Rapid Induction of Histone Hyperacetylation and Cellular Differentiation in Human Breast Tumor Cell Lines following Degradation of Histone Deacetylase-1*

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Quinidine inhibits proliferation and promotes cellular differentiation in human breast tumor epithelial cells. Previously we showed quinidine arrested MCF-7 cells in G1 phase of the cell cycle and led to a G1 to G0 transition followed by apoptotic cell death. The present experiments demonstrated that MCF-7, MCF-7ras, T47D, MDA-MB-231, and MDA-MB-435 cells transiently differentiate before undergoing apoptosis in response to quinidine. The cells accumulated lipid droplets, and the cytokeratin 18 cytoskeleton was reorganized. Hyperacetylated histone H4 appeared within 2 h of the addition of quinidine to the medium, and levels were maximal by 24 h. Quinidine-treated MCF-7 cells showed elevated p21WAF1, hypophosphorylation and suppression of retinoblastoma protein, and down-regulation of cyclin D1, similar to the cell cycle response observed with cells induced to differentiate by histone deacetylase inhibitors, trichostatin A, and trapoxin. Quinidine did not show evidence for direct inhibition of histone deacetylase enzymatic activity in vitro. HDAC1 was undetectable in MCF-7 cells 30 min after addition of quinidine to the growth medium. The proteasome inhibitors MG-132 and lactacystin completely protected HDAC1 from the action of quinidine. We conclude that quinidine is a breast tumor cell differentiating agent that causes the loss of HDAC1 via a proteasome sensitive mechanism.

Histone deacetylase (HDAC) inhibitors comprise a family of related proteins that act in conjunction with histone acetyltransferase proteins to modulate chromatin structure and transcriptional activity via changes in the acetylation status of histones. Histones H3 and H4 are the principal histone targets of HDAC enzymatic activity, and these histones undergo acetylation at lysine residues at multiple sites within the histone tails extending from the histone octamer of the nucleosome core. The association of HDAC proteins with mSin3, N-CoR, or SMRT and other transcriptional repressors has led to the hypothesis that HDAC proteins function as transcriptional co-repressors (reviewed in Ref. 1). The spectrum of genes that show alterations in gene transcription rates in response to decreased HDAC activity is quite restricted (2). Yet, small molecule inhibitors of the enzyme histone deacetylase (HDAC) such as trichostatin A (TSA), superylanilide hydroxamic acid (SAHA), trapoxin, and phenyl butyrate cause major alterations in cellular activity including the induction of cellular differentiation and apoptosis (3–5). Trichostatin A, SAHA, and trapoxin stimulate histone acetylation by acting as direct inhibitors of HDAC enzyme activity (6). Trichostatin A, SAHA, and trapoxin possess lysine-like side chains and act as chemical analogs of lysine substrates. Molecular models based upon the x-ray crystal structure of an HDAC-like protein indicate that trichostatin A and SAHA can bind within the active site of the HDAC enzyme and interact with a zinc metal ion within the catalytic pocket that is critical for enzymatic activity (7). Trapoxin is an irreversible HDAC enzyme inhibitor (8). Much remains to be learned about the biochemical events subsequent to HDAC inhibition that lead to cell cycle arrest, cellular differentiation, and apoptosis. However, a spectrum of biological responses characteristic of HDAC inhibitors has emerged, including cell cycle arrest in G0, elevated p21WAF1 expression, hypophosphorylation of retinoblastoma protein (pRb), hyperacetylation of histones, particularly H3 and H4, and apoptosis. Histone hyperacetylation is directly linked to the activation of p21 transcription and is p53-independent (5). This observation provides an important link between HDAC inhibition and cell cycle arrest because p21WAF1 plays a critical role in causing G1 cell cycle arrest via inhibition of the G1 cyclin-dependent kinase family (9). Overexpression of p21WAF1 has also been associated with apoptosis, but the mechanism of p21WAF1 induction of apoptosis requires further investigation (10).

Cancer therapy that targets the activity of genes or gene products controlling cell cycle progression, differentiation, and apoptosis is a promising new strategy. Because HDAC inhibitors regulate the cell cycle and cause both cellular differentiation and apoptosis, they comprise an interesting group of compounds with potential for development into a new category of clinically significant anti-tumor agents. Single, key protein targets for “gene-regulatory chemotherapy” are difficult to identify due to the existence of parallel, functionally overlap-
ping signaling cascades. For this reason, use of cancer therapeutics that target multiple intracellular signaling pathways, such as observed with the HDAC inhibitors, is an intriguing approach that addresses the problem of redundancy in growth signaling pathways. In this regard, the HDAC inhibitor phenylbutyrate was recently shown to have clinical anti-tumor activity (11).

Quinidine is a natural product therapeutic agent originally used as an anti-malarial and as an anti-arrhythmic agent. Previous studies with human breast tumor cell lines demonstrated that quinidine (90 μM) is an anti-proliferative agent as well. Quinidine arrested cells in early G1 phase and induced apoptosis by 72–96 h in MCF-7 cells (12), but the biochemical basis for the anti-proliferative effect of quinidine was not well understood. To clarify the molecular mechanisms of the anti-proliferative activity of quinidine, we investigated the effects of quinidine on histone acetylation and cell cycle regulatory proteins. In this report, we show that quinidine causes hyperacetylation of histone H4, down-regulation of HDAC1 protein levels, and cellular differentiation in a panel of human breast tumor cell lines. We conclude that quinidine is a novel differentiating agent that stimulates histone hyperacetylation as a result of HDAC1 protein degradation.

MATERIALS AND METHODS

Cell Culture—Permanent cell lines derived from patients with breast carcinomas were used in these studies. MCF-7 cells, passage numbers 40–55, MCF-7/T ras (13), T47D, MDA-MB-231, and MDA-MD-453 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Bio-Whittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, Utah), 2 mM glutamine, and 40 μg/ml gentamicin. Experiments were performed in this medium supplemented with 5% FBS. The cells were maintained at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. After 6 days, cells became about 70–80% confluent and were passaged at a 1:5 ratio (MCF-7) or at a 1:10 ratio (all others). Normal human mammary epithelial cells (HMEC) were obtained from Clonetics, San Diego, CA, and were grown according to directions of the suppliers. Cells were grown from frozen stocks and used for 1–3 passages. Quinidine, TSA, and all-trans-retinoic acid were purchased from Sigma. The cell-permeant protease inhibitors, MG-192 and lactacystin, were purchased from Calbiochem.

Growth Inhibition Assays—Growth inhibition by cell numbers was assayed by plating cells in 35-mm dishes (1–1.5 × 10⁵) on sterile cover slips (1 × 10⁵) containing DMEM, 5% FBS plus quinidine (90 μM). Viable cells were counted using a hemocytometer, and trypan blue (0.02%) exclusion was used as an indicator of viability. Cell growth was also monitored in a 96-well plate format using the One Solution Cell Proliferation Assay (Promega, Madison, WI), which is based upon metabolic bioreduction of a tetrazolium salt with succinate dehydrogenase. The result is the formation of a formazan dye that can be solubilized and measured spectrophotometrically at 490 nm. The plating density for the 96-well dishes (cells/well) was 3000 for MCF-7, T47D, and MDA-MB-231 cells, 2000 for HMEC, 1000 for MCF-7, 500 for MDA-MB-231, and 1500 for T47D cells. The cells were plated at 1000 cells/well (HMEC), 1000 cells/well (MCF-7), and 500 cells/well (MCF-7) for 3 days, 3 days, and 5 days, respectively.

Immunoblotting—Cells were harvested from confluent T-75 flasks and subcultured (1 × 10⁶) in 60-mm dishes. On subcultivation, the confluence of cells was adjusted to 10% and cells were synchronously synchronously synchronized through the cell cycle. To prepare whole cell lysates, the cells were harvested at the times indicated by scraping into ice-cold buffer (50 mM Tris-HCl, 0.25 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, and 0.1 mM Na₃VO₄, pH 7.4). Protease inhibitors (protease inhibitor mixture, Roche Molecular Biochemicals) were added immediately. Cell lysates were centrifuged (3,000 rpm, 10 min, SS-34 rotor) and washed three times with the lysis buffer. Histones were extracted from the crude nuclear pellets using the procedure of Nakajima et al. (16). The pellets were resuspended in 0.1 M ice-cold sterile water containing HCl and concentrated H₂SO₄, to 0.4 x aged. The preparation was incubated at 4 °C for 1 h and then centrifuged (17,000 rpm, 10 min, Sorvall SS-34 rotor). The supernatant containing the extracted histones was mixed with 10 x of acetone, and the precipitate was obtained after an overnight incubation at –20 °C, collected, and air-dried. The acid-soluble histone fraction was dissolved in 50 μl of H₂O and stored at –70 °C.

The protein concentration of the whole cell lysate supernatants or histone preparations was determined using the BCA protein assay (Pierce) and bovine serum albumin as a standard. Equal amounts of protein were loaded onto SDS-polyacrylamide gels. Molecular weights of the immunoreactive proteins were estimated based on the relative migration with colored molecular weight protein markers (Amersham Pharmacia Biotech). Proteins were transferred to polyvinylidene difluoride membranes (NOVEX, San Diego, CA) and blocked at 4 °C using 5% nonfat milk in 0.1% albumin (chicken egg), 5% nonfat dry milk, and 5% FBS overnight. The membranes were incubated with primary antibodies for 3 h at room temperature. The antibody sources were as follows: mouse monoclonal anti-p27 (8, SC-1641), rabbit polyclonal anti-CDK4 (C-25, goat polyclonal anti-p27 (9, 10), SC-6292, Santa Cruz Biotechnology, Santa Cruz, CA; mouse monoclonal anti-pRb (14001A) from Pharmingen (San Diego, CA); mouse monoclonal anti-cyclin D1 (NCL-cyclin D1, 113105) from Novoceastra (Burlingham, CA); mouse monoclonal anti-p16 (AP-1), p21 (WAF1/Ap-1), p53 (AP-6) from Calbiochem; and anti-acetylated histone H4 antibody (rabbit polyclonal, Upstate Biotechnology Inc.). The primary antibodies were diluted at 1:500 in Western blocking solution (0.1% non-fat dry milk, 0.1% albumin (chicken egg), 1% FBS, 0.2% (v/v) Tween 20, in PBS, pH 7.3). The antigen-antibody complexes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat IgG-HP (SC-2020), rabbit IgG-HP (SC-2004), or mouse IgG-HP (SC-2005) from Santa Cruz Biotechnology) at a final dilution of 1:3000 in Western blocking solution. After washing three times with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, and 0.05% (v/v) Tween 20), antibody binding was visualized using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and autoradiography.

In Vitro HDAC Activity Assay—Quinidine HCl was added to a chicken erythrocyte cellular extract, which contained HDAC activity, at concentrations of 90 μM (19). HDAC assays were performed as described in Hendzel et al. (17). Briefly, the cellular extract was incubated with 500 μg of acid-soluble histones isolated from [H]acetate-labeled chicken erythrocytes for 60 min at 37 °C. Reactions were terminated by addition of acetic acid/HCl to a final concentration of 0.120.72 N. Released [H]acetate was extracted using ethyl acetate and quantified by scintillation counting. Samples were assayed three times, and the non-enzymatic release of label was subtracted to obtain the reported values.

RESULTS

Hyperacetylation of Histone H4—Antibodies that recognize acetylated forms of histone H4 have been used as a probe for agents that cause histone hyperacetylation (19). In Western blot experiments, we compared the ability of quinidine to cause

Histone Hyperacetylation in Breast Tumor Cell Lines 35257
Histone Hyperacetylation in MCF-7 cells. A, histones were extracted from cells grown in the presence of 90 μM quinidine for 0.5, 1, 2, 6, 12, 24, or 48 h; histones (20 μg/lane) were electrophoresed in 15% polyacrylamide gels containing 1% SDS and assayed for the presence of acetylated H4 by immunoblotting. B, histones were extracted from cells grown in the presence of 300 nM TSA for 0.5, 6, 12, 24, or 48 h; 20 μg of histone/lane were electrophoresed and analyzed for acetylated histone H4 by immunoblotting. C and D, HDAC1 protein in whole cell lysates was prepared from control MCF-7 cells at 0.5, 9, 12, 24, or 48 h (C) or cells treated with 300 nM TSA 0.5, 12, 24, or 48 h (D); 50 μg of protein/lane were electrophoresed in 12% polyacrylamide gels containing 1% SDS and assayed for HDAC1 protein by immunoblotting. E and F, HDAC1 protein in whole cell extracts from cells grown in the presence of 90 μM quinidine for 15, 20, and 30 min or 9, 12, 24, or 48 h (E) or 0.5, 1, 2, or 6 h (F); extracts were electrophoresed (50 μg protein/lane) and assayed for HDAC1 protein by immunoblotting.

To determine if the rapid loss of HDAC protein in the presence of quinidine were mediated through the 26 S proteasome pathway, MCF-7 cells were treated simultaneously for 30 min with quinidine and MG-132 (30 μM), an inhibitor of the 26 S proteasome. Cells treated with 90 μM quinidine showed a complete loss of HDAC1 protein, which was prevented when MG-132 and quinidine were added simultaneously (Fig. 2A). Treatment with the solvent, Me2SO, or MG-132 in solvent (0.1%) caused a modest reduction in the level of HDAC1 protein. These reductions in HDAC1 did not elicit a detectable stimulation of H4 acetylation, and we suggest that other HDAC enzymes present in MCF-7 cells, insensitive to Me2SO, could compensate for the lost HDAC1 in the maintenance of deacetylated histone H4. This action of quinidine on HDAC1 protein was not reflected in a general decrease in cellular protein content (12), nor were all cell cycle regulatory proteins down-regulated in MCF-7 cells in the presence of quinidine (e.g. p21WAF1 and p53 protein; Fig. 3). Additional studies are required to define the spectrum of proteins affected by quinidine in a proteasome-sensitive manner. Quinidine (90 or 250 μM) did not inhibit the activity of the isolated chicken erythrocyte HDAC1 enzyme in vitro (data not shown) suggesting that quinidine caused histone hyperacetylation by eliciting a rapid and transient loss of HDAC1 protein without a direct inhibition of the HDAC enzyme. The suppression of HDAC protein levels in MCF-7 cells was accompanied by a decrease in HDAC enzyme activity in the cell extracts. Histone acetylation and depressed HDAC1 protein levels persisted for approximately 48 h in the presence of quinidine. When MCF-7 cells were exposed to quinidine for 24 h in the presence of either MG-132 or lactacystin, there was no detectable H4 acetylation (Fig. 2B). These results support the idea that quinidine-induced loss of HDAC1 protein is involved in the H4 acetylation response via a proteasomal sensitive pathway.

G1 Phase Cell Cycle Regulatory Profile in MCF-7 Cells—G1 cell cycle arrest is characteristic of HDAC inhibitors, and reports of alterations in several cell cycle proteins in cells exposed to HDAC inhibitors, particularly the elevation of the p21WAF1 protein, are numerous (21–23). It was of interest to determine whether p21WAF1 and other key cell cycle regulatory proteins such as the retinoblastoma protein (pRb) and the G1 phase cyclin-dependent kinase activator, cyclin D1, were targets of quinidine action in MCF-7 cells. Western blotting analysis showed that by 12 h the levels of p21WAF1 were increased in response to quinidine treatment approximately 11-fold, and this elevated level of protein expression persisted through 48 h.
Fig. 3. G1 cell cycle proteins in MCF-7 cells. Cells released from confluency were plated into control medium or medium containing 90 μM quinidine. Whole cell lysates were prepared 12, 24, or 48 h after plating and assayed by immunoblotting for the cyclin-dependent kinase inhibitors, p21^WAF1, p27 (n = 3), p27 (n = 2, p16 (n = 3), and p53 (n = 3) after electrophoresis of 50 μg of protein/lane through 12% SDS-polyacrylamide gels. pRb protein was immunoprecipitated from 500 μg of whole cell lysate protein using an antibody that recognizes phosphorylated and non-phosphorylated pRb. This entire immunoprecipitate was electrophoresed in a 7.5% SDS-polyacrylamide gel and immunoblotted using this same antibody. Results shown are typical of two independent analyses.

A small, less than 2-fold increase in p27 levels was observed in cells exposed to quinidine for 24–48 h, whereas levels of p16 were unchanged (Fig. 3). Quinidine treatment decreased cyclin D1 and CDK4 protein levels after 12 h of treatment (Fig. 4), indicating that the cyclin-dependent kinase inhibitor, p21^WAF1, as well as an important G1 phase target of p21^WAF1, the cyclin D1-CDK4 complex, are early targets of quinidine in MCF-7 cells. This profile of activity is consistent with the observed cell cycle arrest of quinidine-treated MCF-7 cells in mid-G1 phase (12).

In MCF-7 cell extracts probed using anti-pRb antibodies, two separate but closely migrating bands were distinguishable. The upper band contained more highly phosphorylated pRb, and the lower band contained unphosphorylated or hypophosphorylated pRb. Control cells showed a faint pRb signal at 12 h, the lower band contained more highly phosphorylated pRb, and separate but closely migrating bands were distinguishable. The upper band contained more highly phosphorylated pRb, and the lower band contained unphosphorylated or hypophosphorylated pRb (56). This entire immunoprecipitate was electrophoresed in a 7.5% SDS-polyacrylamide gel and immunoblotted using this same antibody. Results shown are typical of two independent analyses.

Fig. 4. Cyclin D-CDK4 in MCF-7 cells. Confluent MCF-7 cells were subcultured in control medium or medium containing 90 μM quinidine. Whole cell lysates were prepared 0.5, 12, 24, and 48 h after subculture. Equal protein aliquots (50 μg/lane) were electrophoresed in 12% SDS-polyacrylamide gels and assayed for cyclin D1 and CDK protein levels by immunoblotting. Results shown are representative of three independent experiments.

Fig. 5. Proteasome inhibitor modulates retinoblastoma protein levels. Confluent MCF-7 cells were subcultured in the presence of 90 μM quinidine, 30 μM MG-132, or quinidine + MG-132 for 24 h, then harvested, and whole cell extracts (100 μg/lane) were analyzed for pRb. A Coomassie Blue-stained protein is shown as the loading control.

Histone Hyperacetylation in Breast Tumor Cell Lines

In MCF-7 cells exposed to quinidine, the increase in p21^WAF1 and decreased levels of both cyclin D1 and CDK4 (Fig. 4). In addition, Nakanishi et al. (22) showed that p53 is not required for pRb down-regulation by HDAC inhibitors in all cell lines, quinidine-treated MCF-7 cells have elevated p53 levels (5–7-fold) (Fig. 3). Thus, p53 could contribute to the maintenance of the G1 cell cycle arrest in MCF-7 by sustaining p21^WAF1, G1 cell cycle arrest, and apoptosis (25). Wild-type p53 down-regulates pRb levels in MCF-7 cells (26). Although Saito et al. (22) showed that p53 is not required for pRb down-regulation by HDAC inhibitors in all cell lines, quinidine-treated MCF-7 cells have elevated p53 levels (5–7-fold) (Fig. 3). Thus, p53 could contribute to the maintenance of the G1 cell cycle arrest in MCF-7 by sustaining p21^WAF1 protein levels and suppressing pRb protein levels.

Growth Arrest and Cellular Differentiation in Human Breast Tumor Cell Lines—In contrast to MCF-7 cells, normal breast tumor cell lines T47D, MDA-MB-231, and MDA-MB-435 express p53 proteins with distinct point mutations (27). To test for a requirement of p53, this panel of human breast tumor cell lines was exposed to quinidine, and the effects of quinidine on cell growth were compared (Fig. 6). The data shown are viable cell numbers/well, bioreductive metabolism/well, or both. In all four cell lines growth was suppressed in a concentration-dependent manner between 10 and 90 μM quinidine, and maximal growth inhibition was observed at ~90 μM quinidine (data not shown). These data showed that growth suppression by quinidine is a p53-independent response. It is interesting that quinidine was not overtly cytotoxic in HMEC, a line of normal human mammary cells (28).

Evidence that quinidine elicited cellular differentiation in MCF-7 human breast tumor cells in conjunction with the inhibition of cell growth was obtained using maximally effective concentrations of quinidine or retinoic acid (data not shown). Antibodies directed against cytokeratin 18 (29) were used to probe the organization of the cytoskeleton (Fig. 7). In these studies, all-trans-retinoic acid (10 μM) was used to compare the differentiation response (30). Control MCF-7 cells showed expression of cytoplasmic cytokeratin 18 in a disorganized fash-

Histone Hyperacetylation in Breast Tumor Cell Lines

35259
ion. Cells that were treated for 96 h with retinoic acid showed an increase in the intensity of the cytokeratin 18 staining and relocalization of cytokeratin 18 throughout the nucleus as well as the cytoplasm. In contrast, cytokeratin 18 staining occurred in a highly organized pattern in MCF-7 cells treated with quinidine for 96 h and the cells adopted a shape and nuclear localization more typical of columnar epithelium.

Lipid droplets are found in the cytoplasm of normal mammary epithelium (31), and cytoplasmic lipid droplet accumulation occurs in a variety of differentiating cell systems. Induction of differentiation in human breast cancer cell lines by oncostatin M (32), the HER-2/neu kinase inhibitor, emodin (33), overexpression of c-srcB-2 (34), the vitamin D analog, 1α-hydroxyvitamin D5 (35), the HDAC inhibitor, sodium butyrate (36), and retinoic acid (36) is accompanied by the accumulation of cytoplasmic lipid droplets. We utilized a fluorescent stain, Nile Red to monitor lipid droplet formation in mammary tumor cells in response to quinidine. The cells were counterstained with fluorescein-phalloidin that binds actin filaments to assay for changes in the actin cytoskeleton (Fig. 8).

The distribution of actin in four human breast tumor cell lines, MCF-7, T47D, MDA-MB-231, and MDA-MB-435 is seen clearly in the control cells. Three of these lines show strong nuclear staining of actin characteristic of transformed cells, whereas the fourth, MDA-MB-435, shows more cytoplasmic actin. In all cases except MDA-MB-435, the presence of quinidine did not significantly alter the actin cytoskeleton. Lipid droplet accumulation was weak or absent in the control cell.
Histone Hyperacetylation in Breast Tumor Cell Lines

MCF-7 Cytokeratin 18

Control Retinoic Acid Quinidine

FIG. 7. Cytokeratin 18 in MCF-7 cells. Cells were replica-plated (2 × 10^5) on sterile coverslips in 35-mm^2 dishes in medium containing 0.01% ethanol (control), 10 μM retinoic acid, or 90 μM quinidine and grown for 96 h. Cytokeratin 18 detection using a Texas Red-tagged secondary antibody is shown using confocal microscopy. Data shown are typical fields representative of two independent experiments.

Histone hyperacetylation and induction of cellular differentiation by quinidine were seen in a panel of human breast tumor cell lines that were selected for study on the basis of their diversity of genetic backgrounds. The differentiation response to quinidine was independent of the estrogen receptor (ER) status. Cell lines representative of ER-positive and ER-negative human breast carcinoma cells were induced to differentiate in the presence of quinidine. The ER status of the estrogen receptor positive cell lines is MCF-7 (ER-α and ER-β), T47D (ER-α and ER-β), and MDA-MB-231 (ER-β). MDA-MB-435 cells expressed very low levels of ER-β and no ER-α (37, 38). MCF-7 and T47D cells display an epithelial morphology and show similarities with mammary ductal and luminal epithelial cells, respectively (30, 39). MDA-MB-231 cells exhibit an elongated cellular morphology that is also typical of MDA-MB-435 cells. Our results demonstrate that quinidine is a differentiation agent in both types of mammary tumor cells.

HDAC inhibitors reverse the transformed phenotype of NIH3T3ras cells, and this property has been used successfully as a screening assay for the identification of new HDAC inhibitors (40, 41). Quinidine elicited a more differentiated phenotype in MCF-7ras cells, an MCF-7 cell derivative produced by stable transformation with v-Ha-ras, thus demonstrating that quinidine, like other HDAC inhibitors, can reverse an Ha-ras-induced phenotype.

Quinidine induced differentiation independently of wild-type p53. The ability of quinidine to cause differentiation of p53 mutant cell lines is consistent for a role of histone hyperacetylation in the response. HDAC inhibitors typically induce a p53-independent activation of p21WAF1 gene expression (5, 22). Growing MCF-7 and T47D cells express p21WAF1 protein levels (42), and quinidine raised p21WAF1 protein levels in MCF-7 cells approximately 11-fold within 12 h. Although p21WAF1 was reported to be low to undetectable in MDA-MB-231, p21WAF1 was detected in Western analyses of both MDA-MB-231 and T47D cells in a p53-independent fashion in response to serum deprivation, adriamycin, etoposide (42, 43), and quinidine (data not shown). These data support the idea that the p21WAF1 gene is present but inactive in growing MDA-MB-231 cells. Since histone hyperacetylation of the p21WAF1 gene occurs in response to HDAC inhibitors, it might be involved in the pathway of p53-independent activation of p21WAF1 gene expression (5).

The processes of cellular differentiation and cell cycle progression are interdependent. G1 arrest is a necessary but insufficient condition for differentiation in numerous cell types including leukemic cells, keratinocytes, colonic epithelium, and muscle cells. In all of these cells, induction of p21WAF1 protein and G1 cell cycle arrest occurred prior to differentiation (44–50) and was generally independent of p53. We hypothesize that the differentiated state can be viewed as a cellular response to G1 arrest, requiring a change in gene expression profile and suppression of cell death pathways. The response of MCF-7 breast tumor cells to quinidine is consistent with this model.

To begin to understand how quinidine might elicit G1 arrest of MCF-7 cells, we have focused on the action of quinidine as a potassium channel blocking agent. Quinidine enters cells and inhibits cardiac potassium channels by binding to the intracellular face of the ion pore (51). Although the location of the quinidine-binding site on the ATP-sensitive potassium channel is unknown, quinidine is freely permeable across membranes and inhibits the ATP-sensitive potassium channels whether it is applied to the external or internal surface of a lipid membrane bilayer (52).

In the presence of quinidine, MCF-7 cells accumulate at a position 12 h into G1 phase (12). This position, defined by cell cycle arrest and release experiments, precedes the lovastatin arrest point by 5–6 h and is clearly distinct from the restriction...
point described by Pardee (53) near the G1/S transition. The present work showed that quinidine treatment caused elevated levels of p53 and p21\(^{WAF1}\) protein by 12 h (Fig. 3), the point within G1 where MCF-7 cells arrest in response to quinidine (12). When p53 and p21\(^{WAF1}\) proteins were assayed before 12 h, p53 was undetectable, and p21\(^{WAF1}\) was first detected after 8 h of quinidine treatment (data not shown), suggesting a p53-independent induction of p21\(^{WAF1}\) occurred prior to arrest in G1. CDK4 and cyclin D1 protein levels were also reduced, as was CDK4 activity as demonstrated by the abundance of hypophosphorylated pRb protein. Based upon our observations in MCF-7 cells, we conclude that p21\(^{WAF1}\) protein levels become elevated prior to the G1 arrest in response to quinidine and could initiate the G1 arrest. Hypophosphorylated pRb protein is prominent in quinidine-treated MCF-7 cells, and this could act to sustain the G1 state by preventing the transition into S phase. The G1 arrest induced by quinidine in MCF-7 cells was correlated with the blockade of ATP-sensitive potassium channels in MCF-7 cells (12, 54, 55). Direct evidence for the involvement of potassium ions in the G1 arrest was provided using valinomycin, a potassium-selective ionophore to stimulate a G1-S phase transition in the presence of quinidine (12).

In summary, quinidine, a drug that is used therapeutically in the treatment of malarial infections and cardiac arrhythmia, was shown to be useful as an inducer of cellular differentiation in human breast tumor epithelial cells. Quinidine caused histone H4 hyperacetylation and cellular differentiation in human breast tumor cells following the rapid loss of HDAC1 involving a proteasome-dependent pathway. Additional experiments are needed to determine how the action of quinidine upon ATP-sensitive potassium channels initiates the molecular events underlying the differentiation response.

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