HDAC Inhibitor Entinostat Restores Responsiveness of Letrozole-Resistant MCF-7Ca Xenografts to Aromatase Inhibitors through Modulation of Her-2

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Abstract

We previously showed that in innately resistant tumors, silencing of the estrogen receptor (ER) could be reversed by treatment with a histone deacetylase (HDAC) inhibitor, entinostat. Tumors were then responsive to aromatase inhibitor (AI) letrozole. Here, we investigated whether ER in the acquired letrozole-resistant tumors could be restored with entinostat. Ovariectomized athymic mice were inoculated with MCF-7Ca cells, supplemented with androstenedione (Δ4A), the aromatizable substrate. When the tumors reached about 300 mm3, the mice were treated with letrozole. After initial response to letrozole, the tumors eventually became resistant (doubled their initial volume). The mice then were grouped to receive letrozole, exemestane (250 mg/d), entinostat (50 mg/d), or the combination of entinostat with letrozole or exemestane for 26 weeks. The growth rates of tumors of mice treated with the combination of entinostat with letrozole or exemestane were significantly slower than with the single agent (P < 0.05). Analysis of the letrozole-resistant tumors showed entinostat increased ERα expression and aromatase activity but downregulated Her-2, p-Her-2, p-MAPK, and p-Akt. However, the mechanism of action of entinostat in reversing acquired resistance did not involve epigenetic silencing but rather included posttranslational as well as transcriptional modulation of Her-2. Entinostat treatment reduced the association of the Her-2 protein with HSP-90, possibly by reducing the stability of Her-2 protein. In addition, entinostat also reduced Her-2 mRNA levels and its stability. Our results suggest that the HDAC inhibitor may reverse letrozole resistance in cells and tumors by modulating Her-2 expression and activity.

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Introduction

Development of aromatase inhibitors (AI) has significantly improved the treatment outcome of hormone responsive post-menopausal breast cancer. However, not all tumors respond and some eventually acquire resistance. To study the mechanisms of resistance, we have developed a xenograft model that mimics post-menopausal hormone-responsive breast cancer (1–4). Ovariectomized athymic nude mice are inoculated with human hormone-responsive breast cancer cells (MCF-7) stably transfected with the human placental aromatase gene (MCF-7Ca refs. 2, 5, 6). Aromatase expressed in the tumor cells converts androstenedione (Δ4A) into estrogen and provides a non-ovarian source of estrogen, thus simulating the tumors of post-menopausal patients. Using this model, we have established that as a single-agent AI is better than tamoxifen in controlling tumor growth (7–10). Results obtained using this model have been confirmed by several clinical trials (11–15). We also observed that although AI letrozole provides longer control over tumor growth, tumors eventually began to grow and are resistant to further AI letrozole treatment. A cell line was isolated from these long-term letrozole-treated tumors and designated as LTLT-Ca (3, 4). These cells and the tumors had decreased expression of ERα and aromatase compared with parental MCF-7Ca cells. On the other hand, growth factor receptor Her-2 and downstream kinases such as MAPK and Akt were increased. Inhibition of Her-2 using trastuzumab (a humanized antibody against the extracellular domain of Her-2) to block the Her-2 pathway resulted in reversed resistance and restoration of sensitivity to estrogens, antiestrogens, and AIs (16).

Breast cancers that are innately resistant to AIs or antiestrogens lack expression of the ER protein, which is thought to be due to gene silencing (17). HDAC inhibitors have been shown to induce estrogen receptor (ER)α...
expression in an ERα-negative cell line such as MDA-MB-231 (18–20). We recently reported that ER-negative tumors pretreated with HDAC inhibitors such as entinostat became responsive to endocrine therapy with aromatase inhibitors (18). Thus, the entinostat + letrozole combination resulted in marked reduction of tumor growth compared with either entinostat or letrozole treatment alone. Analysis of tumors and cells (MDA-MB-231) revealed induction of ER and aromatase by entinostat. These results were consistent with the actions of entinostat to inhibit histone deacetylase and allow expression of ERα and aromatase, rendering the tumors sensitive to hormones and hormonal therapy. As the ERα and aromatase are reduced in letrozole-resistant tumors by long-term treatment with the drug, we hypothesized that entinostat may also increase ERα and aromatase in AI-resistant tumors by the same mechanism, HDAC inhibition at the ERα and aromatase promoters. However, the results of the current study show that in acquired resistance, entinostat acts by a different mechanism, which results in reduction of Her-2 protein and mRNA.

Several studies have shown that HDAC actions are not limited to histone modifications, as some members of HDAC family acetylate and modulate non-histone proteins, thereby regulating their stability and subcellular localization (21, 22). Hsp90 has been shown to be one of the targets of HDACs. HDAC6 has been shown to induce acetylation of hsp90 and lead to degradation of its client proteins such as Bcr-Abl (23). More recently, inhibition of HDAC1 has been shown to be responsible for inhibition of hsp90 activity, leading to degradation of client proteins such as FLT3 and DNMT1 (24, 25). Our results suggest that inhibition of Her-2 due to inhibition of hsp90 may be one of the mechanisms responsible for reversal of resistance to AIs in the xenograft model. It has been shown that HDAC inhibitors can also induce the decay of mature Her-2 transcript, leading to growth inhibition in Her-2–positive cell lines and tumors (26). In our model, we observed that entinostat treatment reduced the half-life of Her-2 mRNA, which could be another mechanism by which letrozole resistance is reversed. In summary, the mechanism by which entinostat reverses letrozole resistance is through modulation of Her-2.

Materials and Methods

Materials

Dulbecco’s Modified Eagle Medium (DMEM), modified improved minimum essential medium (IMEM), penicillin/streptomycin solution (10,000 IU each), 0.25% trypsin–1 mmol/L EDTA solution, Dulbecco’s PBS (DPBS), and geneticin (G418) were obtained from Invitrogen. Androstenedione (Δ4A) and Matrigel were obtained from Sigma Chemical Company. Antibodies against Her-2 and p-Her-2 were purchased from Millipore; antibodies against p-MAPK, MAPK, p-Erk-1, and p-p90RSK were purchased from Cell Signaling Technology. Antibodies against ERα and aromatase (CYP 19) were purchased from Santa Cruz Biotechnology. Radioactive ligand for aromatase assay 3H-Δ4A (23.5 Ci/mmol) was purchased from Perkin Elmer. MCF-7 human breast cancer cells stably transfected with the human aromatase gene (MCF-7Ca) were kindly provided by Dr. S. Chen (City of Hope, Duarte, CA; ref. 6).

Cell culture

MCF-7Ca cells were routinely cultured in DMEM supplemented with 5% FBS, 1% penicillin/streptomycin, and 700 μg/mL G418. LTLT-Ca cells were developed from MCF-7Ca cells as described earlier (3) from tumors of mice treated with letrozole for 56 weeks and cultured in steroid-depleted medium, containing 1 μmol/L of letrozole. Anastrozole-resistant (AnR) cells were developed as described earlier (27).

Cell line authentication

All cell lines used in this study were authenticated in August 2013, using CellCheck service [microsatellite marker (STR) analysis] provided by Idexx Radil. The cell lines were found to be identical to the genetic profile reported for the MCF-7 cell line (ATCC# HTB-22).

Tumor growth in ovariectomized female athymic nude mice

All animal studies were conducted according to the guidelines and approval of the Animal Care Committee of the University of Maryland, Baltimore, MD. Female ovariectomized (OVX) athymic nude mice 4 to 6 weeks of age were obtained from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed in a pathogen-free environment under controlled conditions of light and humidity; received food, and water ad libitum.

The tumor xenografts of MCF-7Ca cells were grown in the mice as previously described (2, 3, 28). Each mouse received subcutaneous inoculations in one site per flank with 100 μL of cell suspension containing about 2.5 × 10^7 cells/mL in Matrigel. The mice were injected daily with supplemental Δ4A (100 μg/d). Weekly tumor measurements and treatments began when the tumors reached about 300 mm^3. Mice were assigned to groups for treatment so that there was no statistically significant difference in tumor volume among the groups at the beginning of treatment. Letrozole and Δ4A for injection were prepared using 0.3% hydroxypropylcellulose (HPC) in 0.9% NaCl solution. Entinostat was prepared in 30% hydroxypropyl β-cyclodextrin (HPBC) solution to obtain the required concentration. Mice were then injected s.c. 5 times weekly with the indicated drugs [except entinostat was administered orally (po) with Δ4A being given sc]. The doses of entinostat (50 μg/d), letrozole (10 μg/d), and Δ4A (100 μg/d) used are as previously determined and reported (3, 18).

Western blotting

The protein extracts from tumor tissues were prepared by homogenizing the tissue in ice-cold DPBS containing...
protease and phosphatase inhibitors. Total 50 μg of protein from each sample was analyzed by SDS-PAGE as described previously (16, 18, 28, 29). Bands were quantitated by densitometry using ImageJ (NIH). The densitometric values are corrected for β-actin loading control and reported as fold change compared with control underneath each band. The blots shown are representative blot from at least 3 separate experiments (n = 3).

\[ n = \frac{\text{test value}}{\text{control value}} \times 100 \]

\( ^3\text{H}_2\text{O} \) release assay for aromatase activity measurement

For measuring aromatase activity in tumor samples, the tumors were homogenized in ice-cold DPBS containing protease and phosphatase inhibitors. The resulting homogenate was used for aromatase activity assay. The radiometric \(^3\text{H}_2\text{O} \) release assay was conducted as described previously (16, 18, 28–30) using \( [1-\beta^3\text{H}] \Delta^4 \text{A} \) as substrate in presence of molecular oxygen. The activity of the enzyme is corrected for protein concentration in the tumor homogenates.

Chromatin immunoprecipitation assay

For \textit{in vitro} chromatin immunoprecipitation (ChIP) assay, the treated cells were washed with DPBS and fixed with 1% formaldehyde/DPBS for 10 minutes at 37°C after which the cells were washed with ice-cold DPBS containing protease and phosphatase inhibitors. The cells were collected into 1 ml DPBS and pelleted by centrifugation at 6,000 rpm for 5 minutes at 4°C. The cell pellet was resuspended in nuclear lysis buffer (ChIP Kit, Millipore) and incubated on ice for 10 minutes. Samples were sonicated on ice for 10 × 10 second cycles, with 20 seconds pauses between each cycle. The sonicated samples were centrifuged at 14,000 rpm for 10 minutes at 4°C. Sonicated samples were diluted 1:10 with dilution buffer (ChIP kit) before being immunocleared in a solution containing of Protein A or G Sepharose slurry-salmon sperm DNA for 2 hours at 4°C. Immunocleared supernatants incubated overnight at 4°C with pan-acetyl H3 (Millipore) and total H3 antibody (Cell Signaling Technologies). Protein A or G Sepharose beads and salmon sperm DNA were then added and incubated for 1 hour at 4°C. The beads were then washed sequentially with 1 mL each of wash buffers. The protein–DNA complexes were then eluted by twice incubating beads in elution buffer for 10 minutes at room temperature with vigorous mixing. To separate immunoprecipitated protein and DNA, the pooled elutes were incubated at 65°C overnight. The DNA was purified using the Qiaquick PCR Purification Kit (Qiagen).

The yield of target region DNA in each sample after ChIP was analyzed by conventional PCR and real-time quantitative PCR as described earlier (16, 31). The promoter that was analyzed was I.3/I.2, which is the main aromatase promoter used in breast cancer cells lines such as MCF-7 (16, 32) and thus measures the effect of entinostat and trastuzumab on endogenous aromatase in MCF-7 cells. The MCF-7Ca cells are transfected with human placental aromatase cDNA (placental CYP-19 uses promoter I.1 and I.2).

RNA extraction and reverse transcription and PCR

RNA was extracted and purified using the RNeasy Mini Kit (Qiagen) as per manufacturer’s protocol. Analysis of ERα, aromatase (CYP-19), pS2, progesterone receptor (PgR), and Her-2 mRNA expression was carried out by real-time quantitative reverse transcription PCR (RT-qPCR) as described earlier (16, 31) using a Bio-Rad CFX Connect real time system software. Values were normalized to corresponding 18S rRNA values and expressed as the fold increase relative to control.

Coimmunoprecipitation

The association between proteins was measured with coimmunoprecipitation (co-IP). Cell lysates were pre-cleared with either Protein A or G agarose beads. The lysates were then incubated with primary antibody as per manufacturer’s protocol. Next, 30 μL of agarose beads (Millipore) were added and the samples were incubated at room temperature for 1 hour with rotation. The immunocomplex bound beads were precipitated by centrifugation, washed with TBS (with 0.02% Tween 20) 3 times. The immunocomplex was eluted from the beads by adding Laemmli Sample Buffer (Bio-Rad) and boiling for 5 minutes at 100°C. The IP complex was analyzed by Western blotting.

Statistics

For \textit{in vivo} studies, mixed-effects models were used. The tumor volumes were analyzed with S-PLUS (7.0; Insightful Corp.) to estimate and compare an exponential parameter (\( \hat{g} \)) controlling the growth rate for each treatment groups. The original values for tumor volumes were log-transformed. The spline model with a single knot at time = week 15 was used to accommodate the nonlinearity with a piecewise linear model (16, 18, 28, 29). For \textit{in vitro} studies, Western blot analyses and IP were conducted at least 3 times and a representative blot is shown. For real-time RT-PCR studies, the fold-change values were analyzed using one-way ANOVA with Tukey–Kramer multiple comparison test. All \( P \) values less than 0.05 were considered statistically significant. The graphs are represented as mean ± SEM.
Results

Treatment of letrozole-resistant MCF-7Ca xenografts with the combination of entinostat and letrozole or exemestane

To examine whether the mechanism of acquired resistance to letrozole was also due to gene silencing, we used the MCF-7Ca xenograft model. MCF-7Ca xenografts were grown as previously described (3, 16, 28, 29). We inoculated ovariectomized athymic nude mice with MCF-7Ca (engineered to express aromatase) cells. All the mice received Δ^4A supplement, which was converted to estrogen by aromatase in the tumor cells. This provides a non-ovarian source of estrogen that stimulates tumor growth. When the tumors reached about 300 mm^3, the mice were divided into 2 groups such that the average tumor volume was not significantly different between the 2 groups (P = 0.93).

One group (control, n = 10) received only supplemental Δ^4A (100 μg/d) and the second group (n = 50) received Δ^4A (100 μg/d) plus letrozole (10 μg/d). The mice were treated with letrozole for 16 weeks. During this time, the tumors regressed but eventually began to grow. Over the first 10 weeks of treatment, the growth rate of tumors in the letrozole-treated group was significantly slower than that of the control group (P < 0.0001). The tumor volumes of the control and letrozole groups were also significantly different at week 10 (P = 0.01). However, the tumors of letrozole-treated mice then grew and had doubled in size by week 16. Previously, we have shown that tumors adapt to low-estrogen environment that ensues upon letrozole treatment by upregulating alternative signaling pathways such as Her-2. During this time, the tumors first regress and then start to regrow, becoming resistant as they continue to grow in the presence of the drug (3, 28, 33). At this time, the mice were grouped to receive second line treatment with entinostat 50 μg/d, exemestane 250 μg/d, entinostat + exemestane or entinostat + letrozole and one group was continued on letrozole (n = 10 each). The average tumor volumes at the start of second-line treatment were not statistically significantly different among the groups (P values for pairwise comparisons ranging from 0.35 to 0.89). The mice were treated till week 26. The addition of entinostat to letrozole or exemestane treatment significantly reduced the growth rate of tumors compared with each agent alone (P = 0.009 entinostat vs. exemestane; P = 0.048 entinostat vs. entinostat + letrozole) or continued letrozole treatment (P < 0.0001 letrozole vs. entinostat + letrozole). As shown in Fig. 1A, the combination of entinostat with letrozole or exemestane was significantly better in inhibiting tumor growth than in single agents. This suggested that entinostat overcame the acquired resistance of tumors to AIs (Fig. 1A).

HDAC inhibitor entinostat upregulates ERα and aromatase while reducing Her-2/MAPK pathway activation in letrozole-resistant tumors

Tumors of the mice shown in Fig. 1A were examined for changes in protein expression. Consistent with our previous finding, tumors of the mice treated with letrozole exhibited downregulation of ERα and aromatase along with upregulation of Her-2 and MAPK. When treated with ENT, Her-2, p-Her-2, p-MAPK, p-c-Raf, p-MEK1/2, and p-Akt were downregulated along with increase in ERα and aromatase protein expression (Fig. 1B). These changes were seen in tumors of mice treated with ENT alone and in combination with letrozole or exemestane. Furthermore, aromatase activity in the tumors of mice treated with entinostat was significantly greater than those treated with letrozole (P < 0.01) or control (P < 0.05; Fig. 1C).

HDAC inhibitor entinostat increases aromatase expression in an estrogen-dependent manner

Letrozole-resistant tumors of mice treated with entinostat (supplemented with aromatizable Δ^4A), exhibited about 3.5-fold increase in aromatase activity (Fig. 1C) and 1.7-fold increase in aromatase protein expression (letrozole vs. letrozole to entinostat; Fig. 1B). To elucidate the mechanism of upregulation of aromatase, we conducted an in vitro ChIP assay using LTLT-Ca cells. To examine whether the promoter region of the ERα and aromatase (CYP-19) genes were activated following entinostat treatment, we immunoprecipitated chromatin using acetyl histone H3 antibody. The aromatase promoter that was analyzed was L3/II, which is the endogenous aromatase promoter in breast cancer cell lines such as MCF-7 (32). The MCF-7Ca cells are transfected with human placental aromatase cDNA (placental CYP-19 uses promoter I.1 and I.2) with a β-actin promoter. (6, 34). It is known that HDAC inhibitors upregulate silenced genes by acetylating histones to allow transcription. However, in LTLT-Ca cells, ERα promoter was active to the same level as that in the MCF-7Ca cells (Fig. 2). Furthermore, treatment with entinostat alone did not cause any change in promoter activation. However, when supplemented with E2 or Δ^4A, entinostat treatment caused an increase in CYP-19 promoter activation. A similar finding was seen with activation of a known estrogen-inducible gene, pS2. This suggests that entinostat activates aromatase and pS2 in an estrogen-dependent manner. These effects were similar to those observed with trastuzumab (a humanized monoclonal antibody against Her-2) and consistent with our previous findings that inhibition of Her-2 results in increased ER expression (16). On the basis of these observations, we hypothesized that entinostat modulates Her-2, which in turn results in reversal of resistance to letrozole.

HDAC inhibitor entinostat induced ERα mRNA and protein expression in LTLT-Ca cells but not in MCF-7Ca cells

ENT exhibited differential effects in MCF-7Ca versus LTLT-Ca cells. ENT treatment did not change ERα mRNA expression in MCF-7Ca cells (data not shown) but caused reduction in ERα protein levels in MCF-7Ca cells (Fig. 3A, middle row, middle panel) and reduced ERα half-life (middle row right panel compared with middle row left
Her-2 Modulation by Entinostat Reverses Letrozole Resistance

A

MCF-7Ca xenografts

B

Control Letrozole Letrozole→exemestane Letrozole→entinostat Letrozole→ENT + letrozole Letrozole→ENT + exemestane

C

Aromatase activity (fmoles/mg of protein/h)

Her-2 p-Her-2 p-MAPK p-c-Raf p-MEK1/2 MAPK p-mTOR p-Akt CYP-19 ER β-Actin

1 2.1 0.3 0.6 0.8
1 3.2 1.8 0.8 0.3 0.7
1 77 45 34 6.8 7.9
1 1.8 1 0.5 1 0.6
1 1.3 1 0.6 0.8 1.2
1 1 1 1 1 1
1 3.5 2.2 2.5 2.5 0.7
1 13 1 1 5.3 1
1 0.5 1 1 0.9 0.9
1 0.1 0.3 0.8 0.7 0.8

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Her-2 mRNA was also significantly reduced in LTLT-Ca cells when treated with entinostat (Fig. 3B, top row, right panel compared with top row left panel).

Entinostat causes Her-2 degradation via the proteasomal pathway

To elucidate whether entinostat was degrading Her-2 via the proteasomes or the lysosomes, we conducted a rescue experiment with MG-132 (proteasomal inhibitor) or ammonium chloride (lysosomal inhibitor). LTLT-Ca cells were treated with entinostat in presence or absence of MG-132 or NH4Cl or the combination of MG-132 with NH4Cl and Her-2 protein levels were measured by Western blotting. Entinostat treatment reduced Her-2 protein; MG-132, NH4Cl, or the combination of MG-132 with NH4Cl reversed this reduction, although, MG-132 was more effective in this rescue. These results suggest that entinostat degrades Her-2 protein mainly via the proteasomal pathway but also via the lysosomal pathway (Fig. 4A). The results were also confirmed by flow cytometric analysis (Supplementary Fig. S1). This finding is consistent with entinostat treatment increasing the degradation of Her-2 protein and leading to its downregulation through increased ubiquitination (Supplementary Fig. S2). This suggests that posttranslational modifications could be responsible for the rapid effect of entinostat on the protein expression. Entinostat also reduced Her-2 protein levels and activation of its downstream signaling molecules (MAPK and Akt) in 2 other Her-2–positive cell lines, SKBr3 and BT474 (Fig. 4B). However, the reduction in Her-2 was modest in these cell lines, which could be due to the fact that both SKBr3 and BT474 cells have Her-2 gene amplification that is not seen in LTLT-Ca cells (28). On the other hand, 2 other drug-resistant cell lines, namely anastrozole-resistant (AnR) and exemestane-resistant (ExR) cells, showed marked reduction in Her-2 and downstream signaling pathway upon entinostat treatment (Fig. 4C).

Role of hsp90 in modulating Her-2 expression

The above results suggest that entinostat modulates Her-2 through posttranslational modulation. Her-2 is a known client protein maintained in the hsp90 chaperone complex. In addition, HDAC inhibitors are known to hyperacetylate hsp90, leading to degradation of its client proteins (23, 35). We investigated the association of hsp90 with Her-2 in the letrozole-resistant tumors treated with

HDAC inhibitor entinostat reduced Her-2 in LTLT-Ca cells

Treatment of LTLT-Ca cells with entinostat significantly reduced Her-2 protein expression (Fig. 3B, top row). Her-2 mRNA was also significantly reduced with entinostat (Fig. 3C). Entinostat treatment followed by E2, produced a further reduction in Her-2 mRNA (Fig. 3C). Entinostat alone does not cause activation of estrogen-responsive gene such as pS2. However, when followed by E2 treatment, pS2 mRNA is significantly increased (†P < 0.001). A similar trend is also observed in CYP-19 mRNA.

Figure 1. A, effect of entinostat (ENT) alone or in combination with letrozole or exemestane on the growth of letrozole-resistant MCF-7Ca xenografts. Ovariectomized athymic nude mice were inoculated with MCF-7Ca cells. Tumors were allowed to form in the presence of androstenedione (AD, androstenedione). The growth rates of tumors of mice treated with the combination of entinostat with letrozole or exemestane was significantly better than single agent alone (P = 0.0009 ENT vs. ENT + letrozole; P = 0.048 ENT vs. ENT + exemestane; P < 0.0001 ENT vs. ENT + letrozole). B, effect of entinostat alone or in combination with letrozole or exemestane on the tumor protein expression in letrozole-resistant MCF-7Ca xenografts. Protein expression in the tumors was examined by Western immunoblotting as described in Materials and Methods. Blots were probed for β-actin to verify equal loading. The numbers below the blots show densitometric values that are corrected for loading. C, effect of entinostat alone or in combination with letrozole or exemestane on the tumor aromatase activity in letrozole-resistant MCF-7Ca xenografts. The aromatase activity in the homogenized tumors was analyzed as described in Materials and Methods. Tumors of mice treated with letrozole for 15 weeks followed by entinostat (in presence of ΔA) have significantly higher aromatase activity than the letrozole–treated tumors (†P < 0.01) and control tumors (†P < 0.05).

**Figure 2.** ChiP analysis of LTLT-Ca cells to examine the activation of ERα, pS2, and aromatase (PI3/II) promoter. Activation of ERα and aromatase promoter was measured by immunoprecipitating chromatin-bound acetylated histone H3. Blot shows conventional PCR analysis and the numbers below the blot show real-time qPCR analysis. TRZ, trastuzumab; AD, androstenedione.
Entinostat in combination with letrozole or exemestane (Fig. 1A–C). There was increased association of hsp90 with Her-2 in letrozole-resistant tumors compared with control tumors. This association was reduced after treatment with entinostat alone or in combination with letrozole or exemestane (Fig. 5A). Although treatment with...
Entinostat alone caused a marked decrease in Her-2-hsp90 association, the resulting increase in ERα and aromatase may be responsible for lower efficacy of the single agent in inhibiting tumor growth. As such, inhibition of both ERα and Her-2 pathways are required for enhanced inhibition of tumor growth (16). The increased association of Her-2 with hsp90 was also observed in LTLT-Ca cells compared with parental MCF-7Ca cells in vitro (Fig. 5B). In addition, LTLT-Ca cells have a higher level of hsp90 than MCF-7Ca cells (Fig. 5C). Similar results were also obtained in anastrozole (AnR) and exemestane-resistant (ExR) cells (Fig. 5D). These results suggest that entinostat can reduce the hsp90 association with Her-2, leading to degradation of Her-2 protein. As a result of lower Her-2 stability and reduced protein levels, letrozole resistance could be reversed.

**Entinostat reduces Her-2 mRNA stability**

Finally, we examined the effect of ENT on Her-2 mRNA and its stability in LTLT-Ca cells using real-time qRT-PCR. HDAC6 inhibition has been shown to increase the degradation of Her-2 transcript (26). LTLT-Ca cells have a significantly (*P* < 0.001) higher basal level of Her-2 mRNA than MCF-7Ca cells (Fig. 6A); however, the half-life of Her2 mRNA is not significantly different in LTLT-Ca cells versus MCF-7Ca (Supplementary Fig. S3). To examine the effect of ENT on reduction in mRNA levels, LTLT-Ca cells were treated with actinomycin D (5 μmol/L) to inhibit all transcription and RNA was collected at indicated time points. Her-2 mRNA has a half-life of approximately 8 hours in LTLT-Ca cells. Entinostat treatment (1 μmol/L) alone reduced Her-2 mRNA in LTLT-Ca cells in a time-dependent manner, with 50% reduction seen between 4 and 8 hours. However, when new mRNA synthesis was inhibited with actinomycin D and the cells were treated with entinostat, 50% reduction in the Her-2 mRNA was seen as early as 4 hours (Fig. 6A). These findings suggest that entinostat can also increase Her-2 mRNA degradation along with protein degradation.

Previously, investigators have shown that HDAC inhibitors reduce Her-2 protein through hyperacetylation of hsp90 and increased Her-2 mRNA degradation. However, both of these effects were modulated through inhibition of a class IIb HDAC6 (23, 26, 35). However, entinostat is a class I selective HDAC inhibitor. To confirm that the effects seen with entinostat treatment were due to inhibition of a class I HDAC, we treated LTLT-Ca cells with siRNAs against HDAC1 (Fig. 6 B and C). We compared the effect of HDAC1 siRNA on the expression of Her-2,
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HDAC1 (Fig. 6B), and ERα; 18s ribosomal RNA was used as housekeeping gene (Fig. 6C). HDAC1 siRNA treatment reduced Her-2 levels significantly (~40% reduction; *, P = 0.0024; †, P = 0.0021) compared with control. Entinostat was however more potent in reducing the Her-2 mRNA levels (>70% reduction compared with control; †, P < 0.0001) compared with HDAC1 siRNAs (φ, P = 0.026 vs. HDAC1si1 and P = 0.049 vs. HDAC1si2), suggesting that HDAC1 alone may not be involved in Her-2 regulation, although inhibition of HDAC1 by entinostat is responsible for reduction in Her-2.

Treatment of MCF-7Ca xenografts with the combination of entinostat and letrozole does not delay resistance to letrozole

As upregulation of Her-2 is seen as soon as 4 weeks after beginning treatment with letrozole (3) due to increased stability of Her-2 (28), we postulated that addition of entinostat (which causes degradation of Her-2 and reduces its stability) to letrozole may help delay resistance and prolong responsiveness of MCF-7Ca xenografts to letrozole (Fig. 7). Mice bearing MCF-7Ca xenografts were grouped such that the mean tumor volumes were not different across the groups on week 0 (P = 0.67). Mice were treated with letrozole (10 μg/d) or entinostat (50 μg/d) or the combination. All mice received ΔA (100 μg/d); control group received only ΔA. As observed previously, letrozole-treated tumors had significantly lower growth rate than control (P = 0.0001). Similarly, entinostat + letrozole had significantly higher growth rate than control (P < 0.0001). However, growth rates of tumors of mice treated with entinostat + letrozole and letrozole were not significantly different over 17 weeks (P = 0.28). These results suggest that although entinostat is effective in reversing resistance to letrozole, addition of entinostat to letrozole for treating hormone-sensitive tumors may not provide any additional benefit. MCF-7Ca xenografts are very hormone dependent and inhibition of estrogen synthesis by AIs can result in marked tumor inhibition. However, once they become resistant to AIs, other pathways are activated and...
until that time inhibition of those pathways does not impede the growth of these tumors.

Discussion

It has been suggested that epigenetic silencing of ERα could be responsible for de novo resistance to endocrine therapy (17). In our previous study using a triple-negative breast cancer model, we showed that treatment of ER-negative MDA-MB-231 cells and tumors resulted in increased expression of ERα and aromatase (18), which resulted in response of the cells and tumors to the growth inhibitory effects of AI letrozole and mitogenic effects of E2. To evaluate the effect of HDAC inhibition on reduced ERα expression, which results from long-term letrozole treatment and acquired resistance, we treated letrozole-resistant cells and tumors with HDAC inhibitor entinostat. Although the HDAC inhibitor reversed the acquired resistance to letrozole, the mechanism of this reversal was found to be different than that observed in the ER-negative MDA-MB-231 cells, which exhibit de novo resistance. Although ERα protein levels in the LTLT-Ca cells are significantly lower than in the parental MCF-7Ca cells, ERα was not epigenetically repressed in LTLT-Ca cells. ChIP analysis showed that in the presence of letrozole, the ERα promoter was still active but did not require E2 for activation, suggesting ligand-independent activation of ERα. In addition, entinostat activated the

Figure 6. A, RT-PCR analysis of Her-2 mRNA levels in LTLT-Ca cells. Relative levels of Her-2 mRNA after treatment with entinostat (ENT) were analyzed by real-time RT-qPCR. Cells were treated with entinostat (1 μmol/L) alone or in presence of actinomycin D (5 μmol/L) and cells were collected at indicated time points. RNA was isolated, quantified and diluted to 0.08 μg/μL. Total 0.64 mg of RNA was reverse transcribed and Her-2 was amplified using real-time qPCR as described in Materials and Methods. Graph represents relative levels (±SEM) of Her-2 mRNA compared with LTLT-Ca control (set at 1). B and C, RT-PCR analysis of Her-2, HDAC1, ERα, and 18s mRNA levels in LTLT-Ca cells. Relative levels of Her-2, HDAC1, ERα, and 18s mRNA after treatment with entinostat, or siRNA against HDAC1 were analyzed by real-time RT-qPCR. Cells were treated with entinostat (1 μmol/L) or siRNAs against HDAC1 (5 nmol/L) or mock siRNA. The cells were collected after 48-hour incubation at 37°C. RNA was isolated, quantified, and diluted to 0.08 μg/μL. Total 0.64 mg of RNA was reverse transcribed and cDNA was amplified using real-time qPCR as described in Materials and Methods. Graph represents relative levels (±SEM) of Her-2 and HDAC1 mRNA (B) and ERα and 18s mRNA (C). The relative mRNA levels (±SEM) are reported compared with LTLT-Ca control (set at 1).

*, P = 0.0024; †, P = 0.0021; ‡, P < 0.0001 versus control. NS, not significant.
aromatase (PI3/II) promoter in an E2-dependent manner. The results obtained with entinostat were similar to those obtained with trastuzumab; a humanized monoclonal antibody against extracellular domain of Her-2 (16). As entinostat exhibited such similar effects on LTLT-Ca cells and tumors as trastuzumab, we hypothesized that entinostat may modulate Her-2 and thus restore responsiveness of LTLT-Ca cells and tumors to AIs.

Our previous study showed that upregulation of Her-2 in LTLT-Ca cells is the result of longer half-life of Her-2 protein and not gene amplification (28). In this study, we show that entinostat reduced the stability of the Her-2 protein in the LTLT-Ca cells. The results of this study suggest that HDAC inhibitors can overcome resistance to AI letrozole through modulation of Her-2. The treatment of LTLT-Ca cells or letrozole-resistant tumors with entinostat resulted in downregulation of Her-2 along with upregulation of ERα and aromatase. These molecular changes rendered the tumors responsive to AIs such as letrozole and exemestane. The combination of entinostat with letrozole was significantly better at controlling the growth of letrozole resistant tumors compared with single agents. Analysis of the tumors revealed that the Her-2 protein in letrozole-resistant tumors associates more with hsp90, suggesting increased stabilization. Entinostat treatment reduced this association. HDAC1 and 6 are shown to have hsp90 deacetylase activity, and inhibition of these HDACs is responsible for acetylation of hsp90, which leads to inactivation of its chaperone activity, leading to ubiquitination of the client proteins (23, 24). The potential mechanism of Her-2 degradation by HDAC inhibitor entinostat could involve the capacity of HDAC inhibitors to cause disassociation of client protein Her-2 from the hsp90 chaperon complex leading to its destabilization and degradation. This was confirmed by the reduced half-life and increased ubiquitination of the Her-2 protein in LTLT-Ca cells treated with entinostat. In addition, it has been shown that HDAC inhibitors are also capable of rapidly destabilizing mature Her-2 transcript (26). Our studies show that entinostat was also able to reduce Her-2 stability along with its transcription.

These results suggest that non-nuclear effects of HDAC inhibition on the Her-2 protein as well as nuclear effects on Her-2 transcription are responsible for the ability of entinostat to reverse the resistance to letrozole treatment. Entinostat is being investigated in a phase II open-label (ENCORE-303) clinical trial for women with ER-positive breast cancer that are progressing on AI therapy. This preliminary proof-of-concept study showed that the combination provided some benefit, resulting in disease stabilization in heavily hormonally pretreated and relatively hormone-resistant patients with breast cancer (36). The disease stabilization achieved with the addition of entinostat supported the hypothesis that entinostat has the ability to restore sensitivity to AIs. Furthermore, a double-blind, randomized, placebo-controlled phase II study of entinostat in combination with the aromatase inhibitor exemestane (ENCORE 301) showed 27% reduction in the risk of disease progression, translating into a 2-month improvement in progression-free survival (37).

Figure 7. Effect of entinostat (ENT) alone or in combination with letrozole on the growth of MCF-7Ca xenografts. Ovariectomized athymic nude mice were inoculated with MCF-7Ca cells. Tumors were allowed to form in the presence of androstenedione (Δ4-A), aromatizable substrate for estrogen. When the tumors reached ~300 mm3, mice were treated with either letrozole (10 μg/d), entinostat (50 μg/d), or the combination of letrozole and entinostat for 20 weeks. The growth rates of tumors of mice treated with the combination of entinostat plus letrozole was not significantly different than letrozole alone (P = 0.28).
provides further evidence supporting the clinical benefit and tolerability of entinostat in combination with aromatase inhibitors. On the basis of these findings, we hypothesize that the increased half-life of Her-2 protein and increased activation of Her-2 gene, following estrogen deprivation (ensuing letrozole treatment) provides the cells an alternative pathway to escape growth inhibition due to estrogen withdrawal (28). This may result in the cells adapting to growth factor signaling pathways and overcoming growth inhibition by letrozole. Consequently, agents that interfere with the stability of the Her-2 protein and/or mRNA may also extend the responsiveness of hormone-dependent tumors to AIs. As HDAC inhibitor entinostat can modulate expression of Her-2, it may provide additional benefit in the treatment of Her-2–positive or breast cancers that may express ERα (at a lower level) but exhibit resistance to aromatase inhibitors.

Disclosure of Potential Conflicts of Interest
A.H. Brodie has commercial research support from Syndax Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

References

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