The histone deacetylase inhibitor abexinostat induces cancer stem cells differentiation in breast cancer with low Xist expression

Marion A. Salvador\textsuperscript{1,3,4}, Julien Wicinski\textsuperscript{1,3,4}, Olivier Cabaud\textsuperscript{1,3,4}, Yves Toiron\textsuperscript{3,4,5}, Pascal Finetti\textsuperscript{1,3,4}, Emmanuelle Josselin\textsuperscript{1,3,4}, Hélène Lelièvre\textsuperscript{6}, Laurence Kraus-Berthier\textsuperscript{6}, Stéphane Depit\textsuperscript{6}, François Bertucci\textsuperscript{1,3,4}, Yves Collette\textsuperscript{3,4,5}, Daniel Birnbaum\textsuperscript{1,3,4}, Emmanuelle Charafe-Jauffret\textsuperscript{1,2,3,4}, Christophe Ginestier\textsuperscript{1,3,4}

1. Inserm, CRCM, U1068, Laboratoire d’Oncologie Moléculaire, Marseille, F-13009 France; 2. Institut Paoli-Calmettes, Département de Biopathologie, Marseille, F-13009 France; 3. Aix Marseille Université, F-13007, Marseille, France. 4. CNRS, CRCM, 7258, Marseille F-13009 France; 5. Inserm, CRCM, U1068, TrGET, Marseille, F-13009 France; 6. Institut de Recherches Internationales Servier, Paris, France.

Corresponding author:
Christophe Ginestier, CRCM, U1068 Inserm, Department of Molecular Oncology, 27 Bd Leï Roure, BP 30059, 13273 Marseille. Tel: 33 (0)4 91 22 35 09. Fax: 33 (0)4 91 22 35 44. Email: christophe.ginestier@inserm.fr

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Abstract

**Purpose:** Cancer stem cells (CSCs) are the tumorigenic cell population that has been shown to sustain tumor growth and to resist to 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 (BCLs) using ALDEFLUOR assay and tumorsphere 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 non-coding 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 breast cancer patients susceptible to respond to this treatment.
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 HDAC inhibitor 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 HDAC inhibitors may be an effective therapeutic approach to treat breast tumor with low Xist expression.
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 pharmacological inhibitors (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 (CSCs), 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 reduce 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 between histone acetylation/deacetylation by histone acetyltransferases (HAT)/HDACs is one of the main features of the “epigenetic memory” of a cell. Consequently, epidrug therapies modifying the histone code have been proposed for cancer treatment and more recently as potential anti-CSC therapies.

Forty years ago, the anti-cancer properties of HDACis were suggested through their capability to induce differentiation of erythroleukemia cells (10). To date, HDACis have been used as differentiation therapy in several hematological malignancies (11). More recently, HDACis were also reported to induce differentiation in
endometrial stromal sarcoma cells (12), in liver cancer cell lines (13) and in small cell lung cancer cells (14). Differentiation in breast cancer has been described in response to HDACi in different cell line models. Suberoylanilide hydroxamic acid (SAHA) treatment induced a complete differentiation of MCF-7 cells with the induction of milk fat globule protein (15). Several HDACis are being tested in clinical studies as single agents in several solid tumor malignancies (16). These compounds are only efficient at high concentrations. Thus, the effect of HDACis might at least in part be the results of non-specific side effects rather than the consequences of inhibiting HDAC per se. Furthermore, there is no existing biomarker able to predict HDACi efficiency. Both improving our knowledge of HDACi biology and testing more efficient HDACi compounds could greatly impact the therapy success.

We studied the effect of HDAC inhibition on the breast CSC population, using the broad spectrum HDACi abexinostat which has been developed to have a full pharmacological effect at a nanomolar range (17). We identified, in vitro and in vivo, two types of response to abexinostat with either an induction of CSC differentiation in low-dose sensitive BCLs or no effect on the CSC population from high-dose sensitive BCLs. Moreover, we identified the long non-coding RNA (lncRNA) Xist (X-inactive specific transcript) as a potential biomarker predicting BCL-response to HDAC inhibitor. These results open promising perspectives for the use of HDACi as differentiation therapy targeting the CSC population of breast cancer with low Xist expression.
Results

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

A series of 16 BCLs representing the molecular diversity of breast cancers were exposed for 72 hours to increasing concentrations (150nM–2.5μM) of the HDACi abexinostat (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 170nM and 460nM whereas seven were classified as sensitive to high-dose with an IC50 superior to 700nM (IC50s range: 715nM–1650 nM) (Figure 1, Figure S1). These two drug-response profiles were similar when using other HDACi compounds (SAHA and Valproic Acid) (Figure 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 (Figure S3). To exclude an indirect cytotoxic effect of HDACi treatment, we compared our abexinostat response profile to 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 (Figure S3B). We measured histone deacetylase activity in our BCL series before and after abexinostat treatment (Figure S4A, S4B). Intrinsic histone deacetylase activity was not correlated to BCL’s drug-response profile 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 α-Tubulin) after 24 hours of drug exposure (Figure S4C, 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; \textit{t test}) (\textbf{Figure 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 (\textbf{Figure 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 IC50. Low-dose sensitive BCLs were transiently blocked in G1/S phase after 24 hours (p<0.01; \textit{t test}) whereas high-dose sensitive BCLs presented a G2/M cell cycle arrest (p<0.01; \textit{t test}) (\textbf{Figure 2C}, 2D; \textbf{Figure S5A}, S5B). To confirm this result we measured P21 and P27 protein expression using western blot analysis (\textbf{Figure 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.

\textbf{Histone deacetylase inhibitors modulate breast cancer stem cells (CSCs)}

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 non-adherent 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 (IC50s). 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; \textit{t
test) (Figure 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 (Figure S6) (p<0.05; t test).

We next evaluated the effect of two abexinostat structurally-related compounds (S78730, carboxylic acid derivative; S78731, amide derivative) lacking HDAC inhibitory properties. This kind of approach has already been used with other HDACis to demonstrate the specificity of the anti-HDAC activity effect (20, 21, 22). Interestingly, both derivatives did not have any effect on cell growth or on CSC population (Figure S7). Altogether these results suggest that the proportion of breast CSCs is modulated by histone acetylation.

**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 morphological 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 morphological changes were accompanied by a modification of phenotypic profiles. All BCLs analyzed presented a strong overexpression of the luminal marker CK8/18 after HDACi treatment (Figure 4B, 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 (EMT). 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 HDAC inhibitor treatment induces differentiation in low-dose sensitive BCLs, consistent with the decrease of the CSC population observed in these cell lines.
Expression of Xist lncRNA 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 (Figure S2). We established and compared the gene expression profiles of low-dose and high-dose sensitive BCLs. We identified the overexpression of Xist IncRNA (long non-coding RNA) up to 139-fold (p<0.00001, t test; FDR qval: 0.03) in high-dose sensitive BCLs compared to low-dose sensitive BCLs (Figure 5A). We validated the cDNA microarrays results by quantifying Xist expression for each BCL using qRT-PCR. Xist expression level was significantly correlated between both techniques(r=0.84 [0.58-0.95], p=8.7E-05) (figure 5B). We next confirmed that high-dose BCLs tended to be enriched in Xist$^{\text{high}}$ BCLs compared to low-dose sensitive BCLs (p=0.055, Kruskal-Wallis Rank Sum test) (Figure 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 chromosome (24). Several studies have reported genomic instability of X chromosomes (loss of Xi, duplications of Xa) and dysregulation of 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 Xist expression and drug-response to abexinostat, we collected karyotype information for each BCL analyzed (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) (Figure 5D). Altogether these results suggest that Xist IncRNA expression may be used as a biomarker to predict HDACi treatment effect on the breast CSC population.

Abexinostat treatment reduces the CSC population in patient-derived xenografts (PDXs) with low Xist expression
To confirm the impact of abexinostat treatment on the CSC population from breast cancers with low Xist expression, we utilized four different patient-derived xenografts (PDXs) with distinct Xist expression level (CRCM226X, CRCM311X, Xist\textsuperscript{low}; CRCM392X, Xist\textsuperscript{med}; CRCM389X, Xist\textsuperscript{high}) (Figure S8). Cells from these PDXs were transplanted orthotopically into fat pads of 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\textsuperscript{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 (Figure 6A). After three weeks of treatment, the animals were sacrificed and the proportion of ALDEFLUOR-positive CSCs was measured in each residual tumor (Figure 6B). All PDX models presented an increase in the ALDEFLUOR-positive population isolated from docetaxel-treated tumors compared to 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 Xist expression treated with abexinostat presented a two-fold decrease of the ALDEFLUOR-positive population (p<0.05) whereas abexinostat treatment induced an increase of the ALDEFLUOR-positive population of CRCM389X (Xist\textsuperscript{high}). 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 reimplantating 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 and CRCM226X (Xist\textsuperscript{low}), and a delay in tumor regrowth for CRCM392X (Xist\textsuperscript{med}) compared to the cells isolated from placebo-treated tumors (p=0.02) (Figure 6C). In sharp contrast, cells isolated from docetaxel-treated tumors showed a tumor regrowth comparable to placebo-treated tumors. Interestingly, for CRCM389X (Xist\textsuperscript{high}), cells isolated from abexinostat-treated tumors presented a higher regrowth kinetic compared to cells isolated from...
docetaxel- and placebo-treated tumors (Figure 6C). These results suggest that abexinostat treatment targets the CSC population \textit{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 HDAC inhibitors in breast cancer cell lines. These profiles were associated with opposite effects on the breast CSC population. On the 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 demonstrated that HDACis may promote either self-renewal or differentiation of embryonic stem cells depending on the "stem cell status" and dose employed (31). Moreover, this opposite effect of HDACi treatment was previously observed in different malignant diseases where HDACi was described either as a differentiating agent (12, 32, 11, 15, 14, 33, 34, 13) or as an oncogenic factor promoting tumor growth and metastasis formation (35, 36, 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 HDAC inhibitors may be extended to other epidrugs 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 epidrug 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 lncRNA 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 patient-derived xenografts (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 Xi 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, 43, 44, 45, 46). Since 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 HDAC inhibitors. 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 long non-conding RNAs. LncRNAs 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 IncRNAs in cancer biology will help improve cancer understanding and treatment.
Material and methods

Ethics statement. Use of anonymous human tissue samples was exempted from institutional review board. Animal studies were approved by the Inserm office for Laboratory Animal Medicine.

Cell lines. A total of 16 BCLs were used for the study. The characteristic of the BCLs were previously described (53, 54, 55) (Supporting information).

Drugs. BCLs were continuously treated for 72h in adherent conditions with Histone Deacetylase Inhibitors (HDACi): abexinostat (also known as S78454, CRA-024781 or PCI-17481; Servier, Paris, France), SAHA (Cayman) and Valproic Acid (Sigma). For the experiments, abexinostat was prepared in a 23.1mM stock solution, SAHA in a 0.5M stock solution, in dimethyl sulfoxide (DMSO; Sigma) and stored at -20°C. Valproic acid was prepared in a 1M stock solution in phosphate buffered saline (PBS; Gibco) and stored at 4°C. For experiments, cells were treated with respective IC50s. BCLs were also continuously treated for 72h in adherent conditions with 5µM abexinostat derivatives, S78730 (carboxylic acid) and S78731 (amide). S78730 and S78731 were prepared in a 23.1mM stock solution in DMSO and stored at -20°C. DMSO or PBS were used as vehicle control (C<0.1%).

Cell viability and proliferation. Inhibitory concentrations 50 (IC50s) were evaluated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega) as described in the Supporting information.

Histone Deacetylase activity. The effect of abexinostat and abexinostat derivatives treatment on histone deacetylases (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 24h, cells were treated with abexinostat (respective IC50s) or abexinostat derivatives (5µM) or vehicle, and 1h 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 mM HEPES, pH7.1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 100 mM NaF, 1 mM Na3VO4, one tablet of Complete™ inhibitor mix
[Roche] per 25 mL buffer) and loaded onto SDS-polyacrylamide gels. Blots were incubated with respective primary antibodies diluted in tris-buffered saline and Tween20 (TBSt) (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 αTubulin (1/1,000; Sigma), anti-P21 (1/500; AbCam), anti-P27 (1/500; AbCam), anti-αTubulin (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) were plated in adherent conditions in 96-well plates at 10,000 cells per well. After 24h, cells were treated with respective IC50s or vehicle. Caspase activity induction was measured 24, 48 and 72h later according to manufacturer's guidelines.

Cell cycle analysis. Briefly, supernatant and adherent cells were harvested, washed, and suspended in 0.5mL medium containing propidium iodide (40µg/mL) and RNase A (40µg/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 (ALDH) enzymatic activity using an LSR2 cytometer (Becton Dickinson Biosciences) as previously described (27).

Tumorsphere assay. BCLs were grown in adherent condition under abexinostat treatment (IC50s) or vehicle for 72h, 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 LabTeck 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 was collected from our previous study (56) done with Affymetrix U133 Plus 2.0 human oligonucleotide microarrays. The data are deposited with Array Express 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 patient-derived xenografts was extracted using Mini Kit RNA extraction (Qiagen) following recommended instructions. RNA integrity was controled by micro-analysis (Agilent).

**Quantitative Real-Time PCR.** Briefly, 5µg 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 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 $2^{-\Delta\Delta Ct}$ method.

**Animal models.** To explore the efficiency of abexinostat treatment on tumor growth, we utilized four primary human breast cancer xenografts (Patient-derived xenograft, PDX) generated from 4 different patients (CRCM226X, CRCM311X, CRCM389X, CRCM392X). These PDXs were generated from chemo-naïve 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 150mm³, we initiated treatment with abexinostat alone (i.p., 12.5mg/Kg, twice a day, 5/7 days), docetaxel alone (i.p., 10mg/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 (ie, twelve tumors) were injected for each PDX and for each group. After 1, 2 and 3 weeks of treatment, two mice (ie, 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 (ie, 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’s exact test or the t-test for independent samples. The Gaussian mixture model (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 Xisthigh tumors in both BCL groups. A p-value <0.05 was considered significant.
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References


Figure legends

Figure 1. BCLs display two drug-response profiles to abexinostat. Cell viability was measured with an MTS assay after 72 hours of treatment for 16 BCLs (n=12). Dotted line allows determining Inhibitory Concentration 50 (IC50) for each BCL. BCLs sensitive to low-dose of abexinostat are represented with green curves; BCLs sensitive to high-dose of abexinostat are represented with red curves. Error bars represent mean ± SD.

Figure 2. Cell cycle progression was differentially altered by abexinostat according to drug-response profile. A. Measurement of apoptosis induction by measuring caspases 3/7 activation in BCLs panel (excluding MCF7) treated by abexinostat (n=3; *, p<0.01). B. Cell proliferation was estimated using MTS viability test and proliferation rate was calculated in 16 BCLs treated by abexinostat. (high-dose sensitive BCLs: red curve; low-dose sensitive BCLs: green curve) (n=3). C. Flow charts representing cell cycle distribution of a low-dose sensitive BCL (SK-BR-7) and an high-dose sensitive BCL (MDA-MB-436) along 72 hours of treatment. Quantifications of cells in each cell cycle phase are represented in D (n=3; *, p<0.01). Similar results are reported for MDA-MB-231 and HCC1954 BCLs (Figure S6). Error bars represent mean ± SD.

Figure 3. The cancer stem cells (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-B) and tumorsphere formation (C-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.

Figure 4. Abexinostat induces CSC differentiation in low-dose sensitive BCLs. A. Optical microscopy showed that morphology of cells of four representative low-dose sensitive BCLs changed after 72 hours of treatment and cells formed large cells clusters. B. Cell differentiation was monitored by measuring expression of differentiation markers using immunofluorescent staining (green staining); nuclei were counterstained using DAPI (blue staining). Abexinostat treatment induces a modification of phenotypic profile. Scale bar= 10µmeter
Figure 5. Long non-coding RNA Xist predicts response to abexinostat. A. Transcriptomic analysis of low-dose sensitive BCLs vs. high-dose sensitive BCLs. Results are plotted according to their gene differential expression between both BCLs groups (y axis) and their corresponding statistical significance (x axis). Xist IncRNA (red arrow) was the most differentially expressed gene between the two BCL populations with an overexpression up to 139-fold (p<0.00001; FDR qval: 0.03) in high-dose sensitive BCLs. B. BCLs are classified according to increasing Xist expression level measured by cDNA microarrays; face up to cDNA microarrays measurements histogram is presenting Xist expression level measured by qRT-PCR, R=0.84 ([0.58-0.95], p=8.7E-5) (n=3). C. Box plots represent Xist expression level in low-dose and high-dose sensitive BCLs measured by qRT-PCR. High-dose sensitive BCLs are significantly enriched in Xist\textsuperscript{high} BCLs compared to low-dose sensitive BCLs (n=3; p=0.055). D. Repartition of X chromosomes number/cell in both BCLs groups (*, p= 0.0023). Error bars represent mean ± SD.

Figure 6. Abexinostat targets the CSC population in Patient-Derived Xenografts (PDX) with low Xist expression. A. Tumor growth kinetic of PDX treated with docetaxel, abexinostat or placebo (arrow indicates beginning of treatment) is presented for CRCM311X (Xist\textsuperscript{low}), CRCM226X (Xist\textsuperscript{low}), CRCM392X (Xist\textsuperscript{med}), CRCM389X (Xist\textsuperscript{high}) (n=4). B. Evaluation of the ALDEFLUOR-positive population in all four PDXs after 3 weeks of treatment with abexinostat or docetaxel. Results are represented normalized with the proportion of ALDEFLUOR-positive cells in the placebo-treated tumors. (n=4; *, p<0.05) C. 3-weeks treated PDXs were reimplanted into new mice and tumor growth was monitored. Tumor cells isolated from abexinostat-treated tumors were unable to regenerate a tumor for CRCM311X (n=8; *, p=0.02) and CRCM226X (n=4; *, p=0.02) PDX compared to the cells isolated from placebo- or docetaxel-treated PDXs. Error bars represent mean ± SD.
Figure 2

**A**

Caspase 3/7 induction (normalized with vehicle)

- 24h
- 48h
- 72h

- Low-dose BCLs
- High-dose BCLs

**B**

Proliferation rate vs. Days after treatment

- Low-dose BCLs
- High-dose BCLs

**C**

SK-BR-7

- Vehicle
- 6h
- 24h
- 48h
- 72h

MDA-MB-436

- Vehicle
- 6h
- 24h
- 48h
- 72h

**D**

Number of treated cells normalized with vehicle

- SK-BR-7
- MDA-MB-436

Hours after treatment

- G0/G1
- S
- G2/M

* indicates significant differences.
Figure 3

A. Flow cytometry analysis showing the ALDEFLUOR population in treated BrCa-MZ-01 and BAA-A cells with DEAB, DMSO, and abexinostat.

B. Bar graph comparing ALDEFLUOR population normalized with vehicle in low-dose and high-dose BCLs.

C. Images of SUM159 cells treated with DMSO and abexinostat showing the effect on tumor formation.

D. Bar graph showing tumorspheres formed after treatment normalized with vehicle in low-dose and high-dose BCLs.
Figure 5

A

Gene expression (Fold change)

-Log 10 (p-value)

Xist

B

cDNA micro-arrays

qRT-PCR

ZR-75-30
MDA-MB-134
S68
SUM159
MDA-MB-436
SUM149
HCC1937
SK-BR-7
Hs578t
T47D
MDA-MB-231
BrCa-MZ-01
HCC1954
HCC1500
MDA-MB-361
MCF-7

Xist expression level (arbitrary unit)

R=0.84 [0.58-0.95]
p=8.7E-05

C

D

Xist expression (arbitrary unit)

#X chromosome

low-dose BCLs
high-dose BCLs

low-dose BCLs
high-dose BCLs

*
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Marion A Salvador, Julien Wicinski, Olivier Cabaud, et al.

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