples was exempted from Institutional Review Board. Animal studies were approved by the INSERM office (Marseille, France) for Laboratory Animal Medicine.

Cell lines

A total of 16 BCLs were used for the study. The characteristics of the BCLs were previously described (refs. 53–55; Supporting Information).

Drugs

BCLs were continuously treated for 72 hours in adherent conditions with HDACi: abexinostat (also known as S78454, CRA-024781, or PCI-17481; Servier), SAHA (Cayman) and Valproic Acid (Sigma). For the experiments, abexinostat was prepared in a 23.1 mmol/L stock solution, in dimethyl sulfoxide (DMSO; Sigma) and stored at −20°C. Valproic acid was prepared in a 1 mol/L stock solution in PBS (Gibco) and stored at 4°C. For experiments, cells were treated with respective IC50. BCLs were also continuously treated for 72 hours in adherent conditions with 5 μmol/L abexinostat derivatives, S78730 (carboxylic acid) and S78731 (amide). S78730 and S78731 were prepared in a 23.1 mmol/L stock solution in DMSO and stored at −20°C. DMSO or PBS were used as vehicle control (C < 0.1%).

Cell viability and proliferation

IC50 were evaluated using MTS assay (Promega) as described in the Supporting Information.

Histone deacetylase activity

The effect of abexinostat and abexinostat derivatives treatment on HDAC was assessed by measuring residual enzyme activity using HDAC-glow I/II assay (Promega). BCLs were plated in adherent conditions in 96-well plates at 10,000 cells per well. After 24 hours, cells were treated with abexinostat (respective IC50) or abexinostat derivatives (5 μmol/L) or vehicle, and 1 hour later, HDACs inhibition was measured according to manufacturer’s guidelines.

Immunoblotting

Cells were harvested in medium, washed in PBS, lysed in extraction buffer [1% v/v Triton X-100, 50 mmol/L Hepes, pH 7.1, 1 mmol/L EDTA, 1 mmol/L EGTA, 150 mmol/L NaCl, 100 mmol/L NaF, 1 mmol/L Na3VO4, one tablet of Complete inhibitor mix (Roche) per 25 mL buffer], and loaded onto SDS-PAGE. Blots were incubated with respective primary antibodies diluted in TBS and Tween20 (TBS; containing 0.1% Tween20 and 5% nonfat milk) and incubated overnight at 4°C. Then blots were washed, incubated with appropriate secondary antibodies (1/10,000; Dako), and detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Antibodies used were anti-acetylated histone 3 (1/1,000; AbCam), anti-acetylated α-tubulin (1/1,000; Sigma), anti-P21 (1/500; AbCam), anti-P27 (1/500; AbCam), anti-α-tubulin (1/2,000; Sigma).

Caspase activity assay

The effect of abexinostat treatment on apoptotic pathways was assessed by detecting caspase-3/7 activity using Caspase Glo 3/7 assay (Promega). The BCLs panel (excluding MCF7 that lacks functional caspase-3) was plated in adherent conditions in 96-well plates at 10,000 cells per well. After 24 hours, cells were treated with respective IC50 or vehicle. Caspase activity induction was measured 24, 48, and 72 hours later according to manufacturer’s guidelines.

Cell-cycle analysis

Briefly, supernatant and adherent cells were harvested, washed, and suspended in 0.5 mL medium containing propidium iodide (40 μg/mL) and RNase A (40 μg/mL). Analysis of the cell cycle was done on the LSR2 (BD Biosciences) using Diva analysis software.

ALDEFLUOR assay

The ALDEFLUOR Kit (Stem Cell Technologies) was used to isolate the population with high aldehyde dehydrogenase enzymatic activity using an LSR2 cytometer (Becton Dickinson Biosciences) as previously described (27).

Tumorsphere assay

BCLs were grown in adherent condition under abexinostat treatment (IC50) or vehicle for 72 hours, then seeded as single cells in ultra-low attachment plates (Corning) at low density (1,000 viable cells per mL). Tumorspheres were grown in a serum-free mammary epithelium basal medium. The capacity of cells to form tumorspheres was quantified under microscope. Experiments were done in triplicate.

H&E and immunofluorescence staining

BCLs monolayers grown on LabTech slides (Fisher Scientific) were fixed with 4% paraformaldehyde for 15 minutes at room temperature and stained as described in Supporting Information.

Gene expression profiling

RNA expression data were collected from our previous study (56) done with Affymetrix U133 Plus 2.0 human oligonucleotide microarrays. The data are deposited with ArrayExpress under the accession number E-MTAB-1693. We applied supervised analysis based on volcano plot analysis, where fold-change and statistical difference...
between groups were evaluated for each probe set. Probabilities were computed using linear models with empirical Bayes statistic included in the limma R package.

RNA extraction
RNA from BCLs or PDX was extracted using Mini Kit RNA extraction (Qiagen) following recommended instructions. RNA integrity was controlled by micro-analysis (Agilent).

Quantitative real-time PCR
Briefly, 5 μg of RNA extracted from BCLs or PDXs were reverse transcribed in accordance with manufacturer’s instruction (Superscript II reverse transcriptase, Invitrogen). Xist expression level was quantified using TaqMan probes (Hs01077163_m1; Applied biosystems). β-actin (Hs99999903_m1) and gyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs03929097_g1) expression were used for normalization of data. For Xist quantification in PDXs, normal breast cell line HME1 was used as control. Fold-change expression was calculated using the 2−ΔΔCt method.

Animal models
To explore the efficiency of abexinostat treatment on tumor growth, we utilized four primary human breast cancer xenografts (PDX) generated from 4 different patients (CRCM226X, CRCM311X, CRCM389X, CRCM392X). These PDXs were generated from chemo-naive breast tumors with two ER−PR−/ERBB2+ tumors (CRCM311X at the 5th passage and CRCM392X at the 3rd passage), an ER−PR−/ERBB2+ tumor (CRCM226X at the 5th passage), an ER+PR−/ERBB2+ tumor (CRCM389X at the 4th passage). For each PDX, cells from these PDXs were transplanted orthotopically into fat pads of NOD/SCID mice without cultivation in vitro. We injected 1,000,000 (CRCM226X, CRCM311X, CRCM389X) or 125,000 cells (CRCM392X) per fat pads of NOD/SCID mice (with two injected fat pads per mice) and monitored tumor growth. When tumor size was approximately 150 mm3, we initiated treatment with abexinostat alone (i.p., 12.5 mg/kg, twice a day, 5/7 days), docetaxel alone (i.p., 10 mg/kg, once a week; Sigma) or placebo injected with 20% cyclodextrin (i.p., twice a day, 5/7 days; Sigma), and 20% DMSO (i.p., once a week). Six mice (i.e., twelve tumors) were injected for each PDX and for each group. After 1, 2, and 3 weeks of treatment, two mice (i.e., four tumors) from each group were sacrificed according to ethic statements. Tumors were dissociated and cells were analyzed for the ALDEFLUOR phenotype. Cells from 3-week treated mice were reimplanted into two (CRCM226X) or four (CRCM311X) secondary NOD/SCID mice with injection of 1,000 cells for each treated tumor (i.e., four or eight injections per group).

Statistical analysis
Results are presented as the mean ± SD for at least three repeated individual experiments for each group. Statistical analyses used the R software. Correlations between sample groups and molecular parameters were calculated with the Fisher exact test or the t test for independent samples. The GMM was used to attribute BCLs to drug-response groups. Wilcoxon test for independent samples was used to compare different tumor sizes at different time points. The Pearson test was used to evaluate the correlation between Xist gene expression level measured by cDNA microarrays and qRT-PCR. The Kruskal–Wallis rank-sum test was used to compare repartition of Xistlow and Xishigh tumors in both BCL groups. P < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest
M.A. Salvador, J. Wicinski, O. Cabaud, Y. Toiron, E. Josselin, and C. Ginestier have commercial research grant from Institut de Recherches Internationale Servier. No potential conflicts of interest were disclosed by other authors.

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