

# Histone deacetylase inhibitor LAQ824 down-regulates Her-2 and sensitizes human breast cancer cells to trastuzumab, taxotere, gemcitabine, and epothilone B

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## Abstract

Histone deacetylase inhibitors induce hyperacetylation of the amino-terminal lysine residues of the core nucleosomal histones, which results in chromatin remodeling and altered gene expression. Present studies demonstrate that exposure to a novel hydroxamic acid analogue histone deacetylase inhibitor, LAQ824, induced p21<sup>WAF1</sup> and p27<sup>KIP1</sup> and caused growth arrest and apoptosis of human breast cancer SKBR-3 and BT-474 cells that possess amplification and overexpression of Her-2/*neu*. Treatment with LAQ824 depleted the mRNA and protein levels of Her-2/*neu*-encoded Her-2, which was associated with attenuation of pAKT, c-Raf-1, and phosphorylated mitogen-activated protein kinase levels. LAQ824 also induced the acetylation of heat shock protein (hsp) 90, resulting in inhibition of its binding to ATP, which has been shown to impair the chaperone association of hsp 90 with its client proteins, Her-2, AKT, and c-Raf-1. Consistent with this, treatment with LAQ824 shifted the binding of Her-2 from hsp 90 to hsp 70, promoting proteasomal degradation of Her-2. Thus, LAQ824 depletes Her-2 through two mechanisms: attenuation of its mRNA levels and promotion of its degradation by the proteasome. Following LAQ824 treatment, the cell membrane association, autotyrosine phosphorylation, and colocalization of Her-2 with HER-3 also declined. Cotreatment with LAQ824 significantly increased trastuzumab-induced apoptosis of BT-474 and SKBR-3 cells. This was associated with greater attenuation of Her-2, c-Raf-1, and pAKT levels. LAQ824 also enhanced taxotere-induced, epothilone B-induced, and gemcitabine-induced apoptosis of BT-474 and SKBR-3 cells. These findings suggest that LAQ824 is

active against human breast cancer cells and has the potential to improve the efficacy of trastuzumab, taxotere, gemcitabine, and epothilone B against breast cancer with Her-2/*neu* amplification. (Mol Cancer Ther. 2003;2:971–984)

## Introduction

Despite recent progress in our understanding of its genetic and molecular basis, advanced and metastatic breast cancer remains incurable (1). Encouragingly, a number of agents with diverse cellular targets and mechanisms of actions have been developed that are active against breast cancer cells (2). Approximately 25% of breast cancers have amplification and overexpression of Her-2/*neu* oncogene (3), which encodes for a member of the family of epidermal growth factor receptor tyrosine kinases (4). Dimerization of Her-2 with HER-3 results in activation and signaling through the phosphatidylinositol 3-kinase (PI3K)/AKT and Ras-Raf-extracellular signal-regulated kinase (Erk) pathways, promoting cell proliferation and survival, and is associated with poor prognosis in breast cancer (4, 5). Her-2-mediated signaling also confers resistance to apoptosis due to antibreast cancer agents, including tubulin-polymerizing agents (TPAs; *e.g.*, taxotere), as well as antiestrogen treatment with tamoxifen (6–8). Conversely, repression of Her-2 attenuates its antiapoptotic signaling and suppresses Her-2-induced malignant phenotype, making Her-2 an excellent therapeutic target in breast cancer (9, 10). Indeed, a recombinant, humanized, monoclonal anti-Her-2 antibody, trastuzumab (Herceptin), has been shown to dephosphorylate and down-regulate Her-2 levels and signaling as well as to exhibit clinical efficacy against breast cancer (11). Cotreatment with trastuzumab also improves the antibreast cancer activity of chemotherapeutic agents, especially TPAs (12–15). However, resistance to trastuzumab, given alone or in combination with chemotherapeutic agents, is common, involving mechanisms that involve the activation of PI3K/AKT and/or Erk1/2 signaling (16–18). This highlights the need to develop new strategies that would target the mechanisms of resistance and enhance sensitivity of breast cancer to trastuzumab and chemotherapeutic agents (19).

Geldanamycin (GA) and its less toxic analogue 17-allylamino-demethoxygeldanamycin (17-AAG) are inhibitors of the heat shock protein (hsp) 90, which is an intracellular chaperone for a number of client proteins that include protein kinases and nuclear hormone receptors (20–22). Among the protein kinases that are chaperoned by hsp 90 are Her-2, AKT, c-Raf-1, and Bcr-Abl (23–26).

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Depletion of c-Raf-1 and inhibition of Erk1/2 phosphorylation as well as attenuation of pAKT by GA or 17-AAG leads to disruption of the Raf-mitogen-activated protein kinase (MAPK)/Erk kinase-MAPK (Erk1/2) and PI3K/AKT signaling, resulting in cytoskeleton and apoptosis of cancer cells (27). Treatment with GA or 17-AAG has also been demonstrated to down-regulate Her-2 and its downstream signaling (28). This is associated with sensitization of breast cancer cells to chemotherapeutic agents (29). Thus, a hsp 90 inhibitor has the potential to exert dual antibreast cancer effect (*i.e.*, depletion of Her-2 and its signaling as well as attenuation of AKT and c-Raf-1 activity that may be independent of down-regulation of Her-2 signaling). Whether this would translate into superior clinical efficacy of 17-AAG with or without antibreast cancer agents, including Herceptin, TPAs, and gemcitabine, has not been confirmed.

Acetylation, phosphorylation, and methylation are among the key post-translational modifications of the amino-terminal tails of core nucleosomal histones (30). The combinatorial nature of the histone modifications represents a specific epigenetic code called the "histone code" (31). Distinct modifications either increase or decrease the interaction affinity of the nonhistone protein transcriptional complexes with DNA, thereby regulating dynamic transitions between transcriptionally active and silent states of the chromatin (32). Histone acetyltransferases and histone deacetylases (HDAC) catalyze the acetylation and deacetylation of the lysine residues in the histone tails, respectively (33, 34). The acetylation of histones H3 and H4 at specific lysine residues by histone acetyltransferases is associated with transcriptional activation; hence, the histones associated with active genes are highly acetylated (30). Conversely, histones associated with repressed heterochromatin are hypoacetylated due to the activity of HDACs (34). Treatment with HDAC inhibitors (HDI) causes hyperacetylation of the amino-terminal lysine residues in the nucleosomal histones, which has been shown to transcriptionally up-regulate p21<sup>WAF1</sup> (referred to as p21) expression (35). This is associated with cell cycle arrest and apoptosis of cancer and leukemia cells (36–38). The hydroxamic acid analogues (*e.g.*, trichostatin A [TSA] and suberoylanilide hydroxamic acid [SAHA]) have been demonstrated to induce p21 and p27<sup>KIP1</sup> (referred to as p27) as well as to exert potent *in vitro* and *in vivo* anticancer effects (35–39). Depsipeptide (FK228), a natural product HDI, was also shown to cause acetylation of the hsp 90 in lung cancer cells (40). This was shown to destabilize the chaperone complex of hsp 90 with its client proteins and targeted them for degradation by the proteasome (40). Recently, a cinnamic acid hydroxamate, LAQ824, has been demonstrated to act as a potent HDI and exerts anticancer effects at nanomolar concentration (41). In the present studies, we took a more comprehensive look at the effects of treatment with LAQ824 on the mRNA and protein expression of Her-2/*neu* gene and on the cell cycle status and apoptosis of cultured human breast cancer cells. We determined

whether LAQ824 treatment would also induce the acetylation of hsp 90 and disrupt its chaperone association with Her-2, leading to the proteasomal degradation and depletion of Her-2. Furthermore, whether cotreatment with LAQ824 would enhance the apoptosis induced by antibreast cancer agents, including trastuzumab, taxotere, epothilone B (Epo B), and gemcitabine, was also probed in the present studies.

## Materials and Methods

### Reagents

LAQ824 and Epo B were kindly provided by Novartis Corp. (Basel, Switzerland). Taxotere was a gift from Aventis Pharmaceuticals (Bridgewater, NJ). Trastuzumab was a gift from Genentech (South San Francisco, CA). Taxotere and Epo B were made at a concentration of 10 mM in DMSO and stored at  $-20^{\circ}\text{C}$ . 17-AAG was kindly provided by the Developmental Therapeutics Branch of Cancer Therapy Evaluation Program/National Cancer Institute/NIH (Bethesda, MD). Proteasome inhibitor, *N*-acetyl leucyl-leucyl norlucinal (ALLnL), was purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal anti-poly(ADP-ribose) polymerase (PARP) was purchased from Cell Signaling (Beverly, MA). Anti-Bax, anti-cIAP2, and anti-Mcl-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hsp 70 and anti-hsp 90 antibodies were purchased from Stress-Gen Biotechnologies Corp. (Victoria, British Columbia, Canada). Other antibodies for the immunoblot analyses to detect the levels of proteins, including Bcl-xL, pAKT, AKT, p21, and p27, were obtained, as described previously (42, 43).

### Cell Culture

The human breast cancer cell lines BT-474 and SKBR-3 were obtained from American Type Culture Collection (Manassas, VA) and maintained in the recommended culture medium. Culture mediums were supplemented with 10% fetal bovine serum, 100-units/ml penicillin, and 100- $\mu\text{g}/\text{ml}$  streptomycin (Life Technologies, Inc., Grand Island, NY) at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator (44).

### Flow Cytometric Analysis of Cell Cycle Status

The flow cytometric evaluation of the cell cycle status was performed according to a previously described method (42, 44). The percentage of cells in the G<sub>1</sub>, S, and G<sub>2</sub>-M phases were calculated using Multicycle software (Phoenix Flow Systems, San Diego, CA).

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltertrazolium Bromide Assay

Cytotoxic effect of the drugs was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay, as described previously (45). Cells ( $1-5 \times 10^3$  in 100  $\mu\text{l}$ /well) were grown overnight in 96-well plates. Fresh media (100  $\mu\text{l}$ ) containing the designated drug was added for specified exposure intervals. At the completion of incubation, media was replaced with fresh

complete media (100  $\mu$ l). Three hours before the end of the incubation period, 20  $\mu$ l of PBS containing MTT (5 mg/ml) was added to each well. Following this, the plates were centrifuged at  $200 \times g$  for 5 min and the media was removed. The precipitate was then resuspended in 100  $\mu$ l of DMSO. The absorbance was measured on a plate reader at 540 nm. Each experiment was performed in triplicate.

#### Apoptosis Assessment by Annexin V Staining

After drug treatment, cells were resuspended in 100- $\mu$ l 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 min, cells were analyzed by flow cytometry (42, 44). Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with Annexin V) and necrotic cells (stained with both Annexin V and propidium iodide).

#### Morphology of Apoptotic Cells

After drug treatment, cells were washed with PBS and resuspended in the same buffer. Cytospin preparation of  $50 \times 10^3$  cells were fixed and stained with Wright-Giemsa stain (Biochemical Sciences Inc., Swedesboro, NJ). Cell morphology was determined by light microscopy. In all, five different fields were randomly selected for counting at least 500 cells. The percentage of apoptotic cells was calculated for each experiment, as described previously (42, 44).

#### Western Blot Analyses

Western blot analyses of Her-2, c-Raf-1, AKT, pAKT, p21, p27, acetylated histones H3 and H4 proteins, and  $\beta$ -actin were performed using specific antisera or monoclonal antibodies (see above), as described previously (42, 44). Horizontal scanning densitometry was performed on Western blots by using acquisition into Adobe PhotoShop (Adobe Systems Inc., San Jose, CA) and analysis by the NIH Image Program (NIH, Bethesda, MD). The expression of  $\beta$ -actin was used as a control.

#### Immunoprecipitation

Following the designated treatments, cells were lysed in the lysis buffer (1% SDS, 1% Triton X-100, 0.5% deoxycholate, 150-mM NaCl, 2-mM EDTA, 1-mM phenylmethylsulfonyl fluoride, 10- $\mu$ g/ml leupeptin) for 1 h, and the nuclear and cellular debris was cleared by centrifugation. Protein G agarose beads were washed twice with radioimmunoprecipitation assay (RIPA)-1 buffer (50-mM Tris [pH 7.5], 150-mM NaCl, 1-mM EDTA, 1% NP40, 0.1% deoxycholate) and incubated with hsp 90-specific monoclonal antibody (1 in 100; Stressgen) at 4°C for 2 h. After washing the protein G and the antibody mix with RIPA-1 buffer, 100  $\mu$ g of total cell lysates were added and incubated overnight at 4°C. The immunoprecipitates were washed thrice in RIPA-1 buffer and proteins were eluted with the SDS sample loading buffer prior to the immunoblot analyses (44).

#### Immunoprecipitation of Conformationally Changed Bax

Cells are lysed in 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfate (CHAPS) lysis buffer (150-mM NaCl, 10-mM HEPES [pH 7.4], 1% CHAPS) containing protease inhibitors. Immunoprecipitation is performed in lysis buffer by using 500 Tg of total cell lysate and 2.5 Tg of anti-Bax 6A7 monoclonal antibody (Sigma). The resulting immune complexes as well as the supernatants are subjected to immunoblotting analysis with anti-Bax polyclonal rabbit antiserum, as described previously (46).

#### Immunofluorescent Microscopy for Her-2 and HER-3 Expressions

Cells were washed twice in PBS, fixed in 4% paraformaldehyde for 30 min, and permeabilized in 0.5% Triton X-100/PBS for 15 min. After preblocking with 3% BSA/PBS, cells were incubated with primary antibodies at 37°C for 1 h followed by three washes in PBS and incubation with FITC-conjugated or rhodamine-conjugated secondary antibodies for 1 h at 37°C. Cells were washed in PBS before nuclear staining with 0.1  $\mu$ g/ml of 4',6-diamino-2-phenylindole (DAPI) and analysis by a fluorescence microscopy, as described previously (47). Controls for immunofluorescence experiments included use of isotype and subclasses matched monoclonal antibodies when employing monoclonals and preimmune serum when employing polyclonal antibodies.

#### Northern Blot Analysis

Northern blot analysis of Her-2/*neu* mRNA was performed using a previously reported method (43). Total RNA was extracted from treated and untreated cells using RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Total RNA (20  $\mu$ g) was separated on 1% agarose gel containing formaldehyde (0.22 M) and transferred overnight to an uncharged nylon membrane by capillary transfer in  $10 \times$  SSC. Membranes were treated with UV Crosslinker (Stratagene, La Jolla, CA) and subsequently probed with  $^{32}$ P- $\alpha$ [dCTP]-radiolabeled probe. A 1.6-kb fragment of Her-2/*neu* cDNA was used as the probe.

#### Real-Time Quantitative Reverse Transcription-PCR

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen) and real-time reverse transcription-PCR analysis was performed, as described previously (42). Two microliters of total RNA (0.1  $\mu$ g) were added to 23  $\mu$ l of a reaction mix (Taqman RT-PCR Reagent Kit, Perkin-Elmer, Boston, MA) consisting of 19.7  $\mu$ l of H<sub>2</sub>O, 0.15  $\mu$ l of enzyme mix, 0.5  $\mu$ l of forward primer, 0.5  $\mu$ l of reverse primer, 1.0  $\mu$ l of Her-2/*neu* gene-specific oligonucleotide probe, 0.5  $\mu$ l of histone H1 forward primer, 0.5  $\mu$ l of histone H1 reverse primer, and 0.5  $\mu$ l of histone-specific oligonucleotide probe, combined and placed in a 96-well plate. The plate was transferred to an ABI 7700 thermocycler (Perkin-Elmer), and samples were processed at 48°C for 30 min and then at 95°C for 10 min. These were followed by 35 amplification cycles (95°C, 15 s and 60°C, 1 min) during which fluorescent signals of Her-2/*neu* and histone H1 probes were measured, as described previously. Serial dilutions of  $10^6$  to  $10^1$  of Her-2/*neu* plasmid DNA and histone H1 plasmid DNA

were used as a reference standard of fluorescent signals for copy number, as described previously (42). All the probes carried a 5' FAM reporter label and a 3' TAMRA quencher group. All primers and probes were synthesized by PE-Applied Biosystems (Perkin-Elmer).

#### Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) analysis was performed by a slight modification of a previously described method (35). Cells were incubated overnight at a density of  $0.25 \times 10^6$  cells/ml at 37°C with 5% CO<sub>2</sub>. On the next day, cells were cultured with 0, 50, 100, or 250 nM of LAQ824 for 24 h. Formaldehyde was then added to the cells to a final concentration of 1%, and the cells were gently shaken at room temperature for 10 min. Following this, the cells were pelleted and suspended in 1 ml of ice-cold PBS containing protease inhibitors (Complete, Boehringer Mannheim, Indianapolis, IN). Cells were again pelleted, resuspended in 0.5 ml of SDS lysis buffer (1% SDS/1.0-mM EDTA/50-mM Tris-HCl [pH 8.1]), and incubated on ice for 20 min. Lysates were sonicated with 15-s bursts. Debris was removed from samples by centrifugation for 20 min at  $15,000 \times g$  at 4°C. An aliquot of the chromatin preparation (100  $\mu$ l) was set aside and designated as input fraction. The supernatants were diluted 3-fold in the immunoprecipitation buffer (0.01% SDS/1.0% Triton X-100/1.2-mM EDTA/16.7-mM Tris-HCl [pH 8.1]/150-mM NaCl), and 80  $\mu$ l of 50% protein A-Sepharose slurry containing 20- $\mu$ g sonicated salmon sperm DNA and 1-mg/ml BSA in the TE buffer (10-mM Tris-HCl [pH 8.0]/1-mM EDTA) were added and incubated by rocking for 2 h at 4°C. Beads were pelleted by centrifugation, and supernatants were placed in fresh tubes with 5  $\mu$ g of the acetylated histone H3 antibody, acetylated histone H4 antibody, or normal rabbit serum and incubated overnight at 4°C. Protein A-Sepharose slurry (60  $\mu$ l) was added, and the samples were rocked for 1 h at 4°C. Protein A complexes were centrifuged and washed thrice for 5 min each with immunoprecipitation buffer and twice for 5 min each with immunoprecipitation buffer containing 500-mM NaCl. Immune complexes were eluted twice with 250  $\mu$ l of elution buffer (1% SDS/0.1-M NaHCO<sub>3</sub>) for 15 min at room temperature. Twenty microliters of 5-M NaCl were added to the combined eluates, and the samples were incubated at 65°C for 24 h. EDTA, Tris-HCl (pH 6.5), and proteinase K were then added to the samples at a final concentration of 10 mM, 40 mM, and 0.04  $\mu$ g/ $\mu$ l, respectively. The samples were incubated at 37°C for 30 min. Immunoprecipitated DNA (both immunoprecipitation samples and input fraction) was recovered by phenol/chloroform extraction and ethanol precipitation and analyzed by PCR. The Her-2/*neu*-specific primers were used to perform PCR on DNA isolated from ChIP experiments and input samples. The optimal reaction conditions for PCR were determined for each primer pair. Parameters were denaturation at 95°C for 1 min and annealing at 56°C for 1 min followed by elongation at 72°C for 1 min. PCR products were analyzed by 2.5% agarose/ethidium bromide gel electrophoresis. The primer pair used for Her-2/*neu* was forward primer: 5' AAC ACA TCC

CCC TCC TTG ACT ATC 3' and reverse primer: 5' AGC TTC ACT TTC TCC CTC TCT TCG 3'. The size of the amplified product was 330 bp. The ratio of immunoprecipitated DNA to input DNA was calculated for each treatment and primer set. The fold increase after treatment with LAQ824 was calculated from the indicated ratio.

#### Acetylation of hsp 90 and Its Binding to ATP-Sepharose

Untreated or LAQ824-treated cells were lysed in TNESV buffer (50-mM Tris, 2-mM EDTA, 100-mM NaCl, 1-mM sodium vanadate, 1% NP40 at pH 7.5), and total cellular proteins were quantified using the BCA Protein Assay (Pierce Biotechnology, Inc., Rockford, IL). hsp 90 was immunoprecipitated from 200  $\mu$ g of total protein using mouse hsp 90 antibody, and immunoprecipitates were immunoblotted with antiacetyl lysine antibody, as described previously (41). Alternatively, cell lysates were prepared after drug treatment and hsp 90 was affinity precipitated from 200  $\mu$ g of total cellular protein using ATP-Sepharose beads. The concentration of hsp 90 in the precipitates was assessed by Western blot analysis using a polyclonal anti-hsp 90 antibody (41).

#### Preparation of Detergent-Soluble and Detergent-Insoluble Fractions

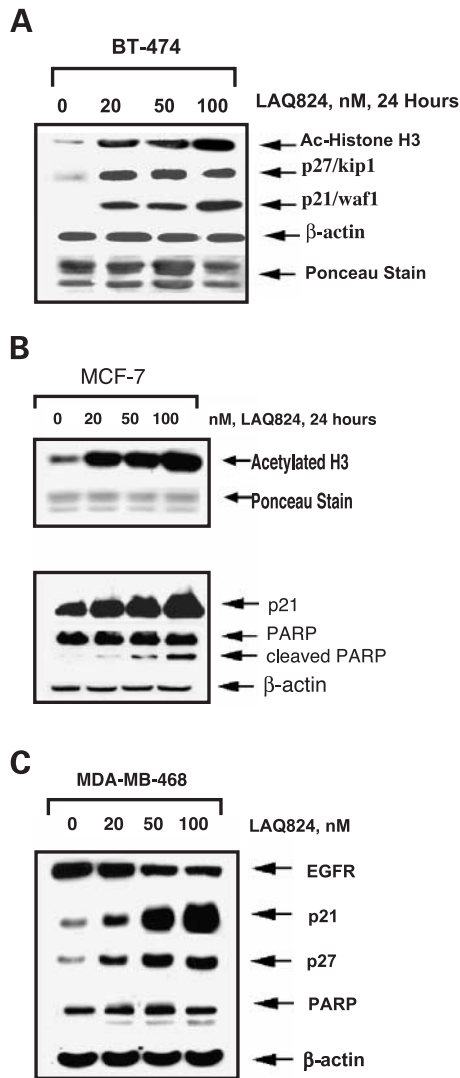
After the designated drug treatments, cells were lysed with TNSEV buffer (50-mM Tris-HCl [pH 7.5], 2-mM EDTA, 100-mM NaCl, 1-mM sodium orthovanadate, 1% NP40 containing 20- $\mu$ g/ml aprotinin, 20- $\mu$ g/ml leupeptin, 1-mM phenylmethylsulfonyl fluoride, 25-mM NAF, and 5-mM *N*-ethylmaleimide; 40). The insoluble fraction (pellet) was solubilized with SDS buffer (80-mM Tris [pH 6.8], 2% SDS, 100-mM DTT, and 10% glycerol). Fifty micrograms of proteins from the NP40 soluble and insoluble fractions were separated on 7.5% SDS polyacrylamide gel and analyzed by Western blotting (44).

#### Statistical Analyses

Data were expressed as means  $\pm$  SEM. Comparisons used Student's *t* test or ANOVA, as appropriate. *P* values of <0.05 were assigned significance.

## Results

We first determined the effects of LAQ824 treatment on histone acetylation, p21, p27, and Her-2 levels, and cell cycle growth arrest and apoptosis of human breast cancer BT-474 and SKBR-3 cells with amplification and overexpression of Her-2. Exposure to 20–100-nM LAQ824 for 24 h increased the acetylation of histone H3 in BT-474 cells (Fig. 1A). This was associated with induction of p21 and p27 expression (Fig. 1A). Similar effects were observed in SKBR-3 cells (data not shown). These results are consistent with the previous reports that another hydroxamic acid analogue, SAHA, induces hyperacetylation of nucleosomal histones associated with p21 but not p27 gene promoter, thereby transcriptionally up-regulating p21 but increasing p27 levels by a nontranscriptional mechanism (35). Fig. 1, B and C, demonstrates that exposure to LAQ824 also results in histone H3 acetylation, increases p21 and p27 levels, and



**Figure 1.** LAQ824 induces histone acetylation, increases p21 and p27, and induces caspase activity in breast cancer cells. BT-474 (A), MCF-7 (B), and MB-468 (C) cells were treated with LAQ824 at the indicated concentrations for 24 h. Following the isolation of histones, histone H3 acetylation was detected by Western blot analysis using an acetylated *Ac-histone H3* antibody. Ponceau staining (*Ponceau Stain*) was used to control for total protein loading. Total cell lysates of the cells were also probed with anti-p21, anti-p27, anti-PARP, and β-actin antibodies.

induces PARP cleavage activity of caspases in MCF-7 and MB-468 breast cancer cell types that do not contain amplification of *Her-2/neu* gene. The effect of LAQ824 on the cell cycle status of BT-474 and SKBR-3 cells is shown in Table 1. The results show that exposure to 100-nM LAQ824 for 24 h markedly increased the percentage of cells in the  $G_2$ -M phase of the cell cycle. We next determined whether the perturbations in cell cycle status of the breast cancer cells induced by LAQ824 were accompanied by the molecular events associated with apoptosis. As shown in Fig. 2A, treatment with LAQ824 induced the conformational change in the cytosolic Bax, detected by immunoblot analysis with the anti-Bax 6A7 antibody, without affecting the total intracellular levels of Bax in SKBR-3 cells.

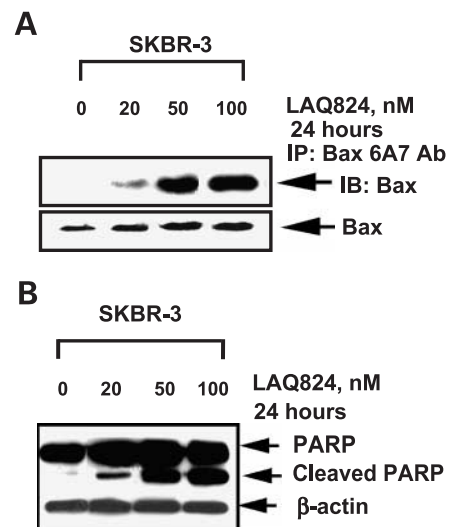
**Table 1.** Cell cycle status of SKBR-3 and BT-474 breast cancer cells after 24-h exposure to LAQ824

	$G_1$	S	$G_2$ -M <sup>a</sup>
SKBR-3			
Control	50.9 ± 3.5	24.7 ± 4.8	24.4 ± 1.7
LAQ824 (20 nM)	50.7 ± 2.3	20.0 ± 5.8	29.3 ± 3.7
LAQ824 (100 nM)	52.6 ± 3.7	1.2 ± 2.2	46.2 ± 5.8 <sup>a</sup>
BT-474			
Control	76.9 ± 2.7	14.2 ± 0.6	9.0 ± 3.3
LAQ824 (20 nM)	55.6 ± 1.6	35.1 ± 0.0	9.3 ± 1.7
LAQ824 (100 nM)	38.5 ± 4.4	32.1 ± 0.5	29.4 ± 3.9 <sup>a</sup>

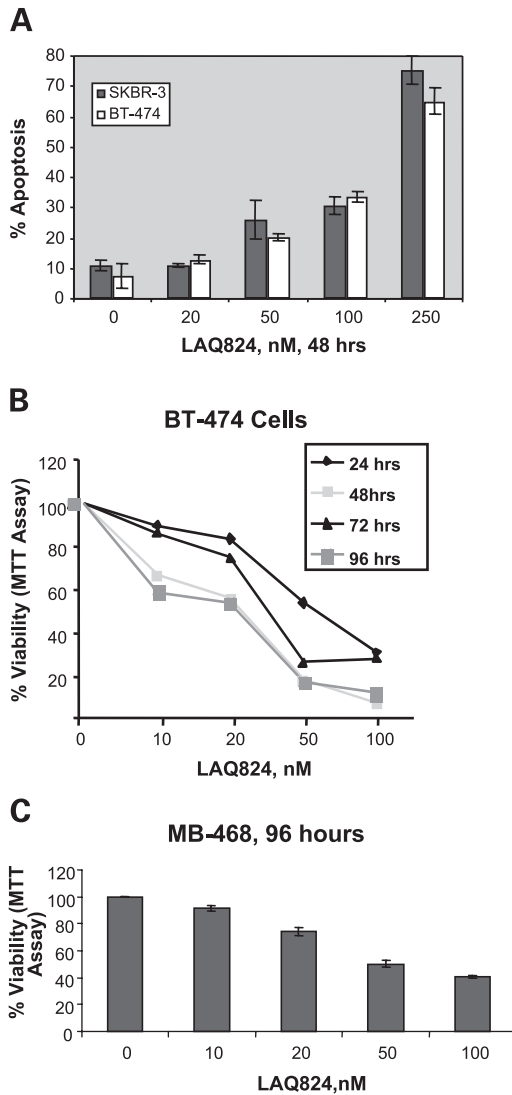
SKBR-3 and BT-474 cells were exposed to 20 or 100 nM of LAQ824 for 24 h. Following this, the cells were stained with propidium iodide, and the DNA content was evaluated by flow cytometry to determine the cell cycle status of the control and LAQ824-treated cells. Values represent the means ± SE of three experiments.

<sup>a</sup>Values of %  $G_2$ -M are significantly different from those observed in untreated control cells.

Exposure to LAQ824 also induced the PARP cleavage activity of caspase-3 in SKBR-3 cells (Fig. 2B). Treatment of BT-474 and SKBR-3 cells with LAQ824 also induced a dose-dependent increase in the percentage of apoptotic cell, as detected by positive staining for Annexin V (Fig. 3A). Exposure to 250 nM of LAQ824 for 48 h induced apoptosis of ~70% of SKBR-3 and BT-474 cells (Fig. 3A). This was also confirmed by morphological assessment of apoptosis (data not shown). A dose-dependent and time-dependent



**Figure 2.** LAQ824 induces Bax conformational change and PARP cleavage activity of caspase-3. SKBR-3 cells were treated with 0, 20, 50, or 100 nM of LAQ824, and the cell lysates were immunoprecipitated with anti-Bax 6A7 antibody (*IP: Bax 6A7 Ab*) followed by immunoblotting with the polyclonal anti-Bax antibody (*IB: Bax*). Total cell lysates were also probed with the polyclonal anti-Bax antibody (A). Alternatively, cell lysates were immunoblotted with anti-PARP antibody to detect the levels of the full-length and cleaved product of PARP (*Cleaved PARP*) as a measure of the generation of caspase-3 activity. β-actin levels served as the control for protein loading (B).



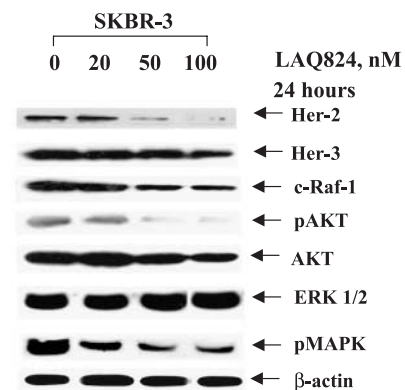
**Figure 3.** LAQ824 induces apoptosis of breast cancer cells. **A**, SKBR-3 and BT-474 cells were exposed to the indicated concentrations of LAQ824 for 48 h. Following this, the percentage of apoptotic cells was determined by Annexin V staining and flow cytometry. *Columns*, means of three experiments; *bars*, SE. **B**, BT-474 cells were exposed for 24–96 h to 0, 10, 20, 50, or 100 nM of LAQ824. Following this, the loss of cell viability was determined by the MTT assay and plotted against LAQ824 concentrations. **C**, MB-468 cells were exposed to 0–100 nM of LAQ824 for 96 h. Following this, the loss cell viability was determined by the MTT assay and plotted against LAQ824 concentrations.

loss of cell viability due to LAQ824, as determined by the MTT assay, was also observed in BT-474 cells (Fig. 3B). In addition, a dose-dependent loss of cell viability due to a 96-h exposure to LAQ824 was also observed in MB-468 cells (Fig. 3C).

Next, we investigated whether the cell cycle and apoptotic effects of LAQ824 were due to inhibition of Her-2 and/or AKT in BT-474 and SKBR-3 cells. Significantly, treatment with 50 or 100 nM of LAQ824 for 24 h markedly depleted Her-2 as well as pAKT, c-Raf-1, and pMAPK levels in SKBR-3 cells (Fig. 4). Exposure to 100-nM

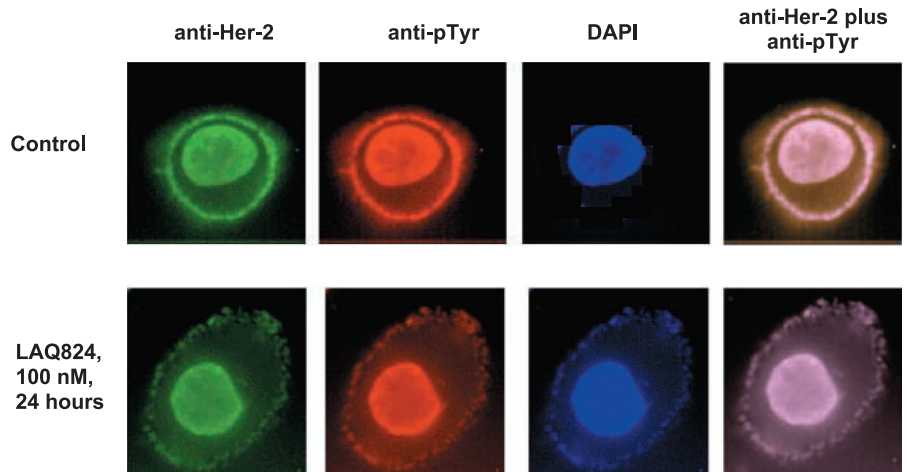
LAQ824 also slightly reduced the levels of HER-3 and AKT (Fig. 4A). Similar effects were observed in BT-474 cells (data not shown). LAQ824-mediated attenuation of Her-2, c-Raf, and pAKT was not simply due to LAQ824-induced caspase activity, because cotreatment with the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone while inhibiting caspase-3 and its PARP cleavage activity did not abrogate the effects of LAQ824 on Her-2, c-Raf, and pAKT (data not shown). We also determined, using indirect immunofluorescence microscopy, the effect of LAQ824 on the tyrosine-phosphorylated Her-2 as well as on its association with HER-3 in the cell membrane. Fig. 5 demonstrates that treatment with 100-nM LAQ824 reduced the diffuse membrane association of Her-2 and its tyrosine phosphorylation in BT-474 cells. Additionally, LAQ824 treatment reduced the colocalization of Her-2 with HER-3 in the cell membrane of BT-474 cells and reduced their distribution from a diffuse to punctuate manner (Fig. 6). These results suggest that treatment with LAQ824 depletes not only the total intracellular levels but also the tyrosine-phosphorylated and membrane-associated levels of Her-2 as well as the HER-3-dimerized forms of Her-2 in BT-474 cells.

Next, we determined whether LAQ824-mediated depletion of Her-2 is due to repression of Her-2/*neu* mRNA. Following treatment with LAQ824 for up to 16 h, the ratios of the mRNA transcripts of Her-2/*neu* to histone H1 genes were determined by a real-time quantitative reverse transcription-PCR technique. LAQ824-treated BT-474 and SKBR-3 cells displayed a marked decline in the ratios of the mRNA transcripts of Her-2/*neu* to histone H1 in a time-dependent manner for up to 16 h (Fig. 7A). As compared with the untreated cells, the ratio in LAQ824-treated cells declined by more than 50% over 16 h. Northern analysis showed a more pronounced decline in the ratio of Her-2/*neu* to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in both BT-474 and SKBR-3 cells



**Figure 4.** LAQ824 depletes Her-2, HER-3, c-Raf-1, AKT, pAKT, and pMAPK levels. SKBR-3 cells were treated with 0, 20, 50, and 100 nM of LAQ824 for 24 h and total cell lysates were used for Western analyses using specific antibodies against Her-2, HER-3, c-Raf-1, pAKT, AKT, Erk1/2, and pMAPK.  $\beta$ -actin levels served as the control for protein loading.

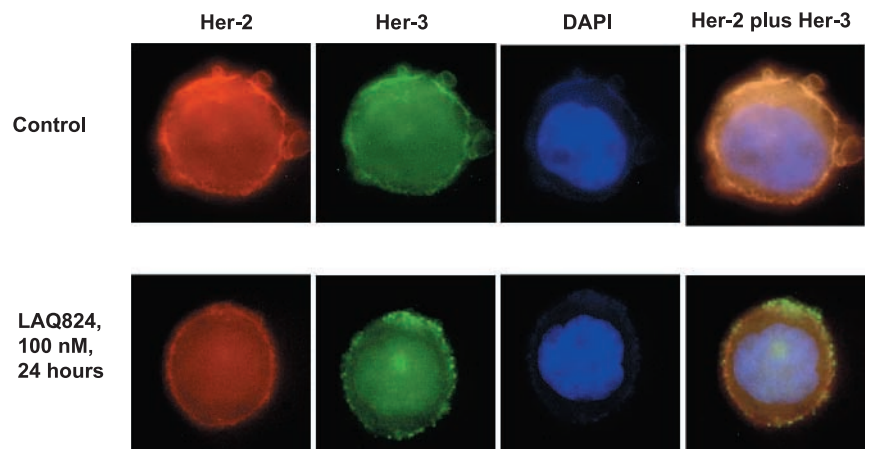
**Figure 5.** Treatment with LAQ824 lowers the cell membrane expression of the tyrosine-phosphorylated Her-2. Indirect immunofluorescent microscopy was performed after staining untreated or LAQ824-treated BT-474 cells with anti-Her-2 and/or antiphosphotyrosine antibody (*anti-pTyr*). DAPI staining was used to highlight the DNA in the nucleus.



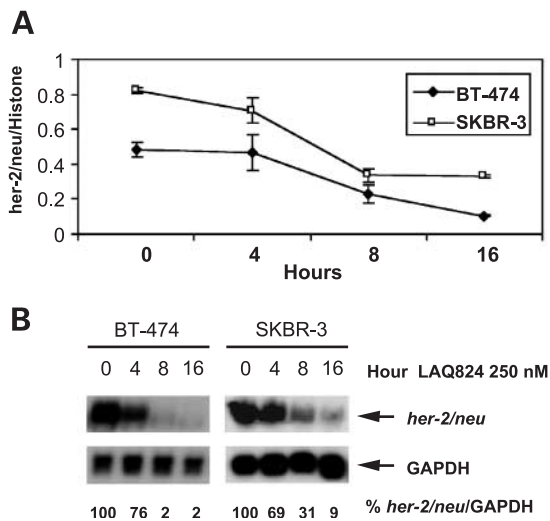
following exposure to LAQ824 for 8 or 16 h (Fig. 7B). LAQ824-mediated decline in Her-2/*neu* mRNA was also dose dependent and occurred prior to an appreciable decline in the Her-2 protein levels (data not shown). We investigated whether treatment with LAQ824 could induce a transcription factor(s) that represses the transcription of Her-2/*neu* message. Fig. 8A demonstrates that cotreatment with cycloheximide restored the levels of Her-2/*neu* mRNA and the ratio of Her-2/*neu* to GAPDH mRNA in SKBR-3 cells depleted by treatment with LAQ824. This suggests that, following LAQ824 treatment, new synthesis of a transcriptional repressor protein is induced by LAQ824, which then inhibits the transcript levels of Her-2/*neu*. This outcome is neutralized by cotreatment with cycloheximide. Next, we used the ChIP assay to assess whether the promoter of Her-2/*neu* is associated with acetylated histones H3 and H4, as is the case for p21. As shown in Fig. 8B, LAQ824 treatment did not increase the association of the promoter of Her-2/*neu* with the acetylated histones H3 and H4 in SKBR-3 cells. This argues against a direct transcriptional repression of Her-2/*neu* due to histone hyperacetylation induced by LAQ824, which

raises the possibility that treatment with LAQ824 induces the levels and/or activity of a repressor of Her-2/*neu*. This possibility is supported by our observation that the decline in the levels of Her-2/*neu* mRNA is reversed by cotreatment with cycloheximide.

Prompted by a report that the HDI Depsipeptide causes acetylation and inhibition of hsp 90 as a chaperone protein for Her-2, we determined the effects of LAQ824 on hsp 90 and its chaperone function for Her-2 in human BT-474 and SKBR-3 cells. Following exposure to LAQ824, immunoprecipitation of hsp 90 followed by immunoblotting with an antibody that recognizes acetylated lysine residues demonstrated marked acetylation of hsp 90 without significantly affecting the levels of hsp 90 in SKBR-3 cells (Fig. 9A). Similar findings were noted in LAQ824-treated BT-474 cells (data not shown). Alternatively, after treatment with LAQ824, cell lysates from SKBR-3 and BT-474 cells were obtained, and hsp 90 was affinity precipitated with ATP-Sepharose. In the precipitates, the levels of hsp 90 were estimated by immunoblot analysis by the anti-hsp 90 antibody. Treatment with LAQ824 attenuated the acetylated hsp 90 that could bind to the ATP-Sepharose in SKBR-3



**Figure 6.** Treatment with LAQ824 reduces the cell membrane colocalization of Her-2 and HER-3. Indirect immunofluorescent microscopy was performed after staining the untreated or LAQ824-treated BT-474 cells with anti-Her-2 and/or anti-HER-3 antibody. DAPI staining was used to highlight the DNA in the nucleus.

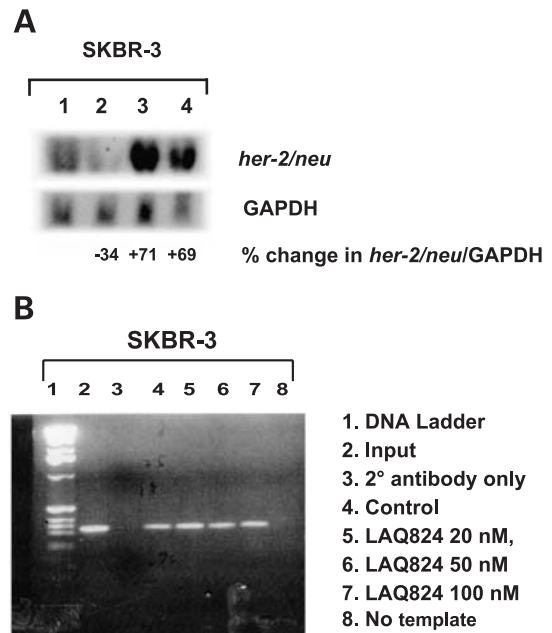


**Figure 7.** Treatment with LAQ824 depletes the mRNA transcript levels of Her-2/neu. **A**, total cellular RNA was isolated from SKBR-3 and BT-474 cells, untreated or treated with LAQ824 (100 nM for 0, 4, 8, and 16 h). Following this, the real-time quantitative reverse transcription-PCR of Her-2/neu and histone H1 was performed to estimate the ratio of the mRNA transcript levels of these genes. Lines, ratio values over time (mean of three experiments). **B**, total cellular RNA from untreated or LAQ824-treated (100 nM for 0, 4, 8, and 16 h) SKBR-3 and BT-474 cells was used for Northern analysis to determine the expression mRNA levels of Her-2/neu. GAPDH mRNA expression was used as the loading control. The Her-2/neu/GAPDH mRNA level ratio as a percentage of untreated control (100%) is also indicated.

and BT-474 cells (Fig. 9B). Thus, LAQ824 clearly increased the acetylation of hsp 90 and reduced its binding to ATP. Next, we evaluated the impact of these modifications on the function of hsp 90 as a chaperone protein. Her-2 was immunoprecipitated from the cell lysates of untreated and LAQ824-treated SKBR-3 cells. The binding of Her-2 to hsp 90 versus hsp 70 was determined. Fig. 10A demonstrates that, similar to the effect of 17-AAG, treatment with LAQ824 shifted the chaperone association of Her-2 from hsp 90 to hsp 70. A similar shift in the chaperone association of c-Raf-1 was also seen in the LAQ824-treated cells (data not shown). These shifts in the binding of Her-2 and c-Raf-1 were associated with induction of hsp 70 but not hsp 90 (Fig. 10B). A densitometric estimation showed that an exposure to 100- and 250-nM LAQ824 for 24 h induced hsp 70 levels by 1.5-fold and 1.6-fold, respectively (Fig. 10B). Previous reports have shown that a shift to the unstable multichaperone complex that includes hsp 70 induces the client proteins Her-2 and c-Raf-1 to accumulate in the detergent-insoluble cellular fraction, and this directs Her-2 and c-Raf-1 for polyubiquitination and proteasomal degradation (48). Following treatment of SKBR-3 cells with 100-nM LAQ824 for more than 8 h, immunoprecipitates of Her-2 showed increased polyubiquitination on immunoblotting with antipolyubiquitin antibody (data not shown). Fig. 10C demonstrates that treatment with LAQ824 for 8 h alone depleted the levels of Her-2 in the detergent (NP40)-soluble fraction, while increasing its levels in the detergent (NP40)-insoluble cellular fraction, prior to degradation by the proteasome. Consistent with this, cotreatment with the

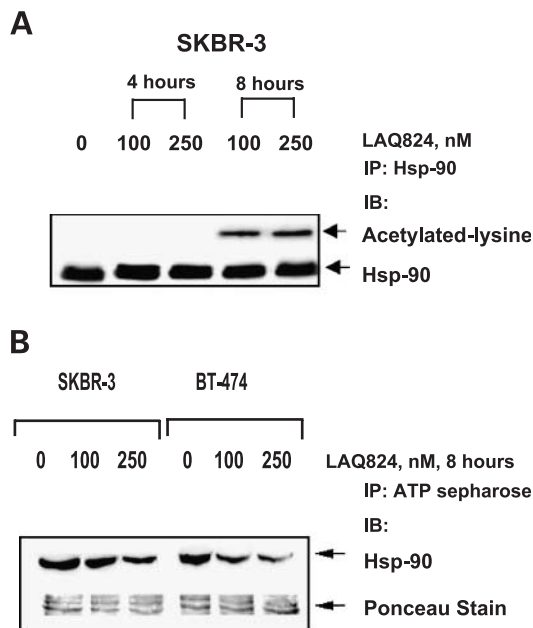
proteasome inhibitor ALLnL increased LAQ824-mediated accumulation of Her-2 in the detergent-insoluble cellular fraction (Fig. 10C). Cotreatment with LAQ824 and ALLnL also led to more accumulation of Her-2 and c-Raf-1 (data not shown) in the detergent-soluble fraction, albeit not as much as in the detergent-insoluble fraction, of SKBR-3 cells. Similar effects of LAQ824 were also observed in BT-474 cells (data not shown). Collectively, these data indicate that, by inducing acetylation of hsp 90, treatment with LAQ824 disrupts the stable chaperone association of Her-2 with hsp 90, which promotes proteasomal degradation of Her-2 and c-Raf-1.

We next determined the effects of LAQ824 on the cytotoxic effects of trastuzumab in BT-474 and SKBR-3 cells. Cotreatment with 20 or 50 nM of LAQ824 for 48 h significantly increased apoptosis induced by 10 or 20  $\mu$ g/ml of trastuzumab in BT-474 (Fig. 11A) and SKBR-3 cells (data not shown). Fig. 11B demonstrates that combined treatment with 50-nM LAQ824 and 20- $\mu$ g/ml trastuzumab



**Figure 8.** Repression of Her-2/neu by LAQ824 requires new protein synthesis. **A**, treatment with LAQ824 represses Her-2/neu mRNA expression, which is reversed by cotreatment with cycloheximide. **A**, samples of 15  $\mu$ g of total cellular RNA were isolated from SKBR-3 cells treated with LAQ824 (100 nM) and/or cycloheximide (100  $\mu$ g/ml) for 8 h and analyzed by Northern blotting. A 1.6-kb fragment of Her-2/neu cDNA was used as a probe, which detected a 615-kb transcript. The same membrane was stripped and rehybridized with a GAPDH probe. Lane 1, untreated cells; lane 2, LAQ824; lane 3, cycloheximide; lane 4, LAQ824 + cycloheximide. The percentage decline or increase in the ratio of Her-2/neu to GAPDH mRNA (% change in her-2/neu/GAPDH) following each treatment, as compared with the ratio in the untreated cells, is as indicated. **B**, LAQ824 does not induce accumulation of acetylated histones in the chromatin associated with Her-2/neu gene. Soluble chromatin was immunoprecipitated with antiacetylated histones H3 and H4 antibodies from untreated and LAQ824-treated SKBR-3 cells, as indicated. PCR primers for a region of the promoter of the Her-2/neu gene were used to amplify the DNA isolated from the immunoprecipitated chromatin. Lanes contain the amplified product, as indicated.

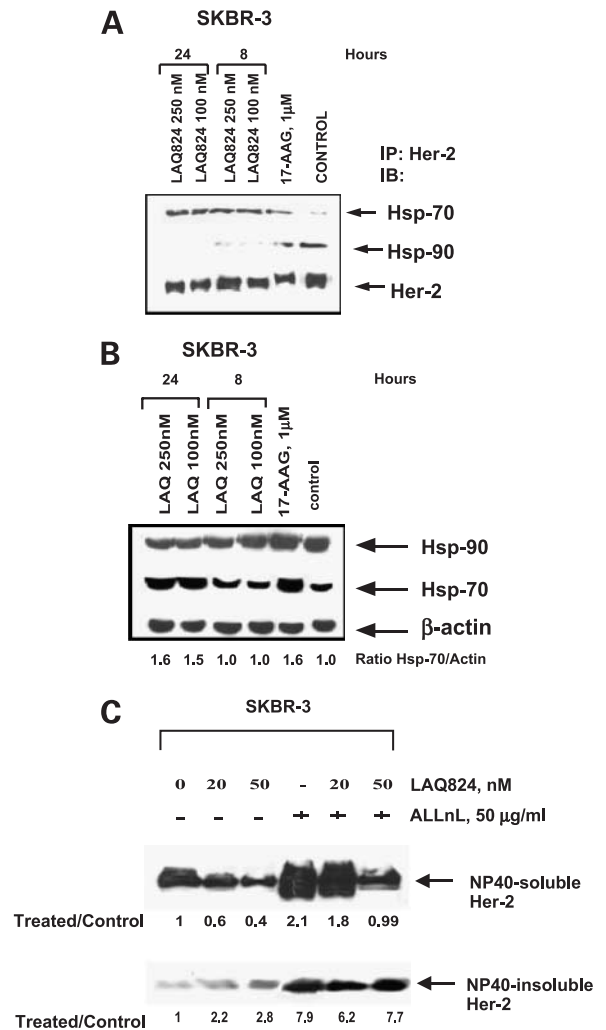




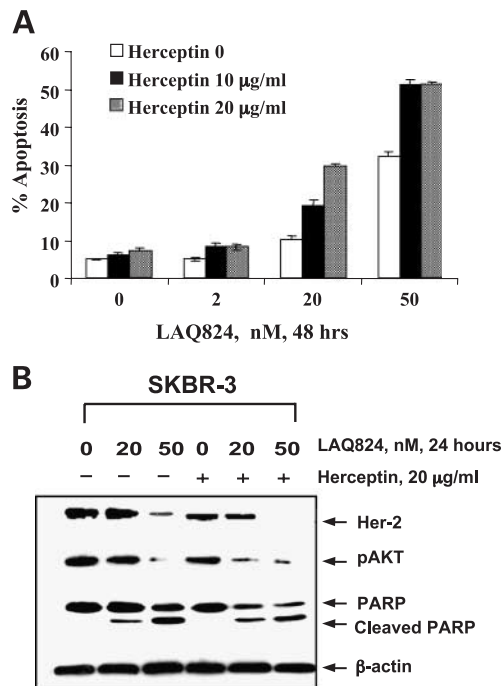
**Figure 9.** LAQ824 acetylates lysine residues on hsp 90 and inhibits its binding to ATP-Sepharose. **A**, SKBR-3 cells were exposed to the indicated concentrations of LAQ824 for 4 or 8 h. Following this, hsp 90 was immunoprecipitated from the cell lysates (*IP: Hsp-90*) and immunoblotted with either anti-hsp 90 or antiacetylated lysine antibody (*IB: Acetylated-lysine*). **B**, cell lysates from SKBR-3 cells were treated with the indicated concentrations and exposure intervals to LAQ824, and hsp 90 was affinity precipitated with ATP-Sepharose (*IP: ATP sepharose*) and immunoblotted with anti-hsp 90 antibody (*IB: Hsp-90*). Ponceau staining was used to confirm equal protein loading.

produced more decline of Her-2 and pAKT levels and greater PARP cleavage activity of caspases in SKBR-3 cells than with either agent alone. Cotreatment with LAQ824 and trastuzumab had similar effects in BT-474 cells (data not shown). Previous reports have shown that down-regulation of Her-2 and its downstream antiapoptotic signaling, mediated through the activity of AKT and Erk1/2, may sensitize cancer cells to chemotherapeutic agents (16, 18, 41, 48). Therefore, we also determined the effects of LAQ824 on taxotere-induced and gemcitabine-induced apoptosis of SKBR-3 and BT-474 cells. Fig. 12A demonstrates that, as compared with treatment with either agent alone, combined treatment with LAQ824 and taxotere for 48 h induced more apoptosis of SKBR-3 cells. The cytotoxic effects of exposure to LAQ824 and/or taxotere for longer intervals were also evaluated by the MTT assay. Fig. 12B shows that a dose-dependent decline in the viability of SKBR-3 cells was observed following combined treatment with LAQ824 and taxotere for 96 h as compared with a treatment with either agent alone. Similar effects of LAQ824 and/or taxotere were also observed against MB-468 cells (Fig. 12C). Gemcitabine is an active anti-breast cancer antimetabolite, which is commonly employed in the treatment of advanced breast cancer (2). Cotreatment with 50-nM LAQ824 also enhanced gemcitabine-induced apoptosis of SKBR-3 cells (Fig. 12D). LAQ824 also sensitized BT-474 cells to taxotere-induced or gemci-

tabine-induced apoptosis (data not shown). Next, we determined the effect of LAQ824 on the cytotoxic effects of the promising, novel anticancer TPA, Epo B, against BT-474 and SKBR-3 cells (46). Fig. 13, A and B, demonstrates that, as compared with treatment with either agent alone, combined treatment with LAQ824 and Epo B for 24 or 48 h induced more apoptosis of BT-474 cells. The cytotoxic effects of exposure to LAQ824 and/or Epo B for longer intervals were also evaluated by the MTT assay.



**Figure 10.** LAQ824 treatment shifts the chaperone association of Her-2 from hsp 90 to hsp 70 and promotes proteasomal degradation of Her-2. **A**, SKBR-3 cells were treated with the indicated concentrations and exposure intervals to LAQ824 or 17-AAG (8 h). Following this, the immunoprecipitates of Her-2 (*IP: Her-2*) from the cell lysates were immunoblotted with anti-hsp 90 (*IB: Hsp-90*), anti-hsp 70 (*IB: Hsp-70*), or anti-HER-2 (*IB: Her-2*) antibody. **B**, cell lysates were immunoblotted with anti-hsp 90 or anti-hsp 70 antibody.  $\beta$ -actin levels served as the control for protein loading. The fold increase in the hsp 70 to  $\beta$ -actin ratio (*Ratio Hsp-70/Actin*) is also indicated. **C**, SKBR-3 cells were treated with the indicated concentrations of LAQ824 and/or ALLnL for 8 h. The detergent-soluble (*NP40-soluble Her-2*) and detergent-insoluble (*NP40-insoluble Her-2*) fractions were immunoblotted with anti-Her-2 antibody. The fold change in Her-2 level in the detergent-soluble and detergent-insoluble fractions of treated over untreated control cells is also indicated.  $\beta$ -actin levels served as the control for equal protein loading (data not shown).



**Figure 11.** Cotreatment with LAQ824 enhances Herceptin-mediated depletion of Her-2 and pAKT levels and increases Herceptin-induced apoptosis of SKBR-3 and BT-474 cells. **A**, BT-474 cells were treated with the indicated concentrations of Herceptin and/or LAQ824 for 48 h. Following this, the percentage apoptotic cells was assessed by Annexin V staining followed by flow cytometry. **B**, after treatment of SKBR-3 cells with the indicated concentrations of Herceptin and/or LAQ824, cell lysates were immunoblotted with anti-Her-2, pAKT, or anti-PARP antibody.  $\beta$ -actin levels served as the control for equal protein loading.

Fig. 13C shows that a dose-dependent decline in the viability of BT-474 cells was observed following a combined treatment with LAQ824 and Epo B for 96 h as compared with a similar treatment with either agent alone. In a dose-dependent manner, LAQ824 also sensitized SKBR-3 cells to Epo B-induced apoptosis (Fig. 13D).

## Discussion

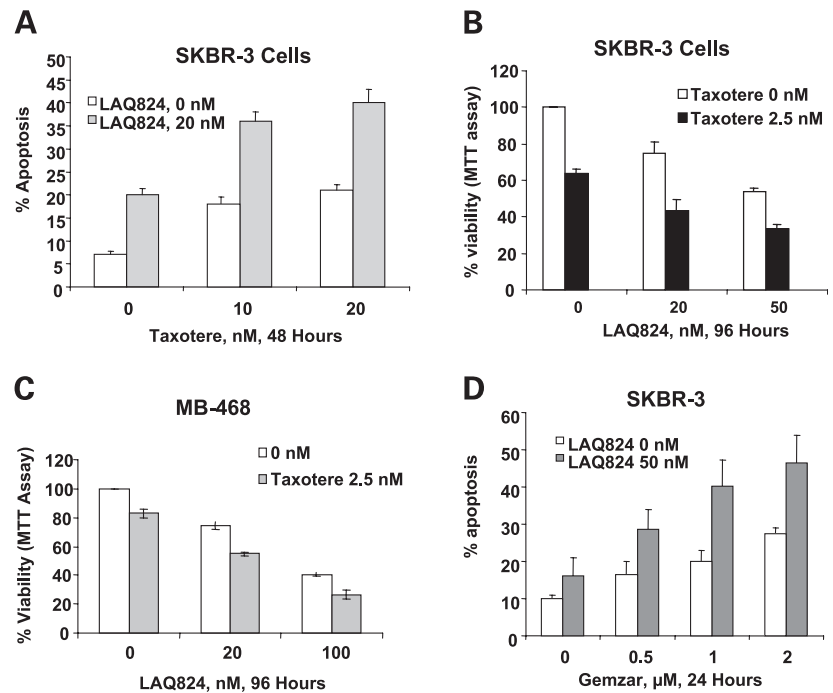
Similar to what has been previously observed following treatment of human leukemia cells with SAHA (38, 39, 43), treatment with LAQ824 also induced the hyperacetylation of histones H3 and H4 and increased expression of p21 in SKBR-3 and BT-474 cells. This is due to association of the promoter of p21 with acetylated histones in the nucleosomes (35). In contrast, increase in p27 levels following treatment with HDI is not due to transcriptional up-regulation. It may occur due to a post-transcriptional mechanism (35). Regardless, LAQ824-induced accumulation of p21 and p27 correlated with cytostasis as well as with induction of Bax conformational change, PARP cleavage activity of caspase-3, and apoptosis of SKBR-3 and BT-474 cells. Present studies also demonstrate for the first time that treatment with LAQ824 not only depletes the mRNA levels of Her-2 but also promotes its degradation by

the proteasome. The mRNA levels of Her-2/*neu* declined markedly after short exposure intervals to LAQ824 as determined by Northern analysis. The results of the ChIP analyses clearly showed that treatment with LAQ824 did not affect the association of Her-2/*neu* promoter with acetylated histones, making it unlikely that Her-2/*neu* transcription was directly affected by LAQ824-induced chromatin modification. Because acetylation has been shown to affect the DNA binding activity of several transcription factors (e.g., p53 and GATA-1; 49, 50), it is possible that treatment with HDIs will repress Her-2/*neu* transcription indirectly through the induction of a transcriptional repressor for Her-2/*neu* and/or by affecting its DNA binding. Present studies indicate that LAQ824-mediated decline in the mRNA levels was reversed by cotreatment with cycloheximide, suggesting that new protein synthesis was involved in the attenuation of Her-2/*neu* transcript levels. A previous report also demonstrated that treatment with TSA inhibits the mRNA synthesis of Her-2/*neu* as detected by a genomically integrated Her-2/*neu* promoter cell screen (51). While exposure to TSA had no effect on the open chromatin configuration of this gene, as monitored by DNase I hypersensitivity, TSA selectively destabilized and enhanced the decay of the mature Her-2/*neu* transcripts (51). While this was not directly studied in the present studies, LAQ824 treatment may also deplete Her-2/*neu* mRNA levels by repressing its transcription and/or reducing its stability and promoting its decay.

LAQ824-mediated decline in the mRNA levels of Her-2/*neu* was associated with the depletion of the total intracellular levels of Her-2 protein. LAQ824 treatment also depleted the cell membrane-associated, tyrosine-phosphorylated levels of Her-2. In addition, the colocalization of Her-2 with HER-3 in the cell membrane also declined. Her-2-HER-3 dimers have been recognized as the most potent Her-2 signaling complex, which leads to the activation of PI3K/AKT and Ras-Raf-Erk1/2 activities, which are involved in cell proliferation, survival, and chemoresistance of breast cancer cells (4, 5). Both transcriptional and post-translational mechanisms have been implicated in Her-2-mediated up-regulation of cyclin D1 (5). Cytosolic sequestration and reduced stability of p27 also occurs downstream of Her-2-initiated signaling through AKT (52–54). Therefore, it is likely that Her-2-mediated signaling through PI3K/AKT and Ras-Raf-Erk1/2 modulates cyclin D1 and p27 levels, which together promote cell proliferation and cell survival (4, 52). Consistent with this, present studies demonstrate that LAQ824-mediated attenuation of Her-2 levels is associated with up-regulation of p27 levels, cytostasis, and apoptosis of breast cancer cells that possess amplification and overexpression of the Her-2/*neu* gene.

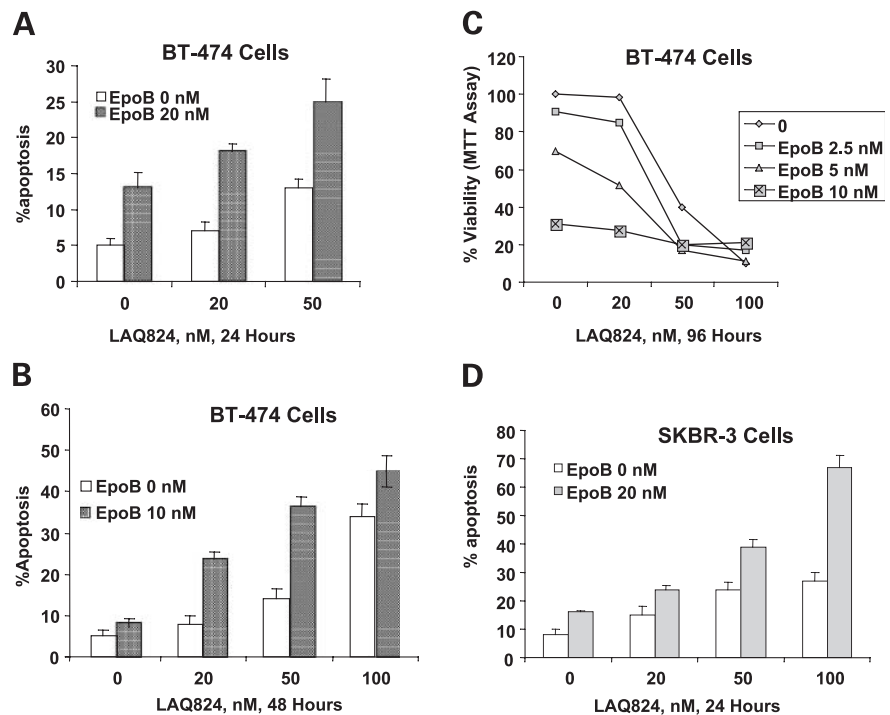
Recent reports and present findings indicate that inhibition of HDACs may also lead to acetylation of proteins other than histones (e.g., a tubulin and hsp 90), which clearly affects their biologic functions (41, 55). In this respect, the name “histone deacetylases” may be a misnomer, and HDIs should be appropriately called

**Figure 12.** Cotreatment with LAQ824 enhances taxotere-induced or gemcitabine (*Gemzar*)-induced apoptosis of SKBR-3 cells. **(A)** SKBR-3 cells were exposed to the indicated concentrations and exposure intervals to LAQ824 and/or taxotere. Following this, the percentage of apoptotic SKBR-3 cells was determined by Annexin V staining and flow cytometry. Cell viability of SKBR-3 **(B)** and MB-468 **(C)** cells was also determined by MTT assay, and the percentage of residual viable is depicted as *bar graphs*. *Columns*, mean percentage of apoptotic cells following treatment with the indicated concentrations of LAQ824 and/or *Gemzar* for 24 h; *bars*, SE **(D)**.



protein deacetylase inhibitors, because proteins other than histones are modified. For example, inhibition of the class 2 and cytoplasmic HDAC-6 by the hydroxamate HDI TSA, but not trapoxin, was recently shown to cause acetylation of a tubulin, which stabilized dynamic microtubules (55–57). Although the specific HDAC(s) inhibited by LAQ824 that may be involved in the acetylation of hsp 90 was not elucidated, the present studies clearly demonstrate that

treatment with LAQ824 increased the acetylation of lysine residues on hsp 90, which reduced the binding of hsp 90 to ATP. This has been shown to disrupt the chaperone association of hsp 90 with its client proteins (*e.g.*, Her-2, c-Raf-1, and pAKT). hsp 90 inhibitor GA, or its less toxic analogue 17-AAG, also inhibits the chaperone function of hsp 90 in a similar manner (21–26). While attenuating their association with the stable multichaperone complex that



**Figure 13.** Cotreatment with LAQ824 enhances Epo B-induced apoptosis of BT-474 and SKBR-3 cells. Following treatment with the indicated concentrations of LAQ824 and/or Epo B for 24 **(A)** or 48 **(B)** h, the percentage of apoptotic BT-474 cells **(A and B)** or SKBR-3 cells **(D)** was determined by Annexin V staining and flow cytometry. Alternatively, following treatment of SKBR-3 cells with the indicated concentrations of LAQ824 and/or Epo B for 24 h, the percentage of residual viable cells was also determined by MTT assay and is depicted as *line graphs* **(C)**.

includes hsp 90, LAQ824 treatment increased the association of Her-2 and c-Raf-1 with the less stable multichaperone complex with hsp 70. For Her-2, this promotes its association with chaperone binding E3 ubiquitin ligase, carboxyl-terminal hsc 70-interacting protein, which efficiently ubiquitinates and down-regulates Her-2 (58). Association of Her-2 and c-Raf-1 with the less stable multichaperone complex with hsp 70 directed the accumulation of Her-2 and c-Raf-1 in the detergent-insoluble cellular fraction, promoting their degradation by the proteasome. This is supported by the observation that cotreatment with the proteasome inhibitor ALLnL with LAQ824 increased the levels of Her-2 and c-Raf-1 in the detergent-insoluble cellular fraction. A similar outcome was previously reported to occur following cotreatment of breast cancer cells with 17-AAG and PS341 (24–26). Therefore, treatment with LAQ824 promotes the proteasomal degradation of Her-2 and c-Raf-1. Although not determined here specifically, LAQ824 is also likely to exert a similar effect on AKT, because following treatment with LAQ824, the levels of pAKT and AKT declined concurrently with Her-2 and c-Raf-1 (Fig. 4). This speculation is supported by the observation that hsp 90 inhibitors GA and 17-AAG also promote proteasomal degradation of Her-2, c-Raf-1, and AKT (21, 22). Recently, Raf-1 and Bcl-2 have been shown to exert independent positive control of cell survival at the mitochondria in breast cancer cells (18, 59). Furthermore, activation of the mitochondrial c-Raf-1 is also involved in the antiapoptotic effect of AKT (60). Therefore, LAQ824-mediated depletion of c-Raf-1 may independently exert a potent proapoptotic effect in breast cancer cells. Collectively, present findings indicate that treatment with LAQ824 can attenuate cell proliferation and antiapoptosis signaling downstream of AKT and Raf-Erk1/2 pathways by depleting Her-2 as well as through Her-2-independent depletion of c-Raf-1 and pAKT.

Treatment with an agent that lowers Her-2 activity and/or levels has been previously shown to enhance the activity of chemotherapeutic agents against breast cancers that possess Her-2/*neu* amplification and overexpression (9, 12–15, 29). Our present findings are consistent with this, demonstrating that cotreatment with LAQ824, which not only depletes Her-2 but also attenuates the levels c-Raf-1, pAKT, and AKT, enhances apoptosis of breast cancer cells induced by chemotherapeutic agents that have diverse mechanisms of action (1, 2). These included an antimetabolite, gemcitabine, and antimicrotubule agents, taxotere and Epo B. Whether the induction of p21 and p27 by LAQ824 plays an important role in LAQ824-mediated potentiation of apoptosis by these agents remains to be established. In this regard, it is noteworthy that only the effect of concurrent administration of LAQ824 with the chemotherapeutic agents was evaluated in the present studies. It is possible that the potentiation of apoptosis due to these agents by LAQ824 will be further augmented if LAQ824 and the chemotherapeutic agents were administered in a different schedule. Previous reports have indicated that although quite active, responses to trastuzu-

mab in breast cancers with amplification and overexpression of Her-2/*neu* are quite variable (11). Our present findings that LAQ824 increased trastuzumab-induced apoptosis of SKBR-3 and BT-474 cells suggest that a combination with LAQ824 may be a promising strategy to improve the efficacy of trastuzumab against breast cancers. The mechanisms of resistance to trastuzumab that have been identified thus far involved Her-2-independent increased activity of AKT (8, 16, 17). This increase was seen downstream of the loss of PTEN or increased levels and activity of insulin-like growth factor-1R (8, 16, 17). Because treatment with LAQ824 attenuates the levels and activity of AKT and c-Raf-1, it is possible that resistance to trastuzumab based on Her-2-independent increased activity of AKT may be overcome by cotreatment with LAQ824 and trastuzumab. Collectively, as shown here, the LAQ824-induced histone modifications that modulate the transcription of p21 and Her-2, coupled with the post-translational effects on Her-2, AKT, and c-Raf-1 mediated through LAQ824-induced acetylation of hsp 90, suggest several LAQ824-based therapeutic strategies that could be tested *in vivo* against advanced breast cancers. Based on present findings, the biologic effects of HDIs in breast cancer cells could also potentially be exploited to improve the therapeutic efficacy of established antibrast cancer therapies.

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