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Award Number: DAMD17-99-1-9447

TITLE: Regulation of C-myc Gene Expression by Potassium Channel Blocker Quinidine in MCF-7 Human Breast Cancer Cell Line

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REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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4/3/02 _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual Summary (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Regulation of C-myc Gene Expression by Potassium Channel Blocker Quinidine in MCF-7 Human Breast Cancer Cell Line

5. FUNDING NUMBERS
DAMD17-99-1-9447

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

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13. ABSTRACT *(Maximum 200 Words)*

C-myc is a protooncogene whose gene product has a regulatory role in cell cycle progression, cellular differentiation, and apoptosis. The abnormal expression of c-myc was reported in 32% of breast cancers, suggesting its importance in the genesis and/or progression of breast cancer. Therefore, it is important to understand the precise molecular mechanisms of c-myc regulation. The goal of this proposal is to elucidate how potassium channel blocking agent, quinidine, regulates expression of c-myc gene in breast cancer cells. Specific aim #1 was to test effects of quinidine on c-myc promoter activity (completed), identify quinidine responsive element (QRE) within c-myc promoter (completed), and study effects of quinidine on proteins/DNA interactions in the QRE (in progress). The aim #2 was to test effects of quinidine on c-myc transcription rate by nuclear run-on assay. In the current report the PI proposed to substitute this aim with the one that tests effects of quinidine on c-myc mRNA stability, due to the technical difficulties to detect small changes in c-myc transcription rate by run-on assay (not initiated). The significance of this proposal is that its results will improve our understanding of c-myc gene regulation and might lead to discovery of new targets for breast cancer therapy.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast cancer, c-myc, quinidine	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

C-myc is a protooncogene whose gene product has a regulatory role in cell cycle progression, cellular differentiation, and apoptosis. C-myc is one of the most common oncogene aberrations in breast cancer [1,2,3]. The abnormal expression of c-myc was reported in 32% of breast cancers [4], suggesting its importance in the genesis and/or progression of breast cancer. Although the molecular mechanisms leading to deregulated expression of c-myc gene in tumors are well understood, the precise regulatory mechanisms of c-myc gene expression remain elusive. Previous research in out laboratory demonstrated that potassium channel blocking agent, quinidine, causes G1/G0 cell cycle arrest and inhibition of growth in MCF-7 human breast cancer cells. Our preliminary results also showed that quinidine suppresses estradiol-stimulated c-myc mRNA levels in MCF-7 cells during 1-24 hrs time course. Quinidine caused rapid (within 1-2 hours) down-regulation of Myc protein in a panel of human breast cancer cell lines (MCF-7ras, MDA-MB-231, MDA-231, MCF-7 and MDA-435, see Figure 1), which followed by the induction of cellular differentiation (Figure 8 in the appended article). Quinidine had no significant effects on growth or Myc protein levels in MCF-10A normal breast epithelial cell line (Figure 4). The goal of this proposal is to elucidate the molecular mechanisms by which potassium channel blocking agent, quinidie, regulates the activity of c-myc gene. Aim #1 was to test the hypothesis that quinidine suppresses c-myc promoter activity in transient reporter gene assay, using c-myc promoter region linked to the luciferase reporter gene. If there is a suppression at this level, then the construction of series of 5'-deletion mutants of c-myc promoter to map a minimal region of the promoter that is sufficient to confer quinidine responsiveness (QRE-quinidine responsive element) was proposed. The last part of the Aim #1 was to test the changes in protein/DNA interaction within QRE in response to quinidine using electrophoretic mobility shift assay (EMSA). Aim #2 was to test hypothesis that quinidine regulates the rate of c-myc transcription, using nuclear run-on assay. The significance of this proposal is that its results will improve our understanding of c-myc gene regulation and might lead to the discovery of new targets for breast cancer therapy.

Body of the Report

Annual Summary

The goal of this proposal is to understand the molecular mechanisms by which potassium channel blocking agent, quinidine, regulates expression of c-myc gene in human breast cancer cells. The specific aim #1 consisted of three parts: 1A was to test effects of quinidine on c-myc promoter in transient reporter gene assay; 1B was to construct a series of 5'-deletion mutants of c-myc promoter to map a minimal region of the promoter that confers quinidine responsiveness (QRE-quinidine responsive element); 1C was to assay protein/DNA interactions in response to quinidine within the QRE using electrophoretic mobility shift assay (EMSA). The specific aim #2 was to test effects of quinidine on the rate of c-myc transcription in nuclear run-on assay.

Aim 1A is completed as described in the annual summary report for 2000.

Aim 1B was partially completed as reported in the annual summary for 2000. The minimal c-myc promoter region of 614 bp (within Frag E construct) that is sufficient for quinidine responsiveness was described. In an attempt to perform a 3'-deletion of this 614 bp region, the PI had difficulties in cutting it with the appropriate restriction enzymes. In order to resolve this problem, Frag-E plasmid was sent to MGIF (Molecular Genetic Facility of University of Georgia) for the sequencing. The results revealed that Frag-E contains 168 bp region of c-myc promoter (from –100 to +68 in respect to P1 promoter start site) instead of 614 bp region, as was originally thought. Since Frag-E was still responsive to quinidine in transient reporter gene assay (annual summary 2000), the PI concludes that QRE (Figure 2A) is located within this 168 bp region of c-myc promoter. This region contains two important regulatory elements: transforming growth factor β1 (TGFβ1) control element (TCE) located between –83 and –63 in respect to P1 [5] and GC-rich region (from –60 to –37) that binds Sp1 and Sp3 transcription factors [6,7] (Figure 2A). Down-regulation of c-myc promoter activity through TCE has been reported to play a key role in TGF beta1 mediated growth inhibition in different cell lines. In order to test the role of TCE in the regulation of c-myc promoter by quinidine, PI carried out a series of experiments comparing effects of quinidine and TGF beta1 on proliferation and Myc protein levels in MCF-7, MDA-231, and MDA-468 human breast cancer cells and MCF10A normal human breast epithelial cells. As demonstrated in the Figure 3, quinidine almost completely inhibited growth of all three cancer cell lines while TGF beta1 had little (MCF-7) or no (MDA-231 and MDA-468) effects on cell growth. In contrast, MCF10A cells were more sensitive to TGF beta1 than to quinidine. Both agents had additive effects on proliferation of MCF10A cells, which indicates that they work through the different pathways. There was a good correlation between inhibition of cell growth and Myc protein levels by quinidine and TGF beta 1 in MCF10A cells (Figure 4). Also, quinidine inhibited growth of MDA-468 cell line (Figure 3) that has a defective TGF beta 1 pathway due to the lack of an important mediator of TGF beta1 signaling, Smad4 transcription factor. Jointly, these results indicate that regulation of c-myc and subsequently cell growth by quinidine and TGF beta 1 is mediated by different mechanisms in the descirbed cell lines. By eliminating TCE as a mediator of quinidine's responses on c-myc promoter, the only regulatory element remaining within the proposed 168 bp QRE is the GC-rich region. This region has been shown to be essential for P1 transcription [6,7] and can bind both Sp1 and Sp3 transcription factors. To assay the involvement of the GC-rich region in regulation of c-myc promoter by quindine, PI next tested effects of

quinidine on the protein levels of both transcription factors (Sp1 and Sp3) in MCF-7 cells. As shown in Figure 2B, quinidine had no effects in either case. From these results the PI concluded that suppression of c-myc promoter activity in response to quinidine is not mediated by the changes in Sp1 or Sp3 proteins levels. This does not imply that Sp1 or/and Sp3 are not involved in the regulation of c-myc promoter activity by quinidine, since binding of these proteins to the GC-rich region but not their levels could be responsive to quindine. This issue will be addressed in the aim 1C.

Aim 1C remains unchanged. It is currently in progress and is planned to be completed by month 26. The changes in protein-DNA interactions in response to quinidine in the GC-rich region of QRE described above will be tested using protocol and experimental design reported in the annual summary 2000 under the aim 1C.

Aim # 2 was to test the hypothesis that quinidine regulates the rate of c-myc transcription using nuclear run-on assay. Our repeated Northern experiments showed only 50% decrease in c-myc mRNA levels in response to quinidine. Although it was sufficient to inhibit cell growth, technically it will be very difficult to detect such small difference by run-on assay. Therefore, the PI proposes to substitute the aim #2 with an alternative approach that has been proposed in the original proposal. The effects of quinidine on c-myc mRNA stability will be tested in the following experimental design. MCF-7 or MCF10A cells will be sub-cultivated in DMEM containing 5% FBS and allowed to attach for one hour. After that, cells will be treated with: 1) actinomycin D to inhibit RNA synthesis or with 2) actinomycin D plus 90 uM quinidine. Total cellular RNA will be isolated every 15 min from both treatment groups during two hours time period. Northern blot experiments will be performed to test effects of quinidine on the half-life of c-myc mRNA. This aim is proposed to be completed by month 26.

Aim # 3 included preparation of reports and manuscripts. This aim remains unchanged. It is currently in progress and will be completed by the end of month 30.

The changes in the time schedules for the completion of the aim # 2 and 3 proposed in the current report are due to the fact that PI is planning to graduate with Ph.D. degree in Pharmacology and Toxicology in December, 2001. The PI has successfully completed Ph.D. course work requirements with a cumulative GPA of 3.9. PI has also successfully passed the qualifying examinations, consisting of: 1) writing and defending the NIH format grant proposal, 2) oral comprehensive exam based on the course work, 3) writing and defending a detailed progress report on the research performed by the PI during the past 4 years.

The following outline summarizes an up-to-date progress for the each specific aim:

Aim 1A	Months 1-2	Complete
Aim 1B	Months 3-24	Complete
Aim 1C	Months 24-26	In progress
Aim 2	Month 26	Not initiated
Aim 3	Months 24-30	In progress

Key research accomplishments:

- Completion of the specific aim # 1B and initiation of the specific aim #1C and aim #3 of the U.S. Army proposal.

Reportable outcomes:

Research Papers

- Qun Zhou, **Melkoumian, Z.K.**, Lucktong, A., Moniwa, M., Davie, J.R., Strobl, J.S. Rapid induction of histone hyperacetylation and cellular differentiation in human breast tumor cell lines following degradation of histone deacetylase-1. *Journal of Biological Chemistry, 2000 Nov 10;275(45):35256-63.*

Abstracts

- **Melkoumian, Z.K.**, McCracken, M.A., Strobl, J.S. Suppression of c-Myc protein levels and induction of cellular differentiation in human breast cancer cells but not in the normal human breast epithelial cells by quinidine. American Society for Cell Biology annual meeting, December 2001 (submitted).

Presentations

- Presented research data at a seminar in the Pharmacology and Toxicology department, West Virginia University.

- Gave two journal club presentations of current scientific literature.

Conclusions:

During the second funded year (2000-2001) PI made a significant progress on the specific aim #1. First two parts of the aim #1, 1A and 1B are completed. The quinidine responsive element of the c-myc promoter was mapped to the 168 bp region (from –100 to +68 in respect to P1 promoter start site). This region contains two important regulatory elements: transforming growth factor β1 (TGFβ1) control element (TCE) located between –83 and –63 in respect to P1 [5,6] and GC-rich region (from –60 to –37) that binds Sp1 and Sp3 transcription factors [7]. In order to test the role of TCE in the regulation of c-myc promoter by quinidine, the PI performed a series of experiments to compare the effects of quinidine and TGF beta1 on Myc protein levels and cell growth in several human breast cancer and normal breast epithelial cell lines. The results suggested that quinidine and TGF beta1 suppress Myc and subsequently cell growth by the different mechanisms. After eliminating TCE as a mediator of quinidine's responses on c-myc promoter, the only regulatory element remaining within the proposed 168 bp QRE is the GC-rich region. The effects of quinidine on proteins/DNA interactions in this region will be tested in the aim 1C using electrophoretic mobility shift assay. This aim is currently in progress and will be completed by month 26. The aim #2 was to test effects of quinidine on c-myc transcription rate by nuclear run-on assay. In the current report the PI proposed to substitute this aim with the one that tests effects of quinidine on c-myc mRNA stability, due to the technical difficulties to detect small changes in c-myc transcription rate by run-on assay (not initiated).

The goal of this proposal is to understand how quinidine regulates expression of c-myc oncogene in human breast cancer cells. C-myc is one of the most common oncogene aberrations in breast cancer [1,2,3]. The abnormal expression of c-myc was reported in 32% of breast cancers [4], suggesting its importance in the genesis and/or progression of breast cancer. One of the major problems of current chemotherapeutic drugs for breast cancer treatment is their lack of selectivity for tumor tissue and associated with it toxicity for normal tissues. Therefore, the findings that quinidine selectively inhibits c-myc gene expression, cell growth and induces cellular differentiation in the different human breast cancer cell lines, but not in the normal human breast epithelial cells is very interesting and exciting. The PI hopes that results from this proposal will improve our understanding of c-myc gene regulation and might lead to the discovery of new targets for breast cancer therapy.

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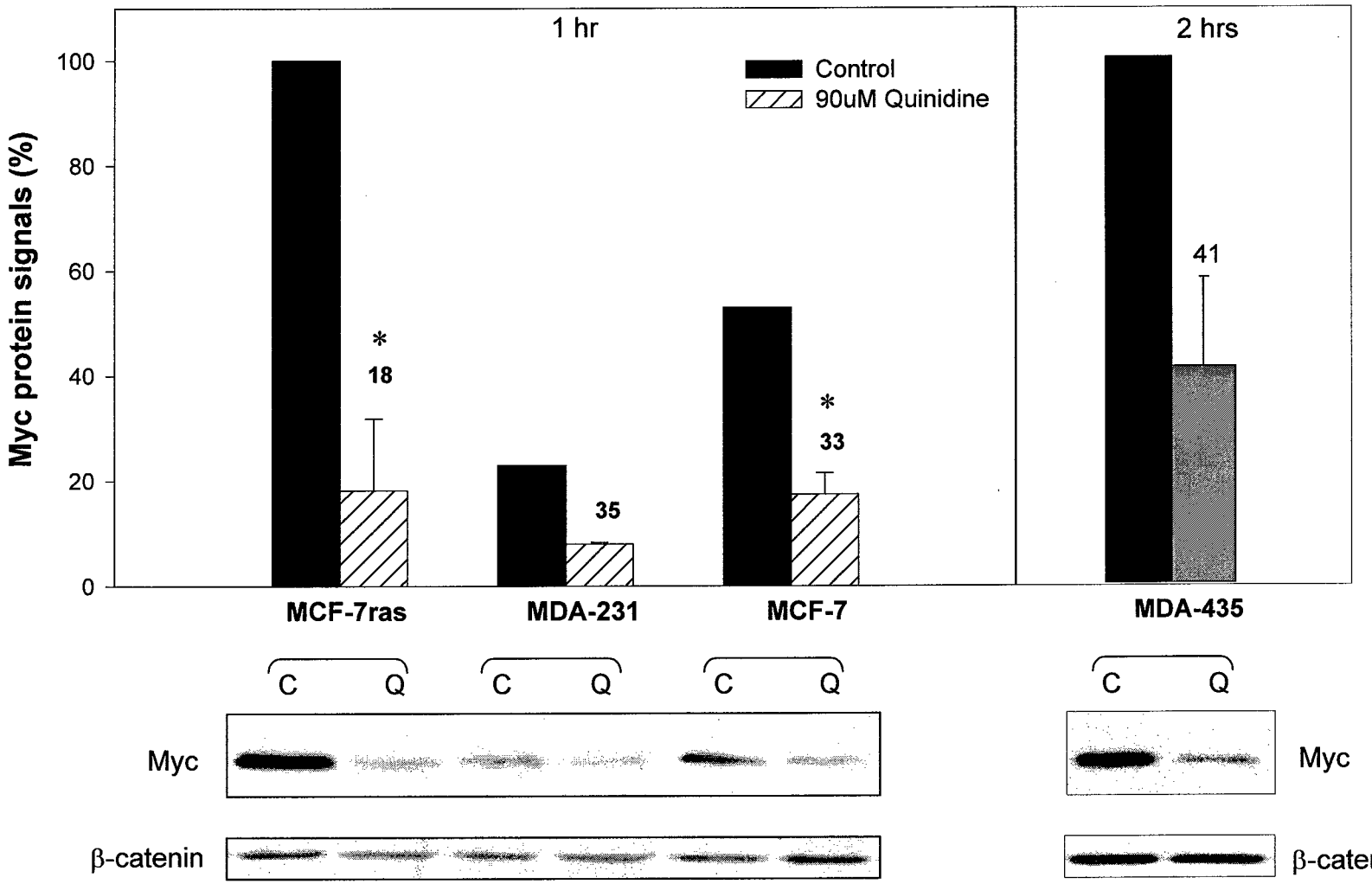
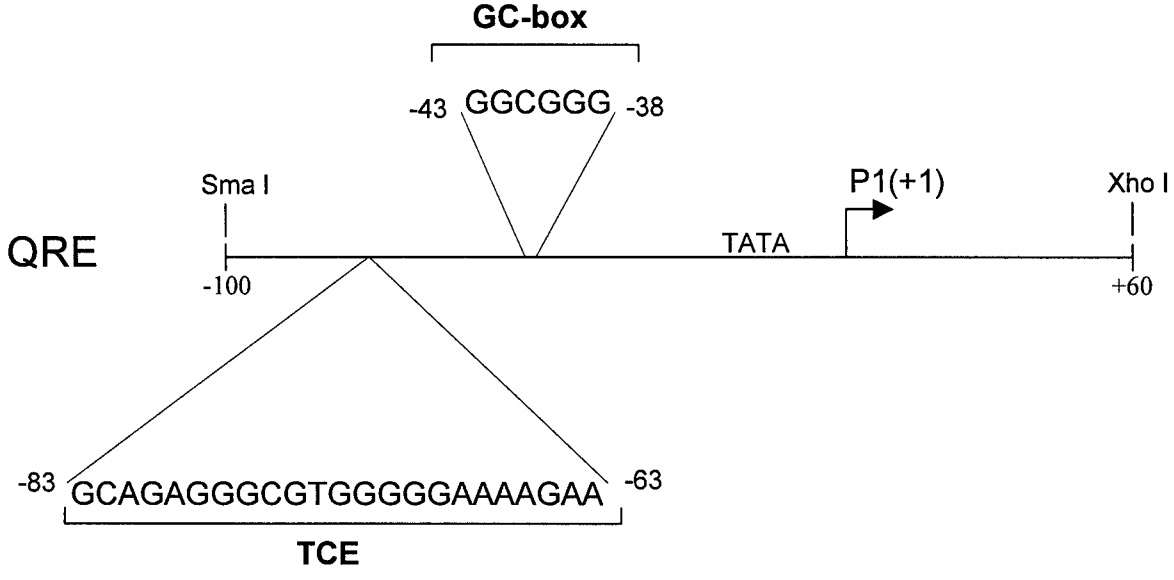
Figure 1. Quinidine suppresses Myc protein levels in human breast cancer cell lines



Myc protein levels in quinidine-treated cells (Q) expressed as a percent of the respective controls (C) for each cell line are shown above the hatched bars. The histogram data are the mean +/- S.D. of n=3 (MCF-7 and MCF-7ras) or n=2 (MDA-231 and MDA-435) experiments. The difference between the percentages of control and quinidine-treated cells was statistically significant for MCF-7ras ($p<0.008$) and MCF-7 cells ($p<0.009$). Myc protein signals were normalized to the β-catenin signals.

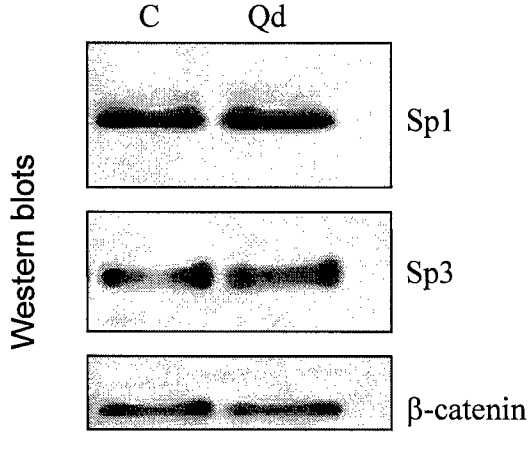
Melkoumian, Z.K.

Figure 2A. The Structure of the 168 bp Quinidine Responsive Element (QRE) of human c-myc gene promoter



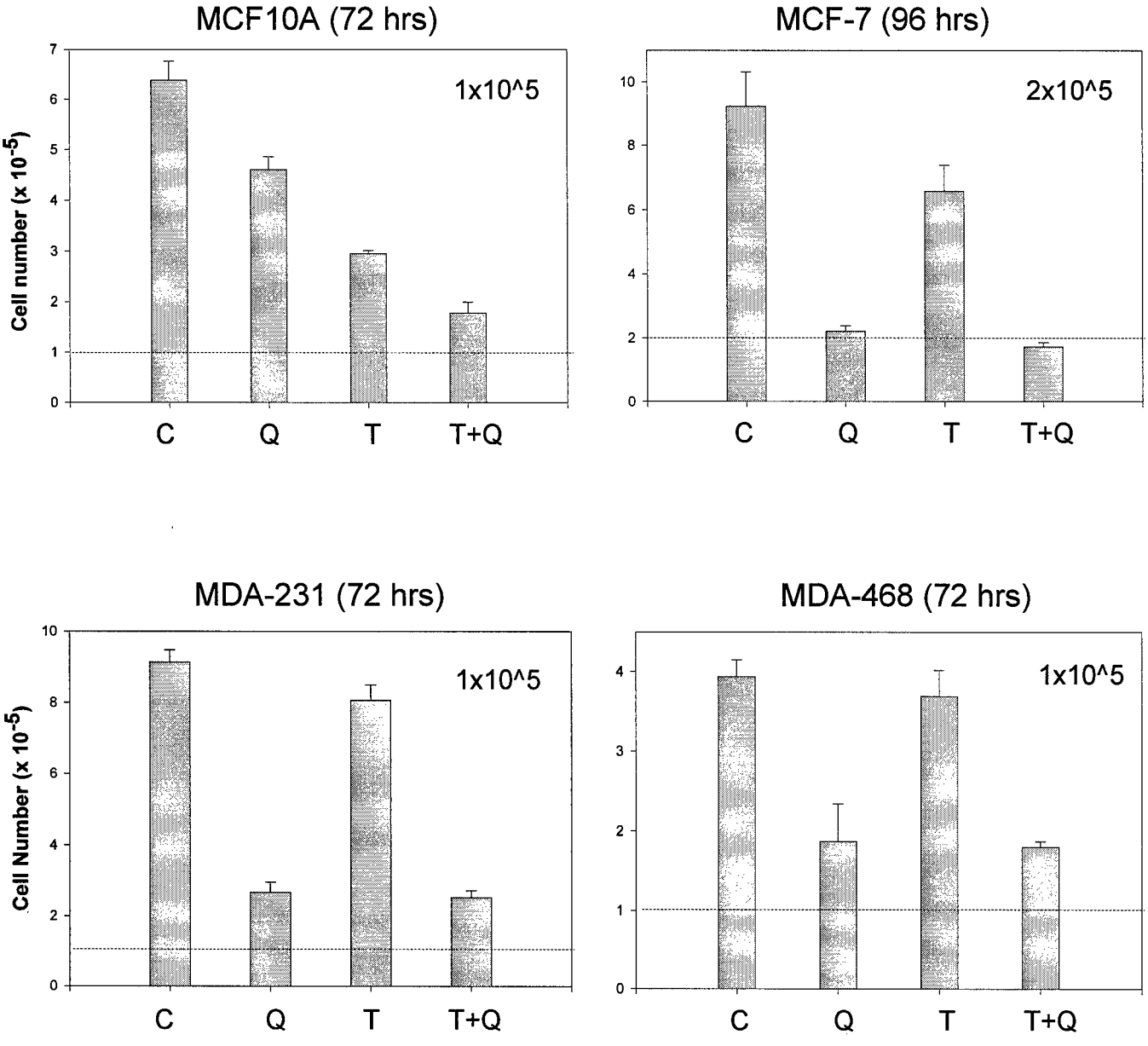
UNPUBLISHED DATA

Figure 2B. Effects of 90 uM quinidine on Sp1 and Sp3 proteins levels in MCF-7 cells



The total cellular proteins from MCF-7 cells treated with +/- 90 uM quinidine were extracted 1.5 hrs after the plating. Western blot assays were performed to test the effects of quinidine on Sp1 and Sp3 proteins levels. The β-catenin signals were used as a loading control.

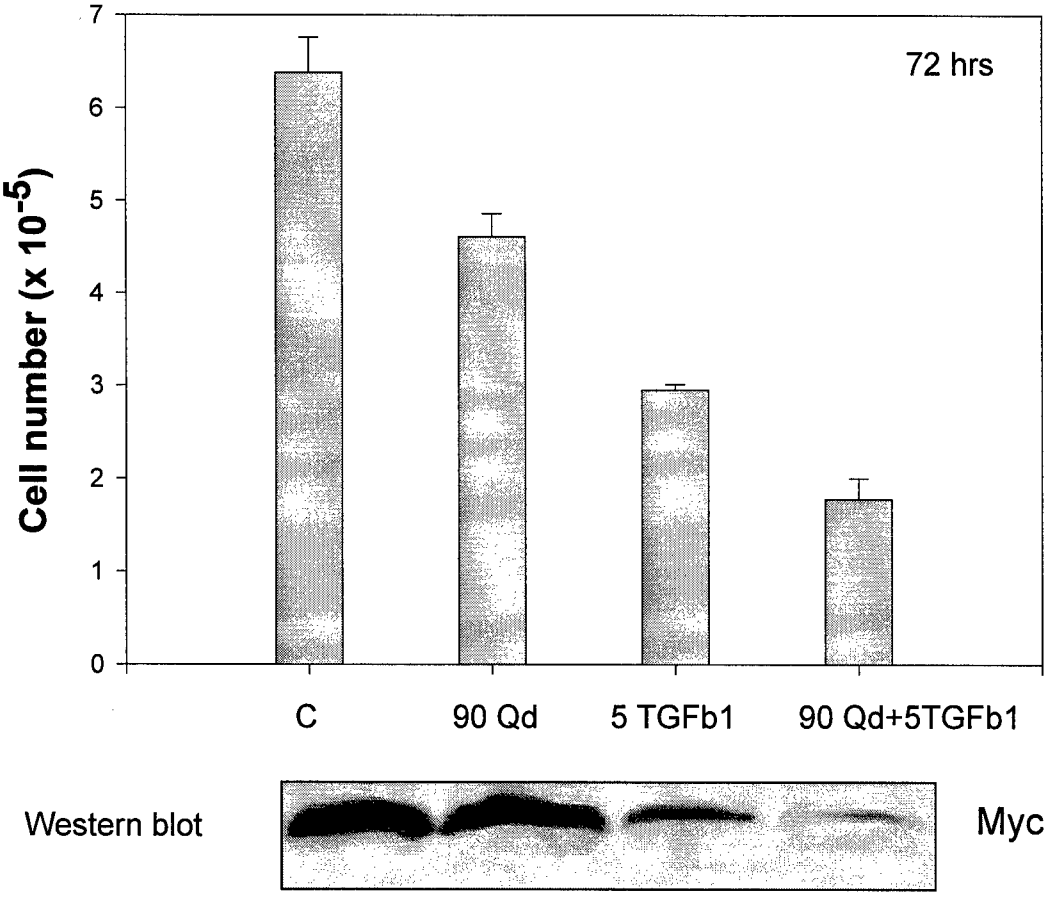
Figure 3. Effects of quinidine and TGF beta 1 on growth of human breast cancer and normal beast epithelial cell lines



C - Control Q - 90 uM Quinidine T - 5 ng/ml TGFb1 T+Q - 90 Qd+5TGFb1

Different human breast cancer (MCF-7, MDA-231, MDA-468) or normal breast epithelial (MCF10A) cell lines were replica-plated in tissue culture dishes at the densities indicated on a top right corner of each graph. Cells were treated with: 1) nothing, 2) 90 uM quinidine, 3) 5ng/ml TGFbeta1, 4) 90 uM quinidine + 5ng/ml TGFbeta1. Cell count was performed by hemocytometer 72 or 82 hours later, as indicated for each cell line.

Figure 4. A correlation between suppression of growth and Myc protein levels in MCF10A cell line



	Contorl	90 uM quinidine	5ng/ml TGFb1	90 Qd + 5TGFb1
Cell count data (% of control)	100 (5.9)	72 (3.9)	46 (0.9)	28 (3.4)
Myc protein signal (% of control)	100 (0)	83 (10.6)	46 (11.2)	33 (14.5)

The data in the table represent mean +/- STDEV of triplicates (cell count assay) or 2 independent experiments (Western blot assay).

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 45, Issue of November 10, pp. 35256–35263, 2000
Printed in U.S.A.

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

Received for publication, April 12, 2000, and in revised form, August 2, 2000
Published, JBC Papers in Press, August 10, 2000, DOI 10.1074/jbc.M003106200

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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 G_1 phase of the cell cycle and led to a G_1 to G_0 transition followed by apoptotic cell death. The present experiments demonstrated that MCF-7, MCF-7*ras*, 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 $p21^{WAF1}$, 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 proteasomal sensitive mechanism.

Histone deacetylase (HDAC)[1] proteins 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 corepressors (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), superoylanilide 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 G_1, elevated $p21^{WAF1}$ 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 $p21^{WAF1}$ plays a critical role in causing G_1 cell cycle arrest via inhibition of the G_1 cyclin-dependent kinase family (9). Overexpression of $p21^{WAF1}$ has also been associated with apoptosis, but the mechanism of $p21^{WAF1}$ 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-

* This work was supported by West Virginia University School of Medicine, the Spurlock Cancer Research Fund, the Susan G. Komen Breast Cancer Foundation, Grants DAMD 17-99-1-9447 and DAMD 17-00-1-0500, and the Medical Research Council of Canada Grant MT-9186. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HDAC, histone deacetylase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PBS, phosphate-buffered saline; pRb, retinoblastoma protein; SAHA, superoylanilide hydroxamic acid; TSA, trichostatin A; HRP, horseradish peroxidase; HMEC, human mammary epithelial cells; ER, estrogen receptor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; CDK, cyclin-dependent kinase.

ping signaling cascades. For this reason, use of cancer therapies 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 phenyl butyrate 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 G_1 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*ras* (13), T47D, MDA-MB-231, and MDA-MD-435 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, 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 93% air, 7% CO_2. 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 proteasome inhibitors, MG-132 and lactacystin, were purchased from Calbiochem.

Growth Inhibition Assays—Growth inhibition by cell numbers was assayed by plating cells in 35-mm^2 dishes (1–1.5 $\times$ 10^5) 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 compound (Owen's reagent) to a colored formazan product that absorbs light at 490 nm. The plating density for the 96-well dishes (cells/well) was varied depending upon the relative growth rates of the cell lines as follows: HMEC (2000), MCF-7 (1000), MDA-MB-231 (500), T47D (1500), and MCF-7*ras* (500). The One Cell Proliferation Assay Reagent was added to each well and incubated for 2 h at 37 °C. Absorbance (490 nm) was read using a Molecular Devices PC340 (Sunnyvale, CA).

Microscopic Imaging—Cells were plated (1×10^5) on sterile coverslips in 35-mm^2 dishes and grown for 96 h in DMEM, 5% FBS supplemented with either 10 μM all-*trans*-retinoic acid (in 0.01% ethanol) or 90 μM quinidine (in H_20). Control cells were grown in medium containing a final concentration of 0.01% ethanol. The presence of ethanol had no effect upon lipid droplet accumulation compared with cells grown in DMEM, 5% FBS. Cells were fixed in 3.7% formaldehyde/PBS, rinsed in PBS (PBS: 140 mM NaCl, 2 mM KCl, 80 mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4, pH 7.0), then treated briefly with 0.4% Triton X-100 in PBS. After rinsing three times in PBS, the cells on coverslips were incubated for 30 min at 37 °C with a primary antibody to cytokeratin 18 (1:1 dilution, provided by Dr. Guillaume van Eys, Maastricht University), rinsed, and incubated (30 min/37 °C) with Texas Red conjugated secondary antibody (goat anti-mouse IgG, Sigma). Alternatively, cells were incubated with fluorescein-phalloidin (1:200 dilution of a-5 μg/0.1 ml solution, Sigma) in the dark for 40 min at room temperature, rinsed, and incubated for 5 min (room temperature) with the fluorescent lipid stain, Nile Red (1:10,000 dilution of a 1 mg/ml acetone solution, Sigma) (14–15). All coverslips were rinsed in PBS and mounted with Fluoromount-G containing 2.5% *N*-propyl galate. Images were obtained using a Zeiss Axiovert 100 M confocal microscope (× 63 objective).

Immunoblotting—Cells were harvested from confluent T-75 flasks and subcultured (1×10^6) in 60-mm^2 dishes. On subcultivation, this confluent population of cells (85% in G_1) synchronously proceeded 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 M NaCl, 0.1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, and 0.1 mM Na_3VO_4, pH 7.4). Protease inhibitors (protease inhibitor mixture, Roche Molecular Biochemicals) were added immediately. Cell lysates were centrifuged in an Eppendorf microcentrifuge (14,000 rpm, 5 min) at 4 °C, and the supernatants were used in immunoblotting experiments.

Histones were prepared from cells grown at a density of 1×10^7/T-162 flask. To harvest the cells, the flasks were placed on ice, and the growth medium was removed. Following a quick rinse with ice-cold PBS, cells were scraped into 1 ml of ice-cold lysis buffer (10 mM Tris-HCl, 50 mM sodium bisulfite, 1% Triton X-100 (v/v), 10 mM $MgCl_2$, 8.6% sucrose, pH 6.5) and nuclei released by Dounce homogenization. The nuclei were collected by centrifugation (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 ml of ice-cold sterile water using a vortex and concentrated H_2SO_4 to 0.4 N was added. 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 ml 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 H_2O 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 upon 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 blocking buffer (1 M glycine, 1% albumin (chicken egg), 5% non-fat 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 (F-8, SC-1641), rabbit polyclonal anti-CDK4 (C-22), goat polyclonal anti-HDAC1 (N-19, SC-6299), all from 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 Novocastra (Burlingame, 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 washing 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-HRP (SC-2020), rabbit IgG-HRP (SC-2004), or mouse IgG-HRP (SC-2005) from Santa Cruz Biotechnology) at a final dilution of 1:3000 in Western washing solution. After washing three times with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 0.5 M 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 (18). 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 [^{3}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 0.12/0.72 N. Released [^{3}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

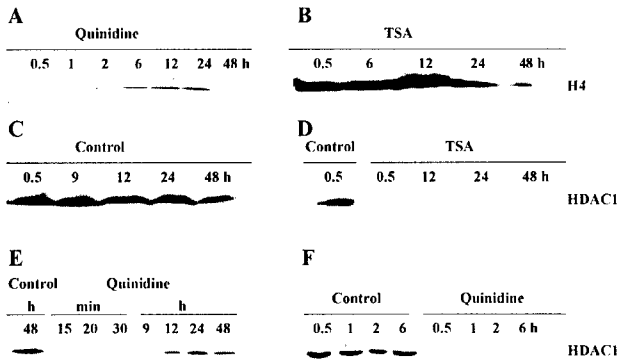

FIG. 1. **Histone hyperacetylation in MCF-7 cells.** *A,* histones were extracted from cells grown in the presence of 90 μM quinidine for 0.5, 1, 2, 6, 12, 24, or 48 h; histones (20 μg/lane) were electrophoresed in 15% polyacrylamide gels containing 1% SDS and assayed for the presence of acetylated H4 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.

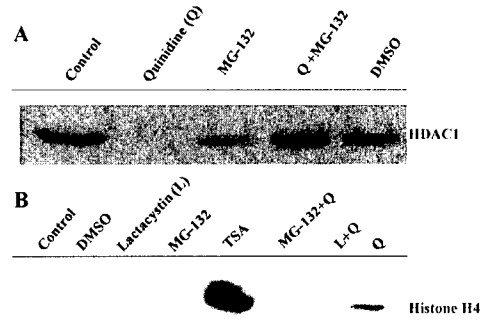

FIG. 2. **Protection of HDAC1 protein by proteasome inhibitors.** *A,* MCF-7 cells were released from confluency and subcultured in normal growth medium supplemented with 30 μM MG-132 in 0.1% Me_2SO (*DMSO*), 90 μM quinidine plus 0.1% Me_2SO or 0.1% Me_2SO alone as indicated. Cells were harvested after 30 min, and Western blot analysis of HDAC1 protein was performed as detailed in methods. *B,* MCF-7 cells were cultured as described above or with lactacystin (3 μM in 0.1% Me_2SO) except were harvested after 24 h and analyzed for the presence of immunoreactive acetylated histone H4.

hyperacetylation of H4 in MCF-7 cells with that of TSA, an established HDAC inhibitor, known to inhibit proliferation in MCF-7 cells (20). H4 acetylation in response to TSA was rapid (within 0.5 h) (Fig. 1*B*) and reached a maximum around 12 h. Some level of H4 acetylation persisted in the TSA-treated cells for 48 h. In cells treated with quinidine (Fig. 1*A*), detectable H4 acetylation was slightly delayed and could be seen at 2 h but not 1 h of treatment. H4 acetylation was maximal between 12 and 24 h but then sharply fell to an undetectable level at 48 h. Hyperacetylation of H4 was a transient response to both agents. Acetylated H4 is present in MCF-7 cells but under the conditions of Western blotting and immunochemical staining was not detected in control cells (data not shown).

HDAC1 is expressed in MCF-7 cells, and this enzyme contributes to the control of histone deacetylation rates (18). Quinidine caused the rapid disappearance of HDAC1 from MCF-7 cells. HDAC1 protein levels in quinidine-treated cells were reduced after 15–20 min compared with control cells, and HDAC1 protein was undetectable between 30 min and 6 h (Fig. 1, *E* and *F*). Partial restoration of HDAC1 protein occurred beginning at 9 h of treatment, but even after 48 h, HDAC1 levels in quinidine-treated cells were still less than control cells. Levels of HDAC1 protein in control MCF-7 cells were relatively constant during this time (Fig. 1*C*). TSA-treated MCF-7 cells also showed reduced levels of HDAC1 protein as early as 30 min after drug addition, and the reduced HDAC1 protein level was maintained through 48 h (Fig. 1*D*). The data indicate that loss of HDAC1 protein might contribute to the H4 acetylation response to both TSA and quinidine. However, HDAC1 protein levels were never reduced by TSA below the level of detection as was observed with quinidine. In light of the more extensive H4 acetylation response to TSA than quinidine, we conclude that the direct inhibition of HDAC1 catalytic activity by TSA remains an important component of the H4 acetylation response *in vivo*. In addition, the time course of the HDAC1 response to quinidine and TSA differ. In response to quinidine, there is initially a more marked decrease in HDAC1 protein levels but a more rapid recovery. TSA treatment caused a sustained reduction in HDAC1 protein levels through 48 h.

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. 2*A*). Treatment with the solvent, Me_2SO, 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 Me_2SO, 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.* $p21^{WAF1}$ 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. 2*B*). These results support the idea that quinidine-induced loss of HDAC1 protein is involved in the H4 acetylation response via a proteasomal sensitive pathway.

G_1 Phase Cell Cycle Regulatory Profile in MCF-7 Cells—G_1 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 $p21^{WAF1}$ protein, are numerous (21–23). It was of interest to determine whether $p21^{WAF1}$ and other key cell cycle regulatory proteins such as the retinoblastoma protein (pRb) and the G_1 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 $p21^{WAF1}$ were increased in response to quinidine treatment approximately 11-fold, and this elevated level of protein expression persisted through 48 h.

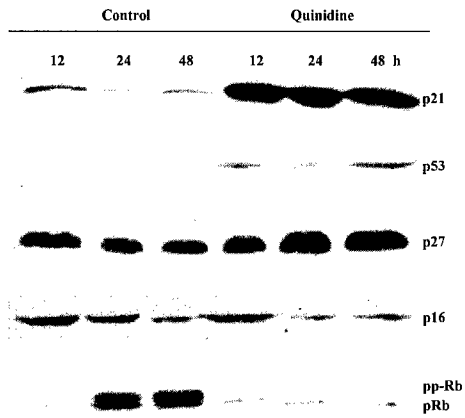

FIG. 3. **G_1 cell cycle proteins in MCF-7 cells.** Cells released from confluency were plated into control medium or medium containing 90 μM quinidine. Whole cell lysates were prepared 12, 24, or 48 h after plating and assayed by immunoblotting for the cyclin-dependent kinase inhibitors, $p21^{WAF1}$ (n = 3), p27 (n = 1), 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 (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.

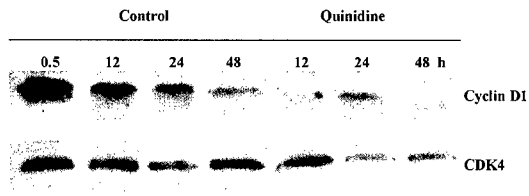

FIG. 4. **Cyclin D-CDK4 in MCF-7 cells.** Confluent MCF-7 cells were subcultured in control medium or medium containing 90 μM quinidine. Whole cell lysates were prepared 0.5, 12, 24, and 48 h after subculture. Equal protein aliquots (50 μg/lane) were electrophoresed in 12% SDS-polyacrylamide gels and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

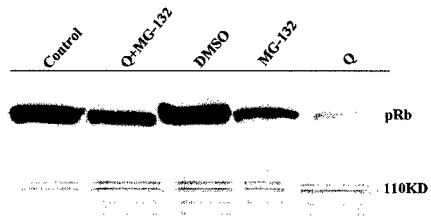

FIG. 5. **Proteasome inhibitor modulates retinoblastoma protein levels.** Confluent MCF-7 cells were subcultured in the presence of 90 μM quinidine, 30 μM MG-132, or quinidine + MG-132 for 24 h, then harvested, and whole cell extracts (100 μg/lane) were analyzed for pRb. A Coomassie Blue-stained protein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

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, $p21^{WAF}$, as well as an important G_1 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-G_1 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, typical of cells in early G_1 phase, and increased expression of both phosphorylated and unphosphorylated pRb at 24 and 48 h. Quinidine-treated MCF-7 cells had no detectable hyperphosphorylated pRb at any time point examined, and total levels of pRb protein failed to increase with progression through G_1 phase as seen in the control, proliferating cells (Fig. 3). The decrease in pRb phosphorylation level was predictable based on the increase in $p21^{WAF1}$ and decreased levels of both cyclin D1 and CDK4 (Fig. 4). In addition, Nakanishi *et al.* (24) showed that $p21^{WAF1}$ can bind pRb protein and block its phosphorylation. However, the actions of quinidine upon $p21^{WAF1}$ and cyclin D-CDK4 activity do not explain why the levels of total pRb protein were so low. Reductions in the cellular content of phosphorylated pRb protein in MCF-7 cells by quinidine is an important additional level of cell cycle control that effectively attenuates progression of cells out of G_1 phase and has been reported in other tumor cell lines in response to HDAC inhibition (22). In Fig. 5 we show data suggesting that the 26 S proteasome pathway regulates the total pRb content. MCF-7 cells incubated for 24 h in MG-132 or MG-132 plus quinidine had more total pRb than cells incubated with quinidine alone. Thus, quinidine promoted the loss of both HDAC1 and pRb, and inhibition of the 26 S proteasome pathway restored the levels of both of these proteins to that seen in the untreated cells. We have no direct evidence that quinidine promotes the proteasomal degradation of either protein. We hypothesize that quinidine may direct degradation of HDAC1 by the proteasome or, alternatively, quinidine might stimulate the proteasomal degradation of other regulatory factor(s) that act to maintain HDAC1 and pRb protein levels.

MCF-7 cells express wild-type p53 protein. Normal p53 is a short lived protein that is maintained at low levels, but in response to cell stress or DNA damage, p53 is stabilized and accumulates in the nucleus where it functions as a transcription factor inducing $p21^{WAF}$, G_1 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 G_1 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, human 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 fashion 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-

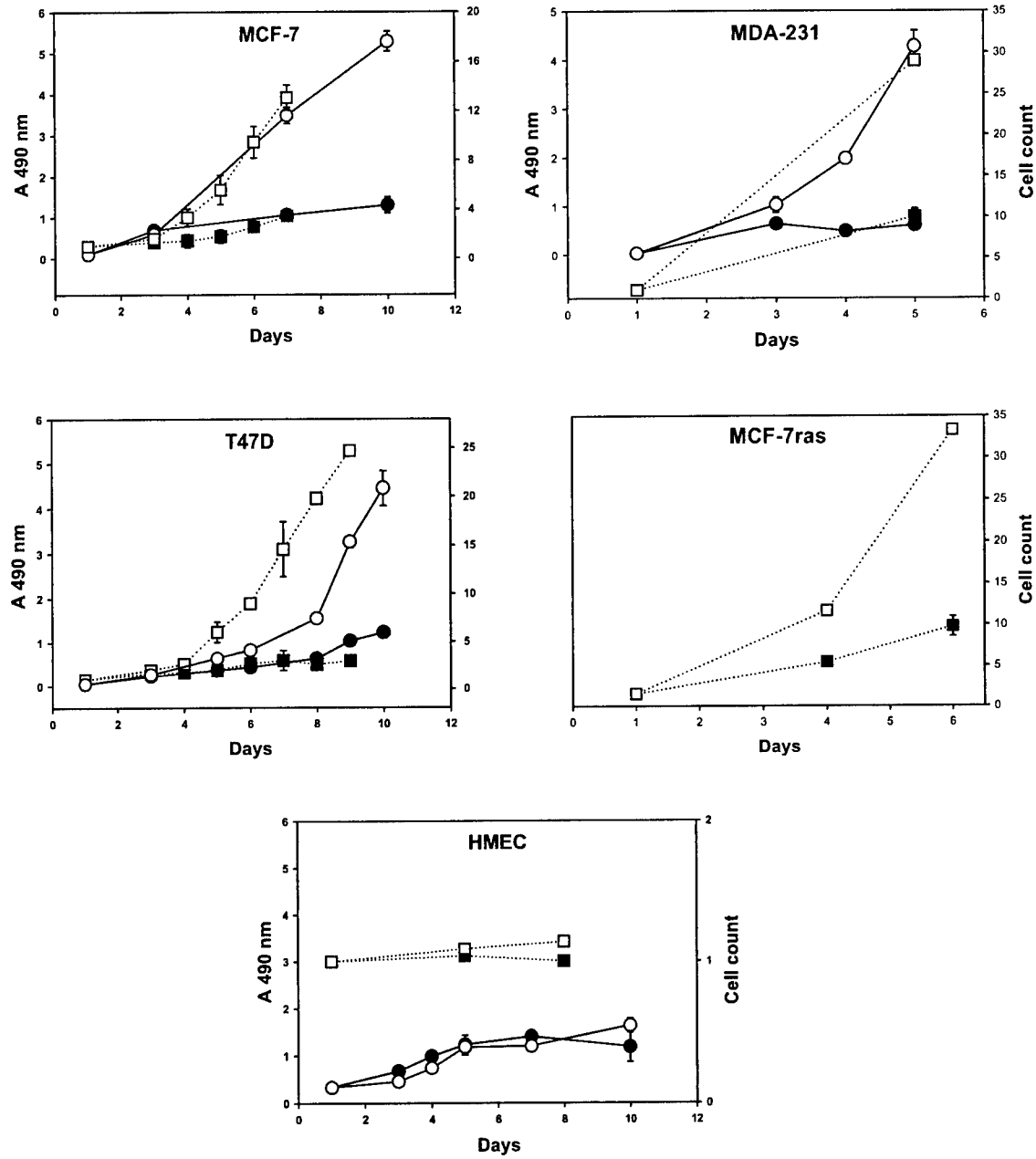

FIG. 6. **Growth of human breast cell lines in quinidine.** MCF-7, MDA-MB-231, T47D, MCF-7*ras* tumor cells, and normal HMEC were replica-plated in 96-well plates in control medium (*open symbols*) or medium containing 90 μM quinidine (*solid symbols*). Cell growth as monitored using the MTS assay is shown with solid lines. Results shown are the average of quadruplicates in one experiment. Quinidine had no effect on MTS metabolism in the MCF-7*ras* cell line (data not shown). Viable cell counts/dish of replica-plated MCF-7, MDA-MB-231, T47D, and MCF-7*ras* tumor cells and normal HMEC cells in 35-mm^2 dishes in control medium (*open symbols*) and medium containing 90 μM quinidine (*solid symbols*) is indicated by *dashed lines*. The cell number (growth curve data) represent the mean and S.E. of three independent experiments for the MCF-7, T47D, and MDA-231 cell lines performed in duplicate dishes (MCF-7 and T47D) or single dishes (MDA-231) for each experiment. MCF-7*ras* data are the mean (± range) of two experiments performed in duplicate dishes. HMEC data are from one experiment performed in single dishes.

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-e,rbB-2 (34), the vitamin D analog, 1-α-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, T-47D, 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

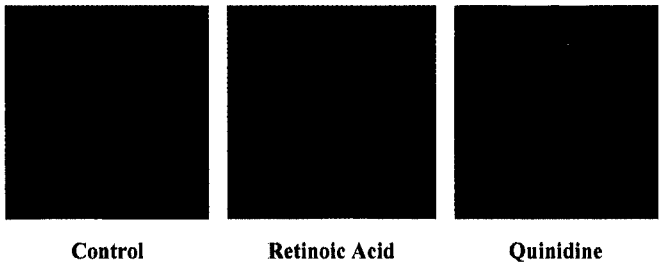

FIG. 7. **Cytokeratin 18 in MCF-7 cells.** Cells were replica-plated (2×10^5) on sterile coverslips in 35-mm^2 dishes in medium containing 0.01% ethanol (control), 10 μM retinoic acid, or 90 μM quinidine and grown for 96 h. Cytokeratin 18 detection using a Texas Red-tagged secondary antibody is shown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

lines and increased by retinoic acid and quinidine. Lipid droplet accumulation was more marked in all four cell lines treated with quinidine than with retinoic acid. These data demonstrate that induction of a more differentiated phenotype is a general response of human mammary tumor cells to quinidine.

Hyperacetylation of Histone H4 in Mammary Tumor Cell Lines by Quinidine—To determine whether differentiation and histone acetylation were linked, we investigated the histone H4 acetylation status of quinidine-treated T47D, MDA-MB-231, and MCF-7$_{ras}$ cells. MCF-7, MCF-7*ras*, T47D, and MDA-MB-231 cells were incubated for 24 h in the presence or absence of quinidine, and then histones were extracted for immunoblotting. Fig. 9 shows that histone H4 was hyperacetylated in all cell lines treated with quinidine. Control cells contained no hyperacetylated histone H4.

DISCUSSION

Quinidine-induced histone H4 hyperacetylation in MCF-7 human breast carcinoma cells can be attributed to the rapid elimination of HDAC1 protein, a response that was blocked by MG-132 and lactacystin, two inhibitors of proteasome-mediated proteolysis. HDAC1 protein was undetectable within 30 min after the addition of quinidine to the medium of MCF-7 cells, and hyperacetylated histone H4 appeared between 1 and 2 h. Levels of HDAC1 protein were completely suppressed between 0.5 and 6 h, and during this time H4 acetylation levels increased. H4 acetylation was maintained at 12 and 24 h, despite the partial restoration of HDAC1 protein at these same time points. These data indicate that quinidine-induced reductions in HDAC1 protein levels are unlikely to explain fully the regulation of H4 acetylation state in MCF-7 cells by quinidine. Additional HDAC enzymes or effects upon histone acetylation rates could possibly play a role as well.

An earlier study showed that over this initial 48-h period, 80% of the MCF-7 cell population had shifted into G_0, a quiescent state marked by the absence of Ki67 antigen immunoreactivity (12). Cellular differentiation manifested as the accumulation of lipid droplets, and a reorganization of the cytokeratin 18 cytoskeleton was evident after this initial 48-h period. Quinidine exhibited all the responses typical of known HDAC inhibitory drugs, with the exception that quinidine had no direct inhibitory effect upon HDAC1 enzymatic activity. We conclude from the current studies that quinidine is a novel differentiating agent that causes histone hyperacetylation, in part, by physical elimination of HDAC1 protein rather than the inhibition of HDAC enzymatic activity.

Histone H4 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 NIH3T3*ras* 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-7*ras* 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 p21^{WAF1} gene expression (5, 22). Growing MCF-7 and T47D cells express p21^{WAF1} protein in moderate to low levels (42), and quinidine raised p21^{WAF1} protein levels in MCF-7 cells approximately 11-fold within 12 h. Although p21^{WAF1} was reported to be low to undetectable in MDA-MB-231, p21^{WAF1} 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 p21^{WAF1} gene is present but inactive in growing MDA-MB-231 cells. Since histone hyperacetylation of the p21^{WAF1} gene occurs in response to HDAC inhibitors, it might be involved in the pathway of p53-independent activation of p21^{WAF1} gene expression (5).

The processes of cellular differentiation and cell cycle progression are interdependent. G_1 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 p21^{WAF1} protein and G_1 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 G_1 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 G_1 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 G_1 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

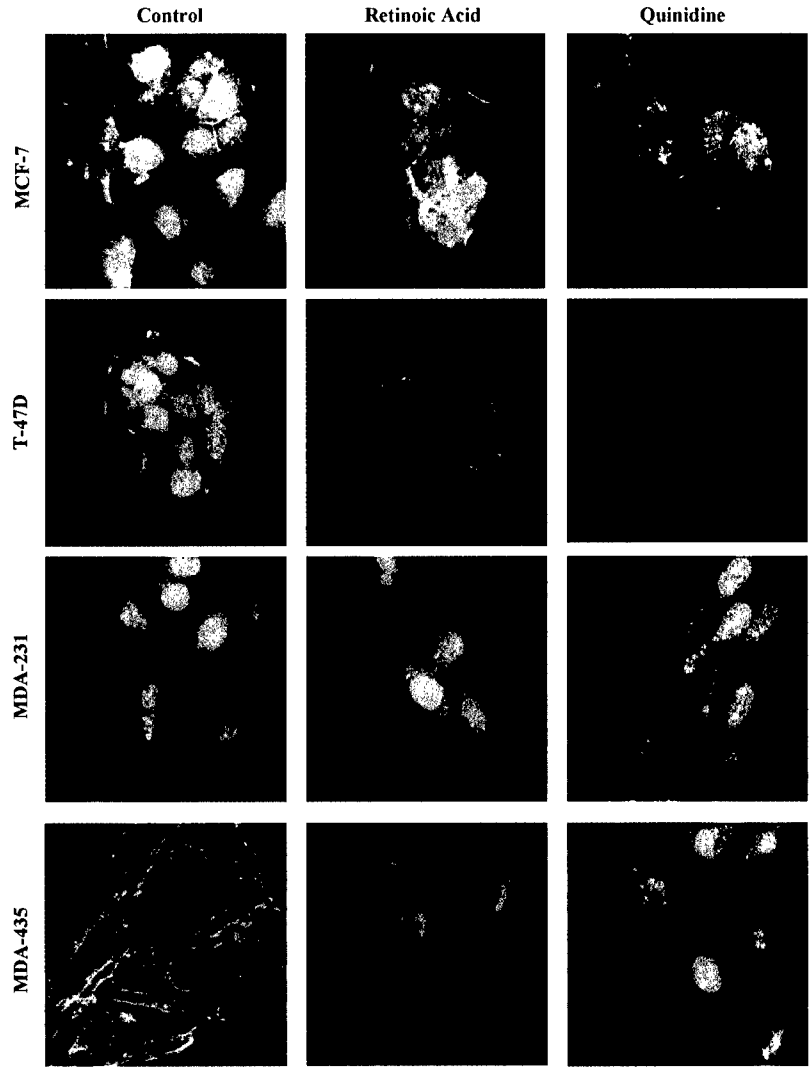

FIG. 8. **Lipid accumulation as an index of cellular differentiation in human breast tumor cell lines.** MCF-7, T47D, MDA-MB-231, and MDA-MB-435 cells were replica-plated ($0.8–3 \times 10^5$) on sterile coverslips in 35-mm^2 dishes in medium containing 0.01% ethanol (control), 10 μM retinoic acid (0.01% ethanol), or 90 μM quinidine (in water). Cells were fixed, permeabilized, and then incubated sequentially with fluorescein-phalloidin to identify actin filaments and Nile Red to identify lipid droplets after 96 h. Images were obtained by confocal microscopy. The results are typical of three experiments conducted in each cell line.

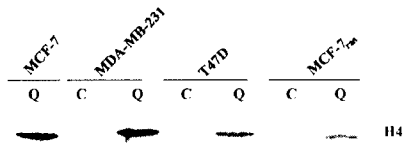

FIG. 9. **Histone H4 hyperacetylation in human breast tumor cell lines.** MCF-7, MCF-7*ras*, T47D, MDA-MB-231, tumor cells were replica-plated (1×10^7/T-162 flask) in control medium (*C*) or medium containing 90 μM quinidine (*Q*). Histones were extracted from the cells after 24 h, and 20 μg/lane of histone proteins were electrophoresed in 15% SDS-polyacrylamide gels. Immunoblotting was performed to detect acetylated histone H4.

point described by Pardee (53) near the G_1/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 G_1 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^{WAF} 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 G_1. 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 G_1 arrest in response to quinidine and could initiate the G_1 arrest. Hypophosphorylated pRb protein is prominent in quinidine-treated MCF-7 cells, and this could act to sustain the G_1 state by preventing the transition into S phase. The G_1 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 G_1 arrest was provided using valinomycin, a potassium-selective ionophore to stimulate a G_1-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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