Activity of Suberoylanilide Hydroxamic Acid Against Human Breast Cancer Cells with Amplification of Her-2

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Abstract  Purpose: We determined the effects of suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, on hsp90 and its client proteins Her-2, AKT, and c-Raf, as well as evaluated the cytotoxic effects of cotreatment of SAHA with trastuzumab or docetaxel in human breast cancer BT-474 and SKBR-3 cells containing amplification of Her-2.

Experimental Design: The cells were treated with SAHA (1.0–5.0 μmol/L) and/or trastuzumab (5–40 μg/mL) or docetaxel (5–20 mmol/L). Following this, apoptosis and the levels of p21(WAF1), p27(KIP1), AKT, c-Raf, and Her-2, as well as of the key regulators of apoptosis were determined. Synergistic interaction between drugs was evaluated by median dose-effect analysis.

Results: Treatment with SAHA up-regulated p21(WAF1) and p27(KIP1) levels, increased the percentage of cells in G2-M phase of the cell cycle, as well as induced apoptosis in a dose-dependent manner. This was associated with up-regulation of the pro-death Bak and Bim, as well as attenuation of the levels of Her-2 and XIAP, survivin, Bcl-2, and Bcl-xL proteins. SAHA treatment induced acetylation of hsp90. This reduced the chaperone association of Her-2 with hsp90, promoting polyubiquitylation and degradation of Her-2. SAHA also attenuated the levels of c-Raf and AKT. Cotreatment with SAHA significantly increased trastuzumab or docetaxel-induced apoptosis of BT-474 and SKBR-3 cells. Additionally, median dose-effect analysis revealed that cotreatment with SAHA and trastuzumab or docetaxel induced synergistic cytotoxic effects against the breast cancer cells.

Conclusions: These preclinical findings support the development of SAHA in combination with docetaxel and/or trastuzumab against Her-2-amplified breast cancer.

Approximately 25% of breast cancers have amplification and overexpression of her-2/neu oncogene (1), which encodes for Her-2, a member of the family of epidermal growth factor receptor tyrosine kinases (2, 3). Active dimers of Her-2 with the other members of the family, e.g., Her-3 and Her-1, result in pro-growth and pro-survival signaling through the phosphoinositide-3-kinase (PI3K)/AKT and/or Ras/Raf/Erk pathways (2, 3). Preclinical studies have shown that Her-2-mediated signaling also confers resistance to anti-breast cancer agents, including tubulin polymerizing agents, e.g., paclitaxel and docetaxel, and the antiestrogen tamoxifen (4, 5). Although Her-2 overexpression has been associated with poor prognosis in breast cancer, it may also be associated with improved clinical outcome following chemotherapy, including tubulin polymerizing agents (6, 7). However, overall, Her-2 has been regarded as an excellent therapeutic target in breast cancer (7–9). Indeed, treatment with the recombinant, humanized, monoclonal anti-Her-2 antibody, trastuzumab (Herceptin), which dephosphorylates and down-regulates Her-2, has exhibited significant clinical efficacy against breast cancer and has been shown to sensitize breast cancer cells to chemotherapeutic agents, especially tubulin polymerizing agents and radiation therapy (10–15). Unfortunately, resistance to trastuzumab, administered alone or in combination with chemotherapeutic agents is common, mediated by mechanisms that often involve the activation of PI3K/AKT and/or Erk1/2 signaling or depletion of p27(KIP1) (referred to as p27; refs. 16–20). This highlights the need to develop new strategies that would target these mechanisms of resistance to trastuzumab, thereby increasing the sensitivity of breast cancer cells to trastuzumab and chemotherapeutic agents.

The reciprocal activities of histone deacetylases and histone acetyltransferases catalyze the deacetylation and acetylation, respectively, of specific lysine residues in the core nucleosomal histones and several transcription factors (21, 22). Treatment with histone deacetylase inhibitors (HDIs), including the hydroxamic acid analogues (HA), e.g., trichostatin A, suberoylanilide hydroxamic acid (SAHA), and LAQ824, which inhibit class I and II histone deacetylases, has been shown to induce hyperacetylation of the histones, which transcriptionally up-regulates p21(WAF1) (referred to as p21) and increase p27...
expression (21–24). This is associated with cell cycle arrest and apoptosis of cancer cells, and is shown to induce the in vivo regression of tumors (21–25). Recently, treatment with the HDIs was also shown to induce acetylation of the heat shock protein 90 (hsp90), which inhibited its ATP binding and chaperone association with its client proteins, e.g., Her-2, mutant p53, AKT, and c-Raf (24, 26). This targets the client proteins for polyubiquitylation and degradation by the proteasome (27, 28). In cancer cells, these effects of HA-HDI treatment were similar to those observed following treatment with the hsp90 inhibitor geldanamycin and its analogue, 17-AAG (27, 28). 17-AAG mediated depletion of c-Raf-1 and inhibition of Erk1/2 phosphorylation, as well as attenuation of p-AKT, leads to disruption of the Raf-Mek-Erk1/2 and PI3K/AKT signaling, resulting in cytostasis and apoptosis of cancer cells (27–31). Treatment with 17-AAG has also been shown to down-regulate Her-2 and its downstream signaling, as well as sensitize breast cancer cells to chemotherapeutic agents (27–29, 32, 33). Taken together, these reports suggest that inhibition of hsp90 by an HA-HDI or 17-AAG exerts anti-breast cancer effects through depletion of Her-2 and its signaling, as well as by attenuation of AKT and c-Raf-1 activity that may be independent of down-regulation of Her-2 signaling (24, 31). SAHA is an orally bioavailable prototype of the HA-HDIs, which has been shown to be relatively safe and active as an anticancer agent (21, 34). In present studies, we determined the effects of SAHA on the levels of Her-2 and the downstream pro-growth and pro-survival signaling proteins. Treatment with SAHA induced acetylation of hsp90, which was associated with decreased binding of Her-2 to hsp90 and increased polyubiquitylation and depletion of Her-2. SAHA treatment also depleted the levels of p-AKT, c-Raf, XIAP, survivin, Bcl-2, and Bcl-xL but induced p27 and Bim. Importantly, cotreatment with trastuzumab or docetaxel with SAHA induced synergistic cytotoxic effects against human breast cancer cells with amplification of Her-2.

**Materials and Methods**

**Reagents and antibodies.** SAHA was kindly provided by Aton Pharma, now wholly owned subsidiary of Merck & Co., Inc. (Flemington, NJ). Docetaxel was a gift from Aventis Pharmaceuticals (Bridgeport, N.J). Trastuzumab was a gift from Genentech Inc. (South San Francisco, CA). Docetaxel was made at a concentration of 10 mmol/L in DMSO and stored at −20°C. Antibodies for the immunoblot analyses to detect the levels of Her-2, c-Raf-1, AKT, p-AKT, ERK1/2, p-ERK1/2, acetylated lysine, p21, p27, acetylated histone H3 and H4 proteins, Bak, Bax, poly-ADP ribose polymerase (PARP), Bim, XIAP, survivin, Bcl-2, Bcl-xL, and β-actin were obtained, as previously described (24, 30, 35–37).

**Cell culture.** The human breast cancer cell lines BT-474, SKBR-3, MCF-7, and MDA-MB-468 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture, as previously described (24).

**Flow cytometric analysis of cell cycle status.** The flow cytometric evaluation of the cell cycle status was done according to a previously described method (35, 36). The percentage of cells in the G1, S phase, and G2/M phases were calculated.

**Apoptosis assessment by Annexin V staining.** After drug treatments, cells were resuspended in 100 μL staining solution (containing Annexin V fluorescein and propidium iodide in a HEPES buffer, Annexin V-FITC, BD Pharmingen, San Diego, CA). Following incubation at room temperature for 15 minutes, cells were analyzed by flow cytometry (35–37).
Fifty micrograms of proteins from the NP40 soluble and insoluble fractions were separated on 7.5% SDS-polyacrylamide gel and analyzed by Western blotting (24, 38).

**Statistical analyses.** Data were expressed as mean ± SE. Comparisons used Student's *t* test or ANOVA, as appropriate. *P* values of < 0.05 were assigned significance.

### Results

**SAHA increases p21 and p27 levels, as well as induces apoptosis of breast cancer cells.** We first determined the effect of SAHA on histone acetylation, as well as on the level of p21 and p27 in breast cancer cells. Exposure to 2.0 or 5.0 μmol/L SAHA for 24 hours increased the acetylation of histone H3 and H4 (data not shown) in BT-474, SKBR-3 and MCF-7 cells (data not shown; Fig. 1A and B). This was associated with induction of p21 in a dose-dependent manner. SAHA treatment increased p27 levels in a dose-dependent manner in BT-474 but not in SKBR-3 or MCF-7 cells. Previous reports have indicated that unlike p21, increase in p27 level following treatment with SAHA is not due to increase in the mRNA transcript levels of p27, but may be due to a posttranscriptional mechanism (23).

**SAHA induces hsp90 acetylation and depletes Her-2, p-AKT, and ERK1/2 levels.** Next, we investigated the effect of SAHA on the levels of pro-growth and pro-survival Her-2, AKT, and c-Raf in the breast cancer cells. Treatment with 2.0 or 5.0 μmol/L SAHA for 24 hours depleted Her-2, p-AKT, AKT, and c-Raf levels in BT-474 and SKBR-3 cells (Fig. 3A). SAHA also mediated the depletion of c-Raf, p-ERK1/2, p-AKT, and AKT in the MCF-7 cells, which lack Her-2 amplification but express ERα and are responsive to estrogen (Fig. 3B). SAHA-mediated depletion of c-Raf in these cell types was associated with decrease in the levels of p-ERK1/2 but not ERK1/2 levels. Additionally, exposure to SAHA also induced p21 but depleted c-Raf, p-AKT, and AKT levels in MB-468 cells that lack both Her-2 amplification and Erα expression (Fig. 3B). Previous reports have shown that the HA-HDIs, e.g., LAQ824, induce acetylation of hsp90, which inhibits the ATP binding and chaperone association of hsp90 with its client proteins, including Her-2, AKT, and c-Raf, leading to their polyubiquitylation and degradation by the 20S proteasome (24, 26). Consistent with these reports, exposure intervals to 2.0 or 5.0 μmol/L of SAHA as short as 8 hours also induced acetylation of hsp90 in the SKBR-3 and MB-468 cells (Fig. 4A), which depleted the binding of Her-2 to hsp90 in SKBR-3 and BT-474 cells (Fig. 4A), similar to LAQ824, SAHA also induced the acetylation of α-tubulin in SKBR-3 (Fig. 4A) and MB-468 cells (data not shown). This may be due to inhibition of histone deacetylase-6, a previously documented α-tubulin deacetylase that is also known to be associated with the microtubules (Fig. 4A; refs. 39–41). Treatment with SAHA increased polyubiquitylation and accumulation of Her-2 in the detergent-insoluble fraction of the cell lysates, directing Her-2 to proteasomal degradation (Fig. 4C and D). As shown in Fig. 4D, cotreatment with the proteasomal inhibitor ALLN augmented SAHA-mediated accumulation of Her-2 in the detergent-insoluble fraction of the cells. Overall, these effects of SAHA caused the depletion of Her-2 levels in SKBR-3 and BT-474 cells (Fig. 3A). Although not investigated in the present studies, treatment with SAHA similar to the other HA-HDIs may also

![Fig. 1. Effects of SAHA on histone acetylation, induction of p21 and p27 expressions, and cell cycle status.](image-url)
deplete the mRNA transcript levels of Her-2, which may contribute to the attenuation of Her-2 in the breast cancer cells (24, 42).

Cotreatment with SAHA and trastuzumab and docetaxel exerts synergistic cytotoxic effects in breast cancer cells. Activated Her-2 dimers trigger pro-growth and antiapoptotic signaling through the activation of PI3K/AKT, which mediates chemoresistance of breast cancer cells (2, 3, 16). Conversely, depletion of Her-2 and AKT activity induces chemosensitization in breast cancer cells (13, 43, 44). Consistent with this, as shown in Fig. 5A, cotreatment with 2.0 or 5.0 μmol/L of SAHA, which attenuates Her-2 and AKT signaling, and trastuzumab induced significantly more apoptosis of BT-474 cells than either agent alone (P < 0.01). Similar findings were also observed in SKBR-3 cells (Fig. 5B). The combinations of SAHA and trastuzumab at various dose levels induced synergistic cytotoxic effects against BT-474 cells, as determined by median dose-effect isobologram analysis (Fig. 5C). Additionally, combined treatment with 1.0 or 5.0 μmol/L of SAHA and 10 or 20 μg/mL trastuzumab caused more depletion of Her-2 and p-AKT levels, but induced p27 and BimEL than treatment with either agent alone (Fig. 5D). Combination of SAHA and trastuzumab also induced more processing of PARP consistent with more apoptosis of BT-474 and SKBR-3 cells (data not shown). Figure 6A and B show that, similar to trastuzumab, cotreatment of SAHA and docetaxel induced significantly more apoptosis than treatment with either agent alone (P < 0.05). The combination also induced synergistic cytotoxic effects against SKBR-3 cells, as determined by median dose-effect isobologram analysis (Fig. 6C). A similar effect was observed against BT-474 cells (data not shown). Exposure of SKBR-3 cells to docetaxel increased p-AKT levels, which were abrogated when treatment with docetaxel was combined with SAHA (1.0 or 5.0 μmol/L; Fig. 6D). Similar abrogation of p-AKT levels was observed in BT-474 cells, following combined treatment with SAHA and docetaxel (concentration ranging between 5.0 and 50 nmol/L; data not shown). The combination of SAHA and docetaxel also induced more processing of PARP than either agent alone, consistent with more apoptosis of SKBR-3 cells (Fig. 6D).

Fig. 2. SAHA induces Bax conformation change and pro-death proteins while attenuating antiapoptotic proteins, as well as inducing PARP cleavage and apoptosis of breast cancer cells. A, SKBR-3 and BT-474 cells were exposed to the indicated concentrations of SAHA for 48 hours. Following this, the percentage of apoptotic cells were determined by Annexin V staining and flow cytometry. Columns, mean; bars, ± SE. B, following treatment with the indicated concentrations of SAHA for 24 hours, immunoprecipitates with 6A7 antibody (recognizes conformationally changed Bax) were immunoblotted with anti-Bax antibody. Alternatively, total cell lysates were immunoblotted with anti-PARP or anti-Bax antibody. β-Actin levels served as the control for protein loading and transfer. C, following treatment with the indicated concentrations of SAHA for 24 hours, total cell lysates of BT-474 were immunoblotted with anti-Bim, Bak, Bcl-2, Bcl-xL, XIAP, or anti-survivin antibody. β-Actin levels served as the control for protein loading and transfer.

Fig. 3. Treatment with SAHA attenuates Her-2, p-AKT, AKT, c-Raf, and p-ERK1/2, in breast cancer cells. A, following treatment with the indicated levels of SAHA for 24 hours, total cell lysates of BT-474 and SKBR-3 cells were immunoblotted with anti-Her-2, c-Raf, p-AKT, AKT, p-ERK1/2, or ERK1/2 antibodies. β-Actin levels served as the control for protein loading and transfer. B, following treatment with the indicated levels of SAHA for 24 hours, total cell lysates of MCF-7 and MB-468 cells were immunoblotted with anti-p21, c-Raf, p-AKT, AKT, p-ERK1/2, or ERK1/2 antibodies. β-Actin levels served as the control for protein loading and transfer.
Discussion

Preclinical in vitro and in vivo studies have shown that treatment with SAHA induces p21 and cell cycle growth arrest, associated with differentiation and apoptosis of breast cancer cells (23, 45, 46). A phase I study of SAHA has indicated that it

Fig. 4. SAHA acetylates hsp90 and inhibits its chaperone association with Her-2, promoting polyubiquitylation and proteasomal degradation of Her-2. A, SKBR-3 and MB-468 cells were exposed to the indicated concentrations of SAHA for 8 hours. Following this, hsp90 was immunoprecipitated from the cell lysates and immunoblotted with either anti-hsp90 or anti-acetyl lysine antibody. Alternatively, total cell lysates from SKBR-3 cells were immunoblotted with anti-acetyl α-tubulin antibody. β-Actin levels served as the control for equal protein loading. B, following treatment with the indicated concentrations of SAHA for 8 hours, immunoprecipitates of Her-2 were immunoblotted with anti-Her-2 or anti-hsp90 antibody. C, following treatment of SKBR-3 cells with the indicated concentrations of SAHA for 8 hours, immunoprecipitates of Her-2 were immunoblotted with anti-polyubiquitin or anti-Her-2 antibody. D, following treatment of SKBR-3 cells with indicated concentrations of SAHA and/or ALLnL for 8 hours, lysates from detergent (NP40)-insoluble cellular fractions were immunoblotted with anti-Her-2 or anti-c-Raf antibody. β-Actin levels served as the control for equal protein loading.

Fig. 5. Combination of SAHA and trastuzumab exerts synergistic cytotoxic effects. BT-474 (A) or SKBR-3 (B) cells were treated for 48 hours with SAHA and/or trastuzumab, and apoptosis was determined by Annexin V staining and flow cytometry. Columns, mean; bars, ± SE. C, analysis of the dose-effect relationship for SAHA (0.5-4 μmol/L) and/or trastuzumab (5-40 μg/mL) for the cytotoxic effects determined by MTT assay after 96 hours of exposure was done according to the median effect method of Chou and Talalay. Following this, the combination index values were calculated. CI < 1, CI = 1, and CI > 1 represent synergism, additivity, and antagonism of the two agents, respectively. D, alternatively, total cell lysates of the cells were obtained following 24 hours of drug treatment and immunoblotted with anti-Her-2, p-AKT, p27, or anti-Bim antibody. β-Actin levels served as the control for equal protein loading.
is a relatively safe agent with clinical antitumor activity (34). This creates the rationale to further evaluate the activity of SAHA as a single agent and in combination with anti-breast cancer agents. Present studies focused on the in vitro activity of SAHA and/or trastuzumab or docetaxel against human breast cancer cells with amplification of Her-2. The results highlight several mechanisms that may contribute to SAHA-mediated cell cycle effects and apoptosis of breast cancer cells. First, SAHA treatment is shown to induce hsp90 acetylation, which inhibits the chaperone association of Her-2 with hsp90, directing Her-2 to polyubiquitylation and degradation by the 20S proteasome. These findings are consistent with our previous reports demonstrating that treatment with other HA-HDIs induce acetylation of hsp90, which promotes polyubiquitylation and accumulation of the misfolded client proteins, including Her-2, AKT, c-Raf, Bcr-Abl and mutant FLT-3, in the detergent-insoluble fraction of the cells leading to proteasomal degradation of the client proteins (24, 35, 47). The chaperone-binding E3 ubiquitin ligase for Her-2 is carboxyl-terminal hsc70-interacting protein (CHIP), which ubiquitylates Her-2 prior to its degradation by the 20S proteasome (48, 49). Similar to hsp90 inhibition by 17-AAG, SAHA-induced acetylation of hsp90 may also promote CHIP-mediated polyubiquitylation and degradation of Her-2, although this was not ascertained in the present studies. Treatment with SAHA also induced α-tubulin acetylation, suggesting that the cytosolic histone deacetylase-6 may be involved in the deacetylation of hsp90, which is inhibited by SAHA (40–42). Which lysine residue(s) on hsp90 is acetylated following SAHA treatment, leading to the inhibition of ATP binding and chaperone association of hsp90 with the client proteins, remains to be determined. Additionally, whether SAHA, following treatment with 17-AAG, also promotes CHIP-mediated polyubiquitylation of Her-2, has yet to be determined.

SAHA-mediated inhibition of the chaperone function of hsp90 also led to proteasomal degradation and depletion AKT and c-Raf, two of the most prominent pro-growth and pro-survival proteins often dysregulated in cancer cells (24, 27, 30). Whereas reduced levels of p-ERK1/2 may be due to decreased c-Raf levels, the depletion of p-AKT has been shown to be due to restoration of the activity of a previously described AKT phosphatase PP1, following depletion of Her-2 due to SAHA (30, 50). Present findings also show that treatment with SAHA attenuated Bcl-2, Bcl-xl, XIAP, and survivin, whereas simultaneously increasing Bak and Bim expression. Collectively, these modulations are likely to lower the threshold for apoptosis in breast cancer cells. Although the precise mechanism(s) responsible for all of these modulations have not been elucidated, depletion of AKT and/or its activity is known to inhibit p27 phosphorylation and increase its nuclear localization (51, 52). Reduced AKT activity also attenuates the phosphorylation of Foxo3A and increases its nuclear localization, which results in transactivation and induction of p27 and the prodeath proteins Bim and TRAIL (53, 54). Taken together, these findings suggest that treatment with SAHA, and other HA-HDIs, inhibits Her-2 and downstream AKT and Ras/Raf/ERK signaling pathways, as well as modulating apoptosis regulators in a manner which could lower the apoptotic threshold to anti-breast cancer agents (24, 43, 55, 56). Consistent with this, SAHA and docetaxel combination induced synergistic cytotoxic effects against breast cancer cells with amplification of Her-2. Combination with other HA-HDIs has also been shown to enhance the activity of anticancer agents with diverse mechanisms of action (24, 57). Molecular perturbations induced by SAHA could also explain why cotreatment with SAHA and
trastuzumab exerted synergistic cytotoxic effects in Her-2-amplified breast cancer cells. A similar outcome was observed when the multiple cyclin-dependent kinase inhibitor, flavopiridol, which also induces molecular perturbations that lower the apoptosis threshold, was combined with trastuzumab against breast cancer cells (58). Although not directly tested here, SAHA-mediated depletion of p-AKT and p-ERK1/2, as well as down-modulation of the apoptotic threshold, may also sensitize breast cancer cells to other targeted agents against Her-2 heterodimers, e.g., pertuzumab (24, 29, 59, 60).

Several reports have highlighted a number of potential mechanisms of resistance to trastuzumab in breast cancer cells. In a majority of patients with breast cancers with amplification of Her-2, this manifests both as primary and acquired resistance to trastuzumab, most likely due to mechanisms that involve loss of PTEN or activation of acquired resistance to trastuzumab, most likely due to amplification of Her-2, this manifests both as primary and cells. In a majority of patients with breast cancers with mechanisms of resistance to trastuzumab in breast cancer Her-2 heterodimers, e.g., pertuzumab (24, 29, 59, 60).

In summary, as shown here, SAHA mediated attenuation of Her-2, pERK1/2, and p-AKT, as well as SAHA-induced potentiation of trastuzumab and docetaxel activity against Her-2-amplified breast cancer cells represent novel findings that can be therapeutically exploited. Combination of docetaxel and trastuzumab is active and commonly employed against breast cancer with amplification of Her-2 (70). Therefore, our findings also support the in vivo testing of SAHA with this combination. To this end, a phase I/II study of SAHA and trastuzumab has been designed and will soon be implemented by a member institution (Eastern Cooperative Oncology Group).

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