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Enhancing the antitumor activity of ErbB blockade with histone deacetylase (HDAC) inhibition

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Molecular inhibition of the ErbB signaling pathway represents a promising cancer treatment strategy. Preclinical studies suggest that enhancement of antitumor activity can be achieved by maximizing ErbB signaling inhibition. Using cDNA microarrays, we identified histone deacetylase (HDAC) inhibitors as having strong potential to enhance the effects of anti-ErbB agents. Studies using a 20,000 element (20K) cDNA microarray demonstrate decreased transcript expression of ErbB1 (epidermal growth factor receptor) and ErbB2 in DU145 (prostate) and ErbB2 in SKBr3 (breast) cancer cell lines. Additional changes in the DU145 gene expression profile with potential interaction to ErbB signaling include down-regulation of caveolin-1 and hypoxia inducible factor 1-α (HIF1-α), and up-regulation of gelsolin, p19(INK4D) and Nur77. Findings were validated using real time RT-PCR and Western blot analysis. Enhanced proliferative inhibition, apoptosis induction and signaling inhibition were demonstrated when combining HDAC inhibition with ErbB blockade. These results suggest that used cooperatively, anti-ErbB agents and HDAC inhibitors may offer a promising strategy of dual targeted therapy. Additionally, microarray data suggest that the beneficial interaction of these agents may not derive solely from modulation of ErbB expression, but may result from effects on other oncogenic processes including angiogenesis, invasion and cell cycle kinetics.

Key words: HDAC inhibitor; ErbB; EGFR; SAHA

The ErbB family of receptor tyrosine kinases represents important mediators of cell growth, differentiation and survival. These cell membrane receptors are activated following dimerization mediated by their respective ligands. Once activated, intracellular tyrosine kinase activity elicits diverse mitogenic and prosurvival signaling mediated through downstream targets including PI3 kinase, AKT, RAS/RAF and MAP kinase, among others. Heterodimerization between family members creates a multilayered network of interactions, which may allow simultaneous activation of signaling pathways unique to each receptor subtype.[1,2]

Of the ErbB family of receptors, molecular inhibition of ErbB1 (epidermal growth factor receptor, EGFR) and ErbB2 have been the most extensively studied as promising anticancer treatment strategies.[3–6] A broad series of agents have been developed that target either the extracellular domain or the intracellular tyrosine kinase domain of the ErbB receptors. Additionally, there has been increased interest in the network interaction between the individual members of the ErbB family. It is hypothesized that ligand dependent horizontal interactions between ErbB receptors can regulate the intensity of mitogenic signaling as well as serve as a mechanism to evade resistance to therapy targeting a single ErbB receptor.[2]

Despite high promise for molecular inhibitors of ErbB signaling as anticancer agents, early clinical trials have shown mixed results. For example, although Phase II trials of the small molecule EGFR inhibitors gefitinib and erlotinib showed highly favorable monotherapy response rates in refractory lung cancer patients,[7,8] subsequent Phase III studies of these agents delivered in combination with cytotoxic chemotherapy as first line therapy showed no overall survival impact.[9] Therefore, considerable effort is currently being focused to better recognize specific molecular footprints of ErbB responsive tumors, and develop novel strategies to potentiate the antitumor activity of ErbB inhibitors.

One class of agents with strong potential for enhancing the effects of ErbB inhibitors are the histone deacetylase (HDAC) inhibitors. inhibitors are a promising new class of anticancer agents with demonstrated activity in a variety of solid and hematologic tumors, including breast, prostate, head and neck, brain, lung and leukemia.[10–12] Encouraging results have been reported from Phase I clinical trials with various HDAC inhibitors, and Phase II trials have been initiated in cutaneous T-cell lymphoma, peripheral T-cell lymphoma and recurrent or metastatic squamous cell cancer of the head and neck (H&N SCC).

We recently conducted preliminary screening experiments using cDNA microarrays to identify a cohort of genes differentially regulated by HDAC inhibition in human breast and prostate cancer. This high throughput analysis identified potential cross-talk with ErbB family members, whose expression was down-regulated following HDAC inhibition. The present study examines the capacity of HDAC inhibition to down-modulate both EGFR and ErbB2 expression and thereby potentiate the antitumor activity of ErbB inhibition.

Material and methods

Chemicals

Cell culture media were obtained from Life Technologies (Gaithersburg, MD). CI-1033 was generously provided by Pfizer Global Research (Ann Arbor, MI). Trichostatin A (TSA) was obtained from Sigma Chemical (St. Louis, MO), suberoylanilide hydroxamic acid (SAHA) from Alexis Biochemicals (Lausen, Switzerland), apicidin from Calbiochem (La Jolla, CA) and valproic acid from Sigma Chemical (St. Louis, MO). Primary antibodies against EGFR, PARP and AKT were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); pEGFR-1068 and pAKT were obtained from Cell Signaling Technologies (Beverly, MA); HER2 and p21/waf-1 were obtained from Neomarker (Freemont, CA) and α-tubulin was obtained from Oncogene Research Products (Cambridge, MA). ECL+ chemiluminescence detection system was purchased from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Abbreviations: CDK, cyclin dependent kinase; CI, combination index; EGFR, epidermal growth factor receptor; GAPD, glyceraldehyde-3 phosphate dehydrogenase; H&N SCC, head and neck squamous cell carcinoma; HDAC, histone deacetylase; HIF1-α, hypoxia inducible factor 1-α; HMBS, hydroxymethylbilane synthase; PARP, poly(ADP-ribose) polymerase; RT, reverse transcriptase; SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A.

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Cell lines

Human LnCaP, DU145 and PC3 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in complete culture media consisting of RPMI (7.4) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Human SKBr3 and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in complete culture media consisting of DMEM (7.4) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. The UM-SCC6 cell line (base of tongue) was provided by Dr. Thomas E. Carey (University of Michigan) and maintained in complete culture media consisting of DMEM (7.4) supplemented with 10% fetal bovine serum, 1% hydrocortisone and 1% penicillin and streptomycin.

DNA microarray

DNA microarray analysis of gene expression was done essentially, as described by the Brown and Derisi Labs (available at www.microarrays.org). The sequence-verified cDNA clones on the human cDNA microarray are available from Research Genetics (www.resgen.com). Purified PCR products, generated using the clone inserts as template, were spotted onto poly-L-lysine-coated microscope slides, using an Omnigrid robotic arrayer (GeneMachines, CA) equipped with quill-type pins (Majer Scientific, AZ).

Cells treated with HDAC inhibitor were solubilized and homogenized in Trizol (Invitrogen, Carlsbad, CA) and total RNA was isolated, according to manufacturer instructions. Once isolated, mRNA was used as a template for cDNA generation using reverse transcriptase (RT). Inclusion of amino allyl-dUTP in the RT reaction allowed for subsequent fluorescent labeling of cDNA using monofunctional NHS ester dyes (as described at www.microarrays.org). In each experiment, fluorescent cDNA probes were prepared from an experimental mRNA sample (Cy5-labeled) and a control mRNA sample (Cy3-labeled) isolated from untreated cells. The experimental cDNA sample was coupled to a monofunctional Cy5 NHS-ester and the reference cDNA sample to a Cy3 NHS-ester (Amersham). The labeled probes were then hybridized to 20K human cDNA microarrays. Fluorescent images of hybridized microarrays were obtained using a GenePix 4000A microarray scanner (www.axon.com, Axon Instruments, CA). The Cy5/Cy3 ratio was collected, and the data sets for each experiment were queried for genes that were differentially expressed in the drug treated versus control cell lines (ratios greater than 1.5 or less than 0.75).[13] Results represent an average of 3 independent experiments.

Quantitative real-time PCR (QPCR)

To further validate microarray findings, we performed quantitative QPCR using the SYBR green dye as previously described.[14] Briefly, 1 μg of total RNA isolated from each sample was reverse transcribed into first strand cDNA. Threshold levels were set for each experiment during the exponential phase of the PCR reaction, using the SDS v 1.7 software (Applied Biosystems, Foster City, CA), and the quantity of DNA in each sample was calculated by interpolating its Ct value versus a standard curve of Ct values obtained from serially diluted cDNA from a mixture of all of the samples, using Microsoft Excel. All standard curves had R^2 values ≥ 0.99 over 3 orders of magnitude. The calculated quantity of the target gene for each sample was then divided by the average calculated quantity of the housekeeping genes glyceraldehyde-3 phosphate dehydrogenase (GAPD) and hydroxymethylbilane synthase (HMBS) corresponding to each sample to give a relative expression of the target gene for each sample. Oligonucleotide primers for EGFR,[15] ErbB2,[16] and HMBS and GAPD[17] were as described. Oligonucleotide primers for caveolin-1, hypoxia inducible factor 1-α (HIF1-α), p19(INK4D) and Nur77 are available upon request. All experiments were performed in duplicate.

Cell cycle analysis

Cells were analyzed after 24 hr exposure to HDAC inhibitors SAHA, TSA and valproic acid. Cells were harvested by trypsinization, washed with PBS, fixed and stored at 4°C before DNA analysis. After removal of ethanol by centrifugation, cells were incubated with phosphate–citric acid buffer at room temperature for 45 min. After centrifugation, cells were then stained with a propidium iodide (PI) solution for 24 hr. Stained nuclei were analyzed for DNA-PI fluorescence using a Becton Dickinson FACScan flow cytometer. Resulting DNA distributions were analyzed by Modfit (Verity Software House, Topsham, ME) for the proportion of cells in sub-G0, G1, S and G2-M phases of the cell cycle.

Cell proliferation assay

Exponentially growing tumor cells were plated in 6-well plates and incubated in medium containing vehicle control, CI-1033, SAHA or both drugs in combination for 72 hr at 37°C. Monolayers were then washed with PBS and fixed/stained with 0.5% crystal violet. Plates were air-dried overnight and dye was eluted with 0.1 M sodium citrate (pH 4.2) in ethanol (1:1). Elution was transferred to 96-well plates, and the absorbance was read at 540 nm to determine cell viability. Results presented are an average of 2 independent experiments, each performed in duplicate.

Analysis of apoptosis by fluorescein-labeled caspase inhibitors

Cells were seeded in 100-mm dishes at a density of 6×10^5 cells/plate and incubated in medium containing vehicle control, CI-1033, SAHA or both drugs in combination for 24 hr at 37°C. Cells were harvested by trypsinization, centrifuged and the cell pellet resuspended to a final concentration of 2×10^6 cells/ml. Caspase activity was analyzed by fluorescence spectroscopy, according to manufacturer protocol (Serologicals, Norcross, GA). Briefly, 300 μl of cells were incubated with 1× fluorescein-labeled pan-caspase inhibitor FAM-VAD-FMK[18] at 37°C for 1 hr in a humidified atmosphere of 5% CO_2. Cells were then washed with buffer and resuspended in 320 μl PBS. A 100 μl aliquot of the cell suspension was transferred to a black 96-well plate in triplicate. Fluorescence was analyzed on a SpectraMax fluorescence plate reader at 550 nm excitation and 600 nm emission wavelengths. Data represent results averaged from 2 independent experiments.

Immunoblot analysis

Following treatment, cells were lysed with RIPA buffer and sonicated in complete proteinase inhibitor cocktail (Roche, Indianapolis, IN) and sodium orthovanadate. Fifteen microgram of protein extracts were mixed with SDS sample buffer and electrophoresed onto a 10% SDS-polyacrylamide gel under reducing conditions. The separated proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was incubated for 1 hr in blocking buffer (Tris--buffered saline with 0.1% Tween (TBS-T) and 5% nonfat dry milk). The membranes were then incubated with specific primary antibodies. After washing 3 times with TBS-T buffer, the membrane was incubated with horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) at 1:5,000 dilution for 1 hr at room temperature. The signals were visualized with the ECL+ detection system and autoradiography. For α-tubulin Western blots, the antibody-probed membranes were stripped with Western Re-Probe buffer (Geno-tech, St. Louis, MO) and blocked in Tris-buffered saline with 0.1% Tween (TBS-T) with 5% nonfat dry milk and incubated with rabbit anti-α-tubulin antibody. Signal quantification was performed using ImageQuant v1.2 (Molecular Dynamics). All results were normalized relative to loading controls.

Statistics

Combination treatment analysis of cellular proliferation was performed by determining a combination index (CI) based on the

TABLE I – CONSOLIDATED LIST OF GENES DEMONSTRATING SIGNIFICANT UP-REGULATION (>1.75) OR DOWN-REGULATION (<0.5) IN THE DU145 CELL LINE FOLLOWING 12 OR 24 HR EXPOSURE TO SAHA (1.0 μM)

	12 hr	24 hr
Gene up-regulation		
Connective tissue growth factor	3.312	6.053
Sialidase 1 (lysosomal sialidase)	3.91	5.027
Amphiregulin (schwannoma-derived growth factor)	3.105	4.801
Nur77	2.513	4.701
Early growth response 1	2.214	4.231
Metallothionein 1L	4.714	4.012
Nebulin	1.245	3.941
H4 histone, family 2	2.166	3.841
H1 histone family, member 0	5.343	3.185
Ubiquitin carrier protein	1.799	2.948
DNA-damage-inducible transcript 3	1.507	2.862
Metallothionein 1H	3.282	2.794
H2B histone family, member Q	1.067	2.779
Vanin 3	0.891	2.666
Legumain	2.277	2.446
Zinc finger protein 205	1.322	2.412
Microtubule-associated protein 1 light chain 3β	1.985	2.374
Phosphatidylinositol 4-kinase type II	2.129	2.158
Calmodulin-I (CALM1)	2.32	2.125
Early growth response 2	1.274	2.11
Immediate early protein	1.232	2.094
Microtubule-associated protein, RP/EB family, member 2	2.503	2.081
BCL2/adenovirus E1B	1.601	2.065
Clusterin	1.531	2.041
Gelsolin	2.1	1.945
P19(INK4D)	1.704	1.912
Heat shock 70kDa protein 1-like	2.806	1.231
Gene down-regulation		
Myosin IB	0.959	0.561
ErbB2	0.462	0.51
DR1-associated protein 1 (negative cofactor 2α)	0.772	0.5
Phosphatidic acid phosphatase type 2B	0.664	0.5
Ubiquilin 1	0.911	0.497
Epiregulin	0.728	0.496
Protein tyrosine phosphatase, receptor type, G	0.982	0.494
NRAS-related gene	0.671	0.489
Neurolysin (metallopeptidase M3 family)	1.06	0.481
SUMO-1-specific protease	0.645	0.479
RAB6A, member RAS oncogene family	0.78	0.478
A kinase (PRKA) anchor protein 2	0.817	0.477
EGFR	0.753	0.476
v-myc	0.74	0.472
Retinoblastoma binding protein 4	0.841	0.47
RAD21 homolog (S. pombe)	0.597	0.463
Zinc finger protein 36	0.628	0.455
Mitogen-activated protein kinase 6	0.537	0.449
Tumor endothelial marker 6	0.604	0.443
Son of sevenless homolog 2	1.153	0.435
EphA1	0.655	0.43
Cyclin D1	0.462	0.427
EBP50-PDZ interactor of 64 kD	0.483	0.418
Transforming growth factor, β2	0.465	0.413
Zinc finger protein 146	0.635	0.412
Cell division cycle 25B	0.621	0.4
Tumor endothelial marker 8	0.734	0.396
Annexin A4	1.152	0.395
Hypoxia inducible factor 1-α	0.536	0.395
Popeye protein 3	0.942	0.39
BCL2-associated athanogene 2	0.61	0.386
RAB31, member RAS oncogene family	0.919	0.379
Cullin 4A	0.618	0.378
Serine/threonine kinase 6	0.537	0.367
DEK oncogene (DNA binding)	0.535	0.362
RAB34, member RAS oncogene family	0.59	0.355
Topoisomerase (DNA) II α 170kDa	0.622	0.326
Ubiquitin-conjugating enzyme UBC3B 0.778	0.324	0.324
v-jun sarcoma virus 17	0.739	0.323
Angiomotin like 2	0.283	0.3
Caveolin 2	0.536	0.279
Caveolin 1	0.622	0.231
CASP8 and FADD-like apoptosis regulator	0.427	0.205
Plasminogen activator, urokinase	0.228	0.183

Boldface represents genes selected for validation.

isobologram model as described by Berenbaum.[19] Briefly, synergy was defined by the CI inequality d*a*/D*a* + d*b*/D*b* < 1, where d*a* and d*b* represent doses of agents *a* and *b* (*i.e.* CI-1033 and SAHA) used in combination, and D*a* and D*b* represent isoeffective doses of the same agents used alone. Combination studies determining apoptosis induction were evaluated using Student's *t* test with the resultant *p* value representing a 2-sided test of statistical significance.

Results

HDAC inhibition attenuates ErbB transcription

To identify genes regulated by HDAC inhibition, we used a 20,000 element (20K) cDNA microarray consisting of known, named genes as well as numerous expressed sequence tags (ESTs).[20] Initial experimentation was performed on LnCaP prostate cancer cells, which demonstrated down-modulation of EGFR expression, following exposure to 3 different HDAC inhibitors (TSA, SAHA and Apicidin) at 24 hr (data not shown). Further array studies were performed on cell lines overexpressing ErbB to examine the effect of the HDAC inhibitor SAHA on ErbB expression. Using the human prostate cancer cell line DU145 (moderate overexpression of both EGFR and ErbB) and the human breast cancer cell line SKBr3 (high-level expression of ErbB2), exposure to SAHA decreased transcript expression (>50%) of both EGFR and ErbB2 in the DU145 cell line, and ErbB2 in the SKBr3 cell line. Other notable genes in the DU145 expression profile with potential interaction to ErbB signaling include caveolin-1, HIF1-α, p19(INK4D) and Nur77 (Table I). To further validate these DNA microarray findings, we carried out real time RT-PCR for the transcript of these particular genes in the DU145 and SKBr3 cell lines. In DU145, we observed >50% down-modulation of both EGFR and ErbB2 transcript at 12 and 24 hr. In SKBr3, a >50% reduction in ErbB2 transcript was observed at 24 hr, with no change in EGFR transcript. We observed a greater than 50% reduction in caveolin-1 and HIF1-α and a 6.8- and 14.3-fold increase in p19(INK4D) transcript at 12 and 24 hr in the DU145 cell line, respectively. Additionally, a 17.3- and 62.7-fold increases in Nur77 transcript were observed in the DU145 cell line at 12 and 24 hr, respectively, and a 7.4-fold increase in the SKBr3 cell line at 24 hr (Fig. 1).

HDAC inhibition modulates ErbB protein expression

As mRNA transcript levels do not always correlate with protein levels, we examined the effect of HDAC inhibition on ErbB protein expression using 3 different HDAC inhibitors. HDAC inhibition by apicidin, TSA and SAHA attenuated levels of ErbB expression in DU145 (EGFR and ErbB2) and SKBr3 (ErbB2) cells at 24 hr. Similar results were obtained using different classes of HDAC inhibitors, including the short chain fatty acid valproate (data not shown). Therefore, this interaction likely represents a general phenomenon involving HDAC inhibitors. To determine if this interaction was applicable to other human cancers, we examined several other cell lines differentially expressing EGFR, including human prostate (PC3) and H&N SCC (data not shown). All cells lines demonstrated attenuated EGFR protein expression following 24 hr of HDAC inhibition (Fig. 2).

Cell cycle kinetics

The influence HDAC inhibition of cell cycle progression was evaluated using flow cytometry (Fig. 3). The HDAC inhibitor SAHA precipitated a G2/M phase arrest with a resulting decrease in the S-phase fraction, using doses that inhibited cellular proliferation (0.5 μM). A dose dependent increase in G2/M phase arrest was demonstrated at cytotoxic doses (1.0–5.0 μM). Similar results were obtained with TSA and valproic acid (results not shown).

HDAC inhibition enhances ErbB antiproliferative effects

The capacity of the HDAC inhibitor SAHA to enhance the antiproliferative effects of ErbB blockade was examined in a variety of human cancer cell lines with increased ErbB expression. The pan-ErbB tyrosine kinase inhibitor CI-1033 was selected to exploit its ability to target the entire family of ErbB oncoproteins.[21] Enhanced inhibition of proliferation was seen in human prostate (DU145, PC3), breast (SKBr3, MCF7)

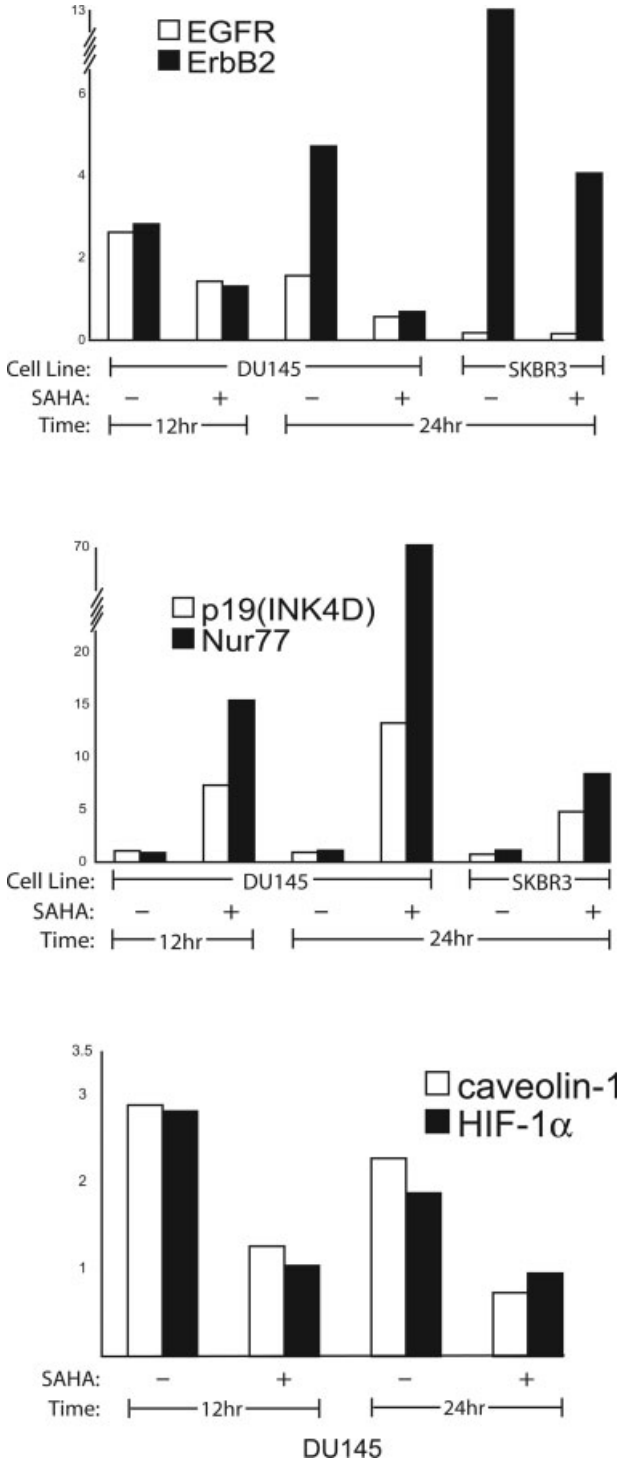

FIGURE 1 – Microarray validation using quantitative SYBR green RT-PCR to determine mRNA transcript modulated by HDAC inhibition in DU145 and SKBr3 cancer cell lines. RT-PCR was performed on each sample in duplicate, and the ratio was calculated relative to the housekeeping genes HMBS and GAPD.

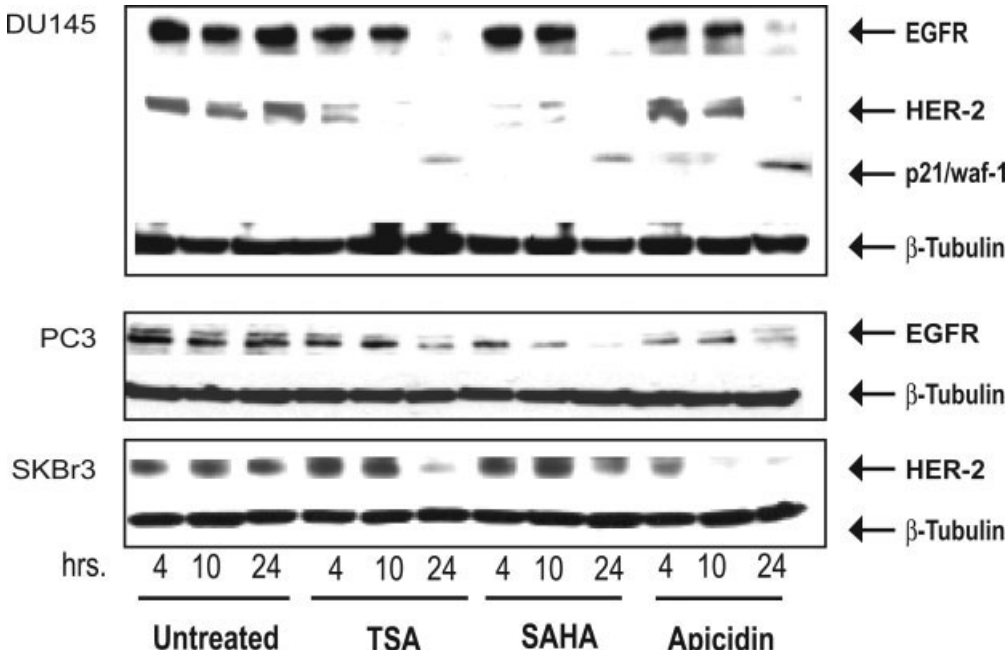

FIGURE 2 – HDAC inhibition down-modulates ErbB expression in prostate and breast cancer cell lines. DU145, PC3 and SKBr3 cells were treated with either TSA (0.3 μM), SAHA (1.0 μM) or Apicidin (1.0 μM) for 4, 10 or 24 hr. Western blot analysis was performed on whole cell lysates, using antibodies against EGFR, ErbB2, p21 (known CDKI upregulated by HDAC inhibition) and α-tubulin, which served as a loading control.

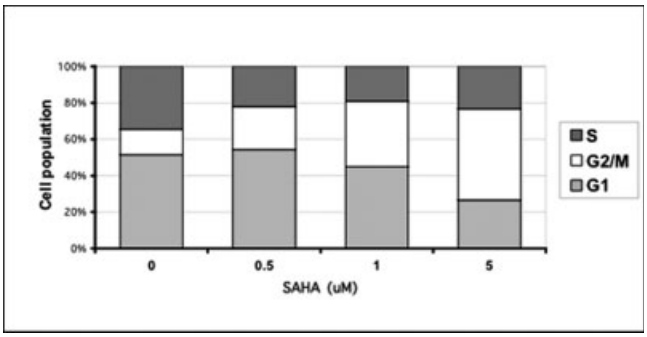

FIGURE 3 – Impact of SAHA on cell cycle phase distribution. DU145 cells were harvested after 24 hr exposure to SAHA at varying doses. Cells were subsequently stained with propidium iodide, and cell cycle distribution was determined by flow cytometry evaluation of DNA content.

and H&N SCC (UM-SCC6) when HDAC inhibition was combined with ErbB blockade (Fig. 4*a*). All cell lines demonstrated a trend of decreased cellular proliferation with the combination of CI-1033 and SAHA, although DU145, which overexpresses both EGFR and ErbB2, was the only line that achieved statistical significance ($p < 0.05$). A 25 and 45% reduction in cell proliferation was observed with independent treatments using CI-1033 (0.5 μM) and SAHA (0.5 μM). When combined, these agents induced a >70% reduction in DU145 cell proliferation. To further examine this interaction between CI-1033 and SAHA in the DU145 cell line, analysis was performed using the CI isobologram, according to methods described by Berenbaum.[19] CI values <1 indicate treatment synergy. Cells were treated with serial dilutions of CI-1033 and SAHA to determine the 70% isoeffective dose of growth inhibition, yielding a CI ranging from 0.79–0.90 (Fig. 4*b*). This suggests that this combination of agents produces a synergistic growth inhibitory effect in the DU145 cells.

HDAC inhibition enhances anti-ErbB downstream signaling

To determine if HDAC inhibitor-mediated down-modulation of ErbB expression translated into enhanced attenuation of cell signaling, we performed Western blot analysis on DU145 whole cell lysates treated with CI-1033, SAHA or both drugs in combination for 48 hr. Cells were then starved in serum free media for 2 hr and stimulated for 10 min with EGF (25 ng/ml). CI-1033 alone induced significant inhibition of both EGFR and AKT signaling. SAHA demonstrated modest inhibition of EGFR signaling, which may be secondary to EGFR down-modulation, and significant inhibition of AKT signaling. By combining HDAC inhibition with EGFR blockade, there was almost complete abrogation (>95% inhibition) of EGFR and AKT signaling (Fig. 5).

HDAC inhibition enhances CI-1033-induced apoptosis

Using a fluorescently labeled pan-caspase inhibitor, 24 hr exposure to either CI-1033 or SAHA alone induced a 3- and 3.5-fold increase in caspase activity, respectively, in DU145 cells. Combining HDAC inhibition with ErbB blockade induced $a > 7$-fold increase in caspase activity (supraadditive, $p < 0.01$) (Fig. 6*a*). This potentiation of apoptosis when combining anti-ErbB agents with HDAC inhibition was further assessed using Western blot analysis to determine cleavage of the death substrate, poly(ADP-ribose) polymerase (PARP). When combined with CI-1033, the HDAC inhibitors TSA and SAHA both demonstrated an increase in PARP cleavage after 24 hr (Fig. 6*b*).

Discussion

The results of the present study suggest the potential of HDAC inhibitors to enhance the antiproliferative and apoptotic effects induced by ErbB blockers. Coadministration of these agents may represent a worthy strategy for more effective molecular targeting of the ErbB oncogenic pathway. In this report, we demonstrate the capacity of HDAC inhibitors to down-modulate EGFR and ErbB2 expression in a variety of human cancer cell lines that moderately to significantly overexpress the ErbB oncoprotein. This interaction between HDAC inhibition and ErbB expression, as well as other tyrosine kinases such as bcr-abl, has been recently reported, and efforts to combine HDAC inhibitors with agents targeting these oncoproteins are currently underway.[22–25]

The potential importance of maximizing ErbB signaling inhibition has been demonstrated in recent reports, which indicate that increased inhibition of phospho-tyrosine activity of EGFR correlates with inhibition of proliferation; although tumor regression is

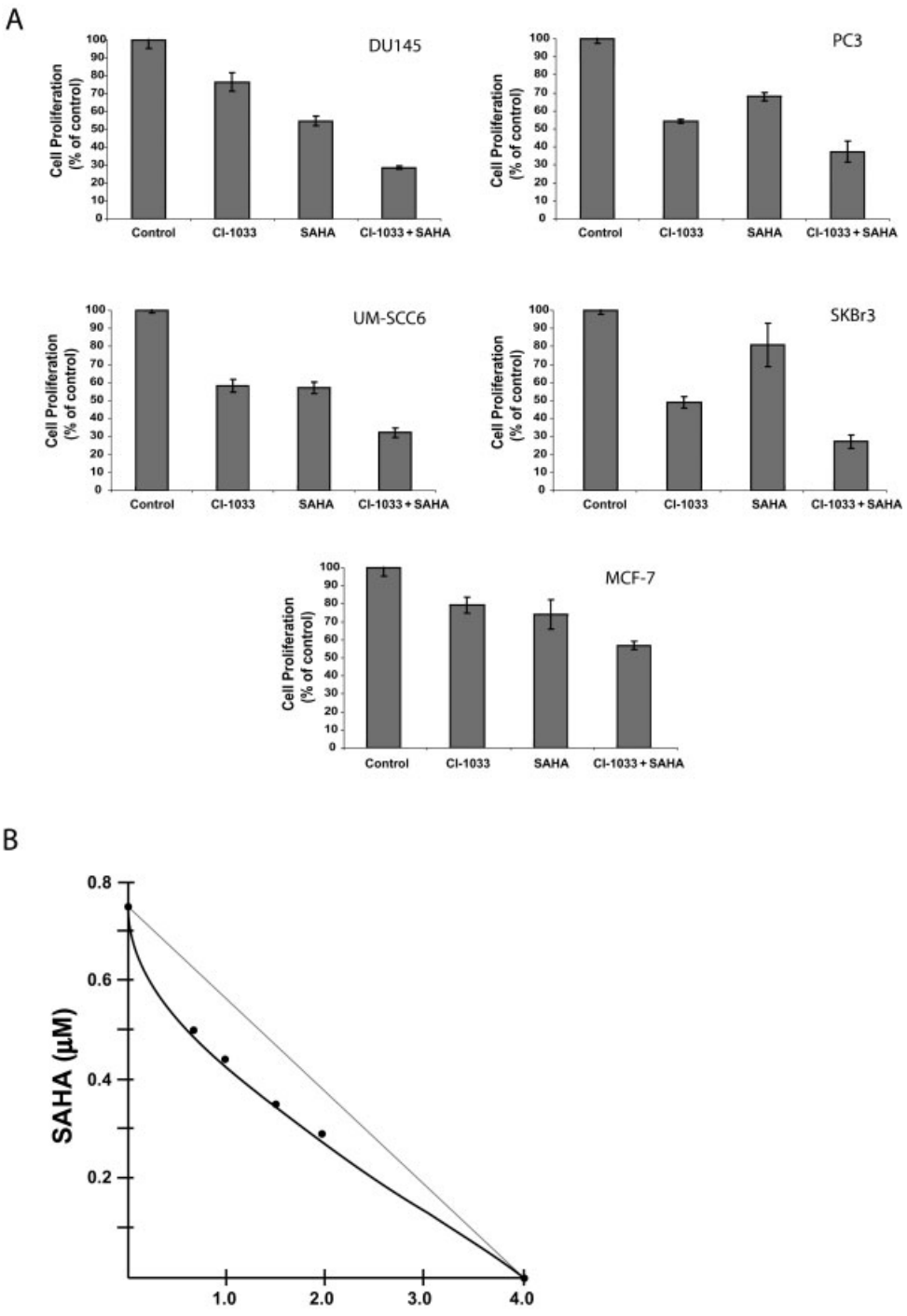

FIGURE 4 – HDAC inhibition enhances ErbB blocker capacity to inhibit cell proliferation. (*a*) Cells were plated in duplicate in 6-well plates and treated with CI-1033, SAHA or both drugs in combination for 72 hr. The dose of SAHA remained constant at 0.5 μM, although CI-1033 dose varied depending on cell sensitivity: UM-SCC6 (0.01μM), SKBr3 (0.1 μM), DU145 (0.5 μM), PC3 (1.0 μM) and MCF7 (1.0 μM). Cells were then stained with 0.5% crystal violet and eluted with 0.1 M sodium citrate (pH 4.2) in ethanol (1:1). Elution was transferred to 96-well plates and the absorbance was read at 540 nm to determine cell viability. (*b*) Isobole showing synergy for inhibition of cell proliferation between CI-1033 and SAHA in the DU145 cell line. The straight line indicates the zero interaction isobole, *i.e.*, the locus of all combinations that would produce this effect if there was no interaction. The 4 combinations tested (●) all reside below this reference line, indicating that less of the drug is required, *i.e.*, the combinations are synergistic.

only demonstrated with >70% decrease in receptor activity.[26] Furthermore, the inhibition of certain ErbB downstream signaling pathways, including AKT and MAPK, may require a much higher concentration of ErbB inhibitor than that needed to simply inhibit receptor phosphorylation.[27–29] This suggests that for effective blockade of critical downstream prosurvival and mitogenic signaling pathways, agents may need to be administered at higher doses. Therefore, combining agents with mechanistic synergy may maximize effective target inhibition and achieve a therapeutic window greater than that allowed by the maximum tolerated doses of individual agents. Similar findings of potential synergy have recently been demonstrated *in vitro* and *in vivo* by maximizing EGFR inhibition, using agents with complementary mechanisms of action, such as monoclonal antibodies and tyrosine kinase inhibitors.[30,31]

The mechanism underlying the influence of HDAC inhibition on ErbB expression is not well characterized. One potential explanation involves the capacity of HDAC inhibitors to cause disassociation of client protein ErbB from the Hsp90 stabilizing complex and enhance association with the destabilizing complex

containing Hsp70.[22,24] Targeting this interaction between client proteins with their molecular chaperones leads to destabilization and rapid selective degradation of the protein kinase. Interestingly, CI-1033 has been demonstrated to serve as a potent inducer of poly-ubiquitylation and degradation of ErbB2. The ubiquitylation and degradation of EGFR and ErbB2 by geldanamycin, an inhibitor of Hsp90, are specifically enhanced by CI-1033 via perturbation of the nucleotide-binding pocket of the receptor tyrosine kinase.[32] This propensity for augmentation of ErbB degradation when combining CI-1033 with HDAC inhibitors may explain how these agents can collaborate in arresting cell growth and apoptosis induction. This model, however, fails to explain the ability of HDAC inhibitors to repress ErbB at the transcript level, which may involve repression of new transcript synthesis and accelerated decay of mature ErbB mRNA.[24,33] We are currently investigating these interactions in further detail.

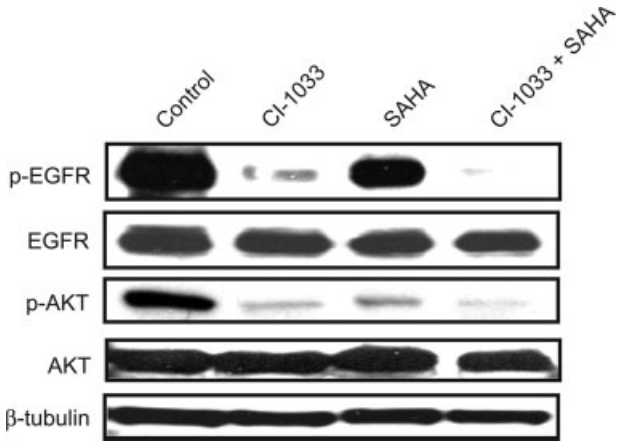

FIGURE 5 – HDAC inhibition enhances ErbB blocker attenuation of EGFR and AKT signaling. DU145 cells were treated with CI-1033 (0.5 μM), SAHA (0.5 μM) or both drugs in combination for 48 hr. Cells were then washed with PBS and starved in serum-free medium for 2 hr, followed by EGF stimulation (25 ng/ml) for 10 min. Western blot analysis was performed on whole cell lysates, using antibodies against EGFR, p-EGFR, AKT, p-AKT and α-tubulin.

We have also shown the ability of HDAC inhibitors to inhibit AKT phosphorylation, which has been identified as a key effector of PI3K-mediated cell survival. In addition to residing downstream of the ErbB signaling pathway, AKT represents another client protein for Hsp90. Recent literature demonstrates that although AKT is downstream of the EGFR signaling pathway, inhibition of its activity may require a much higher dose of drug than needed to inhibit receptor phosphorylation alone.[28,29] Abrogation of AKT signaling by combining CI-1033 with HDAC inhibition may also account for the augmentation of apoptosis observed in the tumor cell lines tested. Additionally, aberrant AKT signaling has been suggested as a key mediator in cellular resistance, including resistance to anti-ErbB agents, chemotherapy and radiation therapy.[34–36] Inhibition of AKT activity by HDAC inhibitors may offer a strategy to overcome these forms of cellular resistance. This potential application of HDAC inhibitors targeting the AKT pathway is currently being explored in our laboratory.

Selective targeting of tumor cells by HDAC inhibitors, as well as specific mechanisms of action, has not been well defined. Our data, and that of other gene profiling studies, suggest that HDAC inhibitors modulate only 2–12% of genes.[37–39] This relatively modest and selected number of genes affected by what one might expect as a global change in histone acetylation may reflect the hierarchical

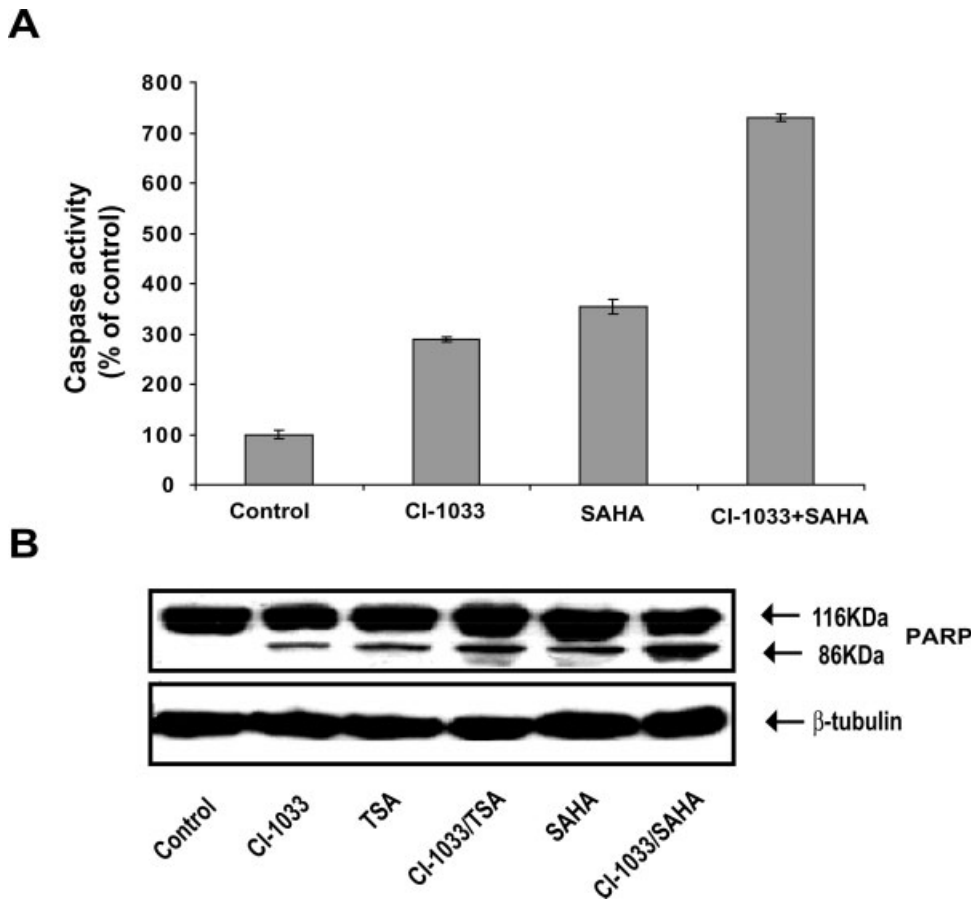

FIGURE 6 – HDAC inhibition enhances CI-1033-induced apoptosis. (*a*) Cells were seeded on 100-mm plates and treated with either vehicle control, CI-1033 (7.5 μM), SAHA (4 μM) or both drugs in combination for 24 hr. Cells were then harvested, stained with fluorescein-labeled pan-caspase inhibitor FAM-VAD-FMK and transferred to 96-well plate in triplicate. Fluorescence was analyzed on a Spectra-Max fluorescence plate reader at 550 nm excitation and 600 nm emission wavelengths. Data represent results averaged from 2 independent experiments, each performed in triplicate. (*b*) Cells were seeded on 100-mm plates and treated with either vehicle control, CI-1033 (7.5 μM), TSA (1 μM), SAHA (4 μM) or CI-1033 combined with TSA or SAHA for 24 hr. Western blot analysis was performed on whole cell lysates, determining cleavage of the PARP.

nature of epigenetic transcriptional control where DNA and histone methylation provide a dominant-repressive effect over histone acetylation.[11] Despite mechanistic uncertainty, genes reported to be affected by HDAC inhibition influence a variety of oncogenic processes including cell cycle progression, proliferation, angiogenesis, invasion, DNA damage repair and hormone resistance. In this regard, our microarray data suggest that in addition to modulatory influence on ErbB expression, HDAC inhibitors may have the potential to affect transcription of other genes associated with oncogenesis, including caveolin-1, HIF1-α, p19(INK4D), Nur77 and proteins linked to RAS signaling, including RAB34 and SOS homolog 2.

Caveolin-1 plays a regulatory role in several signaling pathways, and its activity has been demonstrated to be directly dependent on EGFR/Src signaling.[40–42] Caveolin-1 is highly expressed by prostate cancer cells and has been shown to positively correlate with stage, grade and clinical outcome in prostate cancer. Additionally, it has been demonstrated to be a metastasis-related gene, capable of suppressing apoptosis, and conveying resistance to multiple antineoplastic agents.[43] Our data and other independent reports[44] demonstrate the ability of HDAC inhibitors to attenuate the expression of caveolin-1, representing another potential mechanistic synergy between HDAC inhibition and the ErbB pathway in prostate cancer.

Recent studies have demonstrated the effect of HDAC inhibition on tumor angiogenesis by down-modulation of vascular endothelial growth factor (VEGF) and HIF1-α.[44,45] The VEGF molecule is critical for tumor angiogenesis, is highly expressed in prostate cancer cells and has been shown to positively correlate with stage, grade and clinical outcome in prostate cancer.[44–47] Although not examined in our study, down-regulation of VEGF by HDAC inhibitors may directly inhibit the proliferation of endothelial cells and potentiate the sensitivity of tumor cells to chemotherapy and radiation therapy. Additionally, since ErbB blockade has been demonstrated to independently decrease VEGF transcription,[48,49] *in vivo* combination studies would be of interest in determining potential enhancement of angiogenesis inhibition.

An additional gene of interest that we and others identify to be modulated by HDAC inhibition is HIF1-α.[50,51] HIF1-α is overexpressed in many human malignancies and serves as a potent transactivator of VEGF, thereby promoting cellular survival and growth under hypoxic conditions. The down-modulation of HIF1-α may provide mechanistic explanation for the previously described VEGF regulation by HDAC inhibition. Additionally, HIF1-α and its transcription have been implicated in the neovascularization of solid tumors and radioresistance in colon cancer, cervical cancer and malignant gliomas, therefore representing a potential target with clinical application.[52,53]

The ability of ErbB and HDAC inhibitors to produce cell cycle arrest is well documented. The cyclin dependent kinase (CDK) inhibitor p21, which can regulate cell cycle progression by inhibiting the catalytic activity of CDK, is markedly up-regulated by HDAC inhibitors in many cell types. This was confirmed at the mRNA and protein levels and translated to a dose-dependent increase in G2/M phase arrest. Regulation of p21 appears critical for the activity of HDAC inhibitors, and the ability to substantially up-regulate p21 may govern the fate of the cell, regarding cytostasis or apoptosis following HDAC inhibition.[39,54] In addition to p21 expression, our data also demonstrate the ability of HDAC inhibitors to up-regulate transcription of another CDKI, namely p19(INK4D) in both prostate and breast cancer cell lines. Although the mechanism for this increased expression is not well understood, a similar effect on protein reexpression has been observed when DNA methylation is inhibited in lung cancer cell lines.[55]

In addition to abrogating AKT signaling, the ability of HDAC inhibitors to induce apoptosis may be involved with its potent transactivation of the Nur77 transcript. Nur77 is an orphan receptor and a member of the steroid/thyroid hormone family of receptor proteins,[56,57] which play a key role in apoptosis of T lymphocytes and various other cell types.[58] In prostate cancer cells, Nur77 is rapidly induced following exposure to apoptotic agents and its expression has been demonstrated to enhance tumor cell sensitivity to cytotoxic agents.[59] Apoptosis induction following HDAC inhibition may involve the capacity of Nur77 to induce conformational changes in Bcl-2, converting it from anti- to pro-apoptotic factor.[60] Mechanisms underlying these interactions are currently being evaluated.

In summary, the potential of HDAC inhibitors to modulate expression of a variety of oncopoteins render them a promising class of agents to be examined in concert with other anticancer agents. The current results suggest that, when used cooperatively, anti-ErbB agents combined with HDAC inhibitors may offer a unique strategy of dual targeted therapy by inhibiting the functional activity of the ErbB receptor as well as attenuating overall ErbB expression. This potential for maximizing ErbB inhibition by exploiting this mechanistic synergy may prove valuable for enhancing antitumor activity and overall clinical response. Additionally, preliminary cDNA microarray data suggest that the beneficial interaction of these agents may not derive solely from modulation of ErbB expression, but may result from effects on other oncogenic processes including angiogenesis, invasion and cell cycle kinetics. *In vivo* studies should provide further insight regarding the potential significance of these molecular interactions.

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