2002

Antitumor activity of antimalarials in human breast cancer cells

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Antitumor Activity of Antimalarials in Human Breast Cancer Cells

Qun Zhou

**Dissertation submitted to the School of Medicine
of West Virginia University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Pharmacology and Toxicology**

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2002**

Key Words: Antimalarials, Differentiation, Apoptosis, Histone H4, Breast Cancer

ABSTRACT

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Qun Zhou

Previously we showed that 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 (Wang et al., 1998). The present experiments demonstrate 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. Quinidine did not show evidence for direct inhibition of histone deacetylase enzymatic activity *in vitro*. HDAC-1 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 HDAC-1 from the action of quinidine. These data demonstrate that quinidine is a breast tumor cell differentiating agent that causes the loss of HDAC-1 via a proteasomal sensitive mechanism. Moreover, to determine whether effects of quinidine on cell differentiation and apoptosis is generalizable to quinoline antimalarials, we tested typical quinoline antimalarial drugs for their ability to regulate MCF-7 cell differentiation, hyperacetylation histone H4 and apoptosis. MTS IC₅₀ of amodiaquin, chloroquine, halofantrine, primaquine, quinidine as well as quinine induced hyperacetylation of histone H4 without inhibiting HDAC activity. These results indicate that an alkylamino-substituted antimalarial ring might be important for acetylation of histone H4. Accumulation of lipid droplets was observed in cells treated with chloroquine, primaquine, quinidine and quinine. Only MTS IC₅₀ of chloroquine and quinidine increased p21(WAF1) protein levels and apoptosis. The data imply that p21(WAF1) might play a determining role in apoptosis in MCF-7 cells treated with antimalarials. Chloroquine was most active in stimulating MCF-7 apoptosis, and quinine was most active in promoting MCF-7 cell differentiation. We conclude that distinct mechanisms are responsible for breast tumor cell differentiation and activation of apoptosis by quinoline antimalarials. Alkylamino-substituted antimalarial ring compounds represented by quinidine, quinine and chloroquine will be useful model compounds in the search for more active breast tumor differentiating agents.

ACKNOWLEDGMENTS

I would first like to express my sincerest gratitude to my advisor, Dr. Jeannine S. Strobl, for her advice, guidance and support during my graduate study. Her insight and ideas have nourished and inspired me greatly and have added enormously to my understanding of cancer cell biology. I am grateful as well for her sense of humor and insight into the creative process. Big thanks to Drs. Stephen Graber, William Wonderlin, Marilyn I. Evans and Knox Van Dyke for their encouragement, kindness, help and service on my graduate committee.

I gratefully acknowledge the support of Dr. Robert E. Stitzel. A special thank-you goes out to Dr. Takamitsu Kawai, for his help on the computer language and data analysis. Appreciation is extended to Chris Van Dyke for computer image technical support. I am grateful to my colleagues, Meredith McCracken, Zara K. Melkounian, Anna Martirosyan, and Rayhana Bata for their help and friendship.

Finally, I wish to acknowledge my taproots: my mother and father, my brother and sister and their families, as well as my wife. All of you have enriched my life beyond my wildest dreams. I continue to find this process close to miraculous.

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LIST OF ABBREVIATIONS

AQ	Amodiaquin
BSA	Bovine Serum Albumin
CDK	Cyclin Dependent Kinase
CL	Chloroquine
DTT	Dithiothreitol
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethelenediamine Tetraacetic Acid
ETOH	Ethanol
FBS	Fetal Bovine Serum
HAT	Histone Acetyltransferases
HCl	Hydrochloric Acid
HDAC	Histone Deacetylase
HF	Halofantrine
HBSS	Hank's Balanced Salt Solution
MG-132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
MQ	Mefloquine
MTP	Microtiter Plate
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxmethoxypheyl)-2-(4-sulfophenyl)-2H-tetrazolium
NaB	Sodium Butyrate
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl Fluoride
PQ	Primaquine
PVDF	Polyvinylidene Difluoride
QA	Quinolinic Acid
QL	Quinoline
QD	Quinidine
QN	Quinine
Rb	Retinoblastoma protein
SAHA	Superoylanilide Hydroxamic Acid
TBS	Tris Buffered Saline
TPXT	Trapoxin
TSA	Trichostatin A

I. INTRODUCTION

1. Breast Cancer Therapy

Breast cancer is the most commonly diagnosed invasive malignancy and a leading cause (after lung cancer) of cancer deaths in American women (Greenlee et al., 2000). Most women succumb to breast cancer when their cancers metastasize; long-term survival depends on if the cancer remains localized. The approach to control this malignancy has made significant strides during the last decades of the 20th century. Surgical removal of the primary tumor in patients can reduce breast cancer mortality (Early Breast cancer Trialists' Collaborative Group, 1995); radiation therapy techniques and adjuvant systemic therapy can delay or prevent recurrence and thereby improve survival rates (Hortobagyi, et al., 2000).

1.1. Surgical Therapy

The primary goal of cancer surgery is the complete eradication of local and regional tumor. Traditionally, axillary lymph node dissection (ALND) has been considered as a standard part of the surgical management of patients with breast cancer, which provides accurate prognosis information, maintains local control of disease in the axilla and provides a rational basis for decision about adjuvant therapy (Sakorafas, 2000). Recently, there has been increased interest in extending the local treatment of breast cancer to “incision-less surgery”. The idea is to destroy all the tissue in a radius around the tumor while leaving the remaining breast unaltered using different energy sources such as laser for interstitial hyperthermia (Feyerabend, et al., 1996). This approach has been used in number of patients who are diagnosed with as a small, local and single focus of infiltrating ductal breast carcinoma (Feyerabend, et al., 1996).

1.2. Irradiation Therapy

The objective of radiation treatment of the patient with breast cancer is eradication of the tumor with preservation of the structure and function of normal tissues. For radiation therapy, as for surgery, this means local-regional control of tumor cells, implying also a careful pretreatment evaluation and long-term post-treatment observation.

One radiation treatment is external beam radiotherapy, which is very effective when treating localized disease by reducing the tumor bulk, so reducing the amount of dying tissue and the possibility for infection and odor (Regnard, et al., 1998). The challenge for radiotherapy is for more radiosensitivity that is a measure of the susceptibility to cellular injury by ionizing radiation. Mammalian cells vary in their radiosensitivity as they progress through the mitotic division cycle (Sullivan, et al., 1980). In general, G0 and late S-phase cells are most resistant, and late G2 and mitotic (M) cells are the most sensitive (Sullivan, et al., 1980). Following irradiation with 4-8 Gy (gray), MCF-7 human breast cancer cells exhibit transient delay in progression from G1 into S phase (Nagasawa et al., 1998). The use of combination radiation therapy and chemotherapy have increased strikingly over the past few years. Radiation therapy is directed at the primary tumor masses and regional nodes, whereas chemotherapeutic agents may modify the response of tumor and normal tissues to irradiation. It has been reported that cytotoxic drugs that are S-phase-specific could synergize with radiation therapy to overcome radioresistance in the S phase (Sondel et al., 1986).

1.3. Adjuvant Therapy

There are two major types of systemic therapy: cancer chemotherapy and biological therapy (immunotherapy). There is considerable overlap between these two types of therapy, and they are increasingly employed together in various combinations (Trail et al., 1993).

Chemotherapy involves the use of cytotoxic drugs and hormones to induce tumor cell death. The typical chemotherapeutical agents include cyclophosphamide, doxorubicin, methotrexate and tamoxifen

Cyclophosphamide is used to treat a wide range of human malignancies. Bioactivation of cyclophosphamide yields 4-hydroxycyclophosphamide, which then interconverts with its tautomer aldophosphamide. Through an elimination mechanism, aldophosphamide fragments to acrolein and phosphoramidate mustard (PM) (Anderson et al., 1995). It has been reported that PM and acrolein are reactive species toward DNA. The covalent DNA adducts formed by PM are intra/interstrand cross-link DNA adducts and mono adducts at the N7 position of guanine (Anderson et al., 1995). The DNA adducts from PM and acrolein have been found in rodents after administration of cyclophosphamide (Hemminki 1985). The alkylating agent cyclophosphamide is a potent immunosuppressive drug that has been synthesized. The proliferating lymphoid cells are primarily targeted by this agent. It is a known carcinogen and is associated with induction of therapy-related leukemia and bladder cancer (Ortiz et al., 1992). *In vivo* genotoxic activity including chromosome aberrations, sister chromatid exchange, and gene mutation has been reported in both animals and humans after administration of cyclophosphamide (Anderson et al., 1995).

Doxorubicin is considered one of the most active drugs in breast cancer, with response rates of 40% when given as a first-line single agent (Houstone et al., 1993). This drug is capable of binding to DNA, usually by interposition between base pairs with subsequent uncoiling of the DNA helix.

Intercalation impairs DNA synthesis and RNA synthesis which inhibit tumor cell proliferation (Houstone et al., 1993).

Methotrexate (MTX) is one of the most commonly used agents in the treatment of solid malignancies as well as leukemias and lymphomas. However, the toxicities of MTX to bone marrow and gastrointestinal tract accompany this therapy (Moscow 1998). This drug is a member of the antifolate class of compounds that exerts its cytotoxicity by blocking DNA/RNA synthesis pathways, MTX binds to the active catalytic site of dihydrofolate reductase (DHFR), and blocks the synthesis of the reduced form of folate that acts as a one-carbon unit donor in biochemical syntheses. Lack of this cofactor interrupts the biosynthesis of thymidylate and purine nucleotides, thereby interfering with the formation of DNA and RNA (Moscow 1998).

Endocrine Therapy is an effective treatment for breast cancer patients. Normally, estrogens function by binding to estrogen receptor. Subsequent to ligand (estrogen)-ER binding, ligand-ER complexes undergo conformation change, dissociate from the chaperone heat shock protein Hsp90 and become active complexes (Hurd and Moudgil 1998). In the nucleus, ER-ligand complexes bind to a transcriptional complex and an estrogen response element on DNA (Beato et al., 1996). Recent evidence suggests that the activated ligand-ER complexes participate a switching between binding of a multisubunit co-repressor complex containing factors (not completely identified yet) with histone deacetylase activity (HDAC) and binding of a co-activator complex containing factors, SRC (steroid receptor co-activators)/NcoA (nuclear receptor co-activators), with histone acetylase activity (HAT) such as p300/CBP(CREB (cyclic AMP-responsive element binding)-binding protein) and PCAF (p300/CBP- associated factor)(Grunstein 1997). Ligand-ER binding results in the replacement of the repressor complex, by a co-activator SRC-1/NcoA-1 complex with multiple HAT proteins which catalyze histone acetylation (Torchia et al, 1998). The net effect is that estrogen activates gene expression and increases cell proliferation (Katzenellenbogen, 1997).

In about one-third of advanced breast cancers, estrogens stimulate tumor growth, whereas estrogen deprivation causes tumor regression. Inhibition of estrogenic stimulation is the main target in endocrine treatment of breast cancer. This is achieved by either blocking the estrogen receptor or the inhibition of estrogen production.

Tamoxifen (TAM) is a typical agent for hormone therapy, which binds to estrogen receptors, competes with endogenous estrogens, and occludes the coactivator association between the activation function domain 2 (AF2) and the receptor ligand-binding domain (LBD), leading to inhibition of tumor cell growth (Shiau et al., 1998). Inhibition of ER by TAM blocks the secretion of polypeptide growth factors such as epidermal growth factor and transforming growth factor α , leading to growth arrest of ER-positive cells (Taylor et al., 1984). In addition to its cytostatic effects mediated through the ER in ER-positive MCF-7 cells, TAM delays G1 cell cycle transit leading to an accumulation of non-dividing cells in G0/G1 phase (Sutherland, et al., 1983). The cell cycle delay is ER-independent, since TAM also inhibits the growth of the ER-negative cell line BT20 by accumulation of cells in the G0/G1 phase (Osborne et al., 1989). TAM cytotoxicity is proposed to be mediated by the induction of apoptosis (Perry et al., 1995).

A doxorubicin based chemotherapy combined with tamoxifen proved superior to tamoxifen alone (Fisher et al., 1990). This finding was supported by in vitro studies. Clinical studies suggest an additive or even synergistic effect of doxorubicin and tamoxifen, especially in postmenopausal women, where addition of tamoxifen to chemotherapy increases the recurrence-free survivability in breast cancer patient. This benefit is more pronounced for ER positive patients than for ER negative ones (Osborne et al., 1989).

Femara (letrozole). Aromatase is an enzyme that catalyzes the conversion of the androgens androstenedione and testosterone to estrone and estradiol. The cytochrome P-450 enzyme complex aromatase is the main target for inhibition of estrogen biosynthesis. In recent years, several new

aromatase inhibitors including Femara have been developed. Femara is the selective aromatase inhibitor and recently licensed by the FDA for advanced breast cancer treatment in postmenopausal women where tamoxifen therapy has failed (Harvey 1998). It is a potent inhibitor of cell proliferation and more effective than tamoxifen in MCF-7 cells (Brodie et al., 1998).

Immunotherapy involves the use of biological molecules known as biological modifiers. The HER2 receptor is the protein product of protooncogene *HER2* (also known as *neu* and as *c-erbB-2*), and is a member of EGFR (epidermal growth factor receptor) family of transmembrane tyrosine kinases. Upon binding of ligand to the EGFR, HER2 is recruited as the preferred partner of these ligand-bound receptors into an active, phosphorylated heterodimeric complex that activates several signaling pathways involved in the proliferation and survival of breast cancer cells (Riese et al., 1998). Overexpression of HER2 in breast cancer patients is associated with poor prognosis (Riese et al., 1998).

HER2 has been proposed to contribute to antiestrogen-resistance because tumors expressing high levels of HER2 exhibit lower clinical response rates after antiestrogen therapy (Houston 1999). HER2-overexpressing estrogen receptor positive breast cancer cells failed to respond to tamoxifen (Elledge et al., 1998). HER2 overexpression activates the MAPK (mitogen-activated protein kinase) signaling pathway, which causes phosphorylation of ER, and ligand-independent ER activation with loss of the inhibitory effect of tamoxifen (Bunone et al., 1996). Immunotherapy using a monoclonal antibody against HER2, has proved to be clinically efficacious in breast cancer patients (Baselga et al., 1996). Herceptin, an antibody against HER2, increased the inhibitory effect of tamoxifen against HER2 overexpressed BT-474 human breast cancer cells in culture (Witters et al., 1997). Herceptin targets the HER2 ectodomain and blocks HER2 cleavage preventing the production of an active truncated HER2 fragment (Molina et al., 2001). Treatment with herceptin and doxorubicin showed

greater inhibition of cell proliferation than that observed with any agent alone in BT-474 cells (Baselga et al., 1998).

Summary. Adjuvant therapy has led to a reduction in the risk of relapse of only 20-40%, depending on the chemotherapeutic agents used (Hortobagyi et al., 2000). Frequent toxic effects, including nausea, vomiting and alopecia, are usually connected with adjuvant therapies. In addition, the emergence of multidrug resistance poses a considerable obstacle to success of breast cancer chemotherapy. Therefore, the development of new target-selective ‘smart’ drugs that are less toxic and circumvent drug resistance, is an important goal.

2. Antimalarials

Malaria is the common devastating human infection in the developing countries. There are nearly 2.5 million deaths each year (Phillips 2001). The human malaria parasites have *P.falciparum*, *P.vivax*, *P.ovale* and *P.malariae* species (Galinski et al., 1996). Much of human mortality is caused by infection with *P. falciparum* (Phillips 2001). Although this mosquito-born infection has been eradicated from the USA, immigration from and travel to endemic regions still cause health problems.

The asexual erythrocytic cycle produces more merozoites that are released with the destruction of the red blood cell after 48 or 72 h; for the human malaria parasites, depending on the species, merozoites then immediately invade additional erythrocytes (Hawking et al., 1968). For the past 50 years, quinoline-containing drugs have been the main class of antimalarial agents in use. In most cases, these agents are targeted at the asexual erythrocytic stage of the parasite. The quinoline-containing drugs include the cinchona alkaloids which are quinine and quinidine, the aminoalcohol quinine analogue mefloquine, halofantrine, primaquine, chloroquine and its relative amodiaquin (White 1994).

2.1. Antimalarial drugs

The structures of quinoline antimalarials are shown in Figure1. Most of the aminoquinoline compounds behave like quinine in many respects, but each drug has individual properties.

Quinidine has a quinoline group through a secondary alcohol linkage to a quinuclidine ring. A vinyl side chain is attached to the quinuclidine ring while a methoxy side chain is linked to the quinoline ring. Quinine has the same structure as quinidine except for the steric configuration of the secondary alcohol group. Quinidine is more potent as an antimalarial and more toxic than quinine. It has been reported that quinine inhibits incorporation of adenosine into parasitic DNA, whereas the mechanism by which quinidine acts as an antimalarial is not known (Whinstanley et al., 1993).

Mefloquine is a 4-quinolinemethanol derivative that has four optional isomers with about the same antimalarial potency. Mefloquine is selective against mature *trophozoite* and *schizont* forms of malarial parasites. The exact mechanism of action of mefloquine is unknown. It does not intercalate with DNA (Davidson et al., 1997).

Chloroquine is a 4-alkylamino substituted quinoline that also possesses a chlorine substitution at the 7-position of the ring. Chloroquine is a more potent antimalarial than either quinidine or quinine, but despite chloroquine's structural similarities with quinine and quinidine, chloroquine has a different mechanism of antimalarial activity, and chloroquine-resistant malaria responds to quinine treatment (White, et al., 1994). Chloroquine's antimalarial action is mediated through binding to a Hb (hemoglobin) degradation product which becomes toxic to the parasite. It selectively inhibits haem polymerization which is an essential defense mechanism to detoxify haem (Wellems, et al., 1992).

Amodiaquin is a analog of chloroquine, which is no longer used as an antimalarial because its use increases hepatic toxicity and agranulocytosis (White et al., 1994).

Primaquine is a quinoline derivative that is synthesized with methoxy and substituted 8-amino groups. The great clinical value of primaquine is active against the primary exoerythrocytic forms of *P. falciparum*. The 8-aminoquinolines exert the most potent antimalarial effect (Tarlov et al., 1962).

Halofantrine The (+) and (-) enantiomers of halofantrine are equally active against malaria (Carroll et al., 1978). Halofantrine acts against the asexual erythrocytic stage of *Plasmodium*; it is primarily used as an alternative to quinidine and mefloquine to treat acute malarial attacks caused by chloroquine-resistant and multidrug-resistant strains of *P. falciparum* (Basco et al., 1992). Halofantrine also acts on parasite mitochondria to form toxic complexes, which destroy biomembranes (Warhurst et al., 1987).

2.2. Cancer Treatment by Antimalarials

In the past twenty years, the potential of antimalarial drug such as chloroquine to improve cancer therapy has been investigated. Chloroquine treatment (20mg/ml) in cultured mouse myeloid leukemia M1 cells for 4 days induced differentiation, inhibited DNA and RNA polymerases, and interfered with lysosomal function (Takenaga, et al. 1980; Morrow, et al., 1989). Chloroquine inhibited cell proliferation in a dose and time-dependent manner in malignant B cells obtained from a patient with B-cell chronic lymphocytic leukaemia, and induced apoptosis as demonstrated by a decrease in the amount of bcl-2 protein, increased caspase-3 activation and DNA fragmentation (Lagneaux et al., 2001).

Chloroquine also acts as an effective potentiator of hyperthermia, which is an adjunctive treatment modality in neural crest tumors such as melanoma and murine neuroblastoma (Morrow, et al., 1989; Thomas 1990). In C3H/He mice bearing transplantable mammary adenocarcinomas (C3HBA), 5-fluorouracil (5-FU) and chloroquine phosphate increased complete remissions compared with 5-FU treatment alone (Shoemaker, et al., 1978; Shoemaker, et al., 1979). Chloroquine therapy also enhanced radiosensitization in patients with chest wall necrosis; the acute radiation reaction was also

greater in chloroquine treated animals (Utley, et al., 1997). Moreover, chloroquine was used to reduce the toxicity of the anti-brain tumor drug TFCRM107 by selectively suppressing toxicity to the vasculature without altering the antitumor efficacy (Hagihara et al., 2000).

Chloroquine increased the effect of Aluminium Chloride Phthalocyanine (ALCIPc), a second generation sensitizer for the photodynamic therapy of EMT-6 mouse mammary tumor cells. In the photodynamic cancer therapy model, ALCIPc was incorporated together and formed the polymeric micelles by dialysis. Chloroquine, a weak base, raised the internal pH of acidic organelles such as the lysosome. Endosomal lysosomal acidity is important for the pH-sensitive polymeric micelles to be fully effective (cytotoxicity) (Taillefer, et al., 2001). Chloroquine and its analogues have the potential for enhancing the efficient gene transfer into various gynecological tumors by their endosomal buffering capacity, therefore these compounds are also used in gene therapy applications (Keil, et al., 2001). Interestingly, chloroquine and its derivative hydroxychloroquine have been reported to possess anti-HIV-1 activity in lymphocytic and monocytic cells. The anti-HIV effect by chloroquine has been confirmed in vivo in two clinical trials (Boelaert et al., 2001). Combination therapy with the antimalarial agent chloroquine may be an interesting option for treatment for cancer and HIV infected individuals in the developing countries.

Quinoline (QL)

Quinolinic Acid (QA)

Primaquine (PQ)

Quinine (QN)

Quinidine (QD)

Amodiaquin (AQ)

Chloroquine (CL)

Mefloquine (MQ)

Halofantrine (HF)

Figure. 1. Chemical Structures of Antimalarials

3. Mechanism for Regulating Tumor Cell Growth

The cell cycle is regulated by the sequential activation of cyclin-dependent kinases (CDKs). The activation of CDKs and subsequent phosphorylation of specific substrates govern progress through the cell cycle on multiple levels (Figure 2). CDKs are inactive in the absence of cyclin binding; therefore, cyclin abundance is a major determinant of cyclin-CDK activity (Sherr 1996). Each cyclin is typically present for only a restricted portion of the cell cycle, and changes in cyclin abundance are sufficient to alter the rate of cell cycle progression (Hunter et al., 1994). Specific CDK-inhibitory (CKI) proteins including p15, p16, p21(WAF1/cip1) and p27 target CDK4 that associates with the D-type cyclins in G1 phase. The cyclin-dependent kinase inhibitor p21(WAF1) causes cell arrest in G1 through inhibition of CDK complexes, which are required for the G1 to S transition (Xiong et al. 1993). p21(WAF1) may have additional roles in cell cycle and cancer, because it is able to inhibit DNA replication and repair, independently of its ability to inhibit CDK activity (Michieli et al., 1994). It has been reported that p21(WAF1) may play a role in the differentiation of epithelial cells and act as a tumor-suppressor gene (Halevy et al., 1995 and Chen et al., 1995). p21(WAF1) immunoreactivity has been recently observed in human neoplastic tissues, where its expression is related to cell proliferation and differentiation (Doglioni et al., 1996). High p21(WAF1) expression could be related to normal or increased p53 function, and low p21(WAF1) expression could be related to p53 gene deletion or mutation. p21(WAF1) expression may reflect different p53 functional states. This idea is based on the observation that p21(WAF1) expression in human breast cancer is low when p53 is mutated (Bukholm et al., 1997).

One of the key endogenous substrates of CDK4 in G1 phase is the retinoblastoma protein (Rb). Its phosphorylation is an important step in the transition between the G1 and S phases of the cell cycle because sufficiently phosphorylated Rb releases a transcription factor E2F1 that drives cells into S

phase (Slansky et al., 1996). A diverse body of evidence now indicates that pRb stands in the midst of G1/S cell cycle regulatory pathway that suffers disruption during the pathogenesis of very many human tumors. Disruption of the pRb/E2F1 interaction could be achieved by pRb functional inactivation, viral oncoprotein binding, or CDK phosphorylation during tumor progression. As a direct consequence, E2F1 is liberated from control and the progression of cells into late G1 and S becomes uncontrolled. Therefore, drugs which restore pRb normal function are potential antitumor agents.

4. Ubiquitin-mediated Protein Degradation

Endogenous proteins are selectively targeted by the ubiquitin/proteasome system for degradation by means of a complex system of protein-protein recognition elements. Degradation of a protein via proteasomes occurs by two distinct and successive steps. First, the protein is conjugated to ubiquitin. In the second step, the 26S proteasome recognizes the ubiquitin-conjugated protein, and degrades it. The ubiquitin system is in both cytosolic and nuclear, and has high selectivity for normal short-lived proteins and misfolded proteins (Kisselev, et al., 1999). Many cell cycle regulatory proteins, including p27 and c-myc have been shown to be phosphorylated, ubiquitinated, and degraded by the proteasome (Montagnoli et al., 1999; Gregory et al., 2000). Thus, the activity of the proteasome performs an important cell cycle regulatory function.

Figure 2. Cell Cycle Regulatory Proteins for G1 to S Phase Transition. Rb (retinoblastoma); CKI (cyclin dependent kinase inhibitor); CDK (cyclin dependent kinase); p (phosphorylation).

5. Apoptosis

Apoptosis or programmed cell death is a major mechanism for maintaining cell homeostasis in normal development and adult life. It is also an important determinant of the response of tumors to chemotherapeutic agents. Morphological features of apoptosis include chromatin condensation, cell shrinkage, nuclear fragmentation and blebbing with the formation of membrane-bound apoptotic bodies. These events are the result of complex cellular biochemical pathways (Vaux et al., 1999). Disregulation of apoptosis has been identified in many human diseases and is recognized as an important event during carcinogenesis and in tumor progression. Thus, manipulation of apoptotic pathways is a promising approach for the treatment of various human cancers.

Activation of caspases is a central mechanism of apoptosis. Caspases are a family of cysteine proteases, which cleave specific substrates using a cysteine-aspartate recognition sequence. Caspases are synthesized as pro-enzymes and are activated by internal cleavage. Caspases are the executioners of cell death.

Apoptosis usually involves the release of cytochrome c from mitochondria by many different stimuli including tumor necrosis factor (TNF), chemotherapeutic and DNA-damaging agents (Reed 1997). Chemotherapeutic-induced cytochrome c release is caspase-independent in contrast to TNF, which is receptor-mediated apoptosis and depends on activation of caspase-8 (Sun et al., 1999). Activation of cytochrome c-caspase-9 pathway by chemotherapeutic agents leads to tumor cell apoptosis (Brown et al., 1999, and Tada-Oikawa et al., 1998). In the cytosol, cytochrome c combines with Apaf-1 in the presence of ATP to activate caspase-9, which in turn activates effector caspases such as caspase-3, -6 and -7 (Zou et al., 1997; Green et al., 1998). The activation of caspase-3 and subsequent degradative events are responsible for many of the biochemical and morphological changes associated with the apoptotic phenotype including DNA cleavage (Sun et al., 1999). Defects in this pathway which suppress apoptosis may contribute significantly to the development of breast cancer

and resistance to chemotherapeutic agents. Chemotherapeutic resistance represents a major limitation in the ability of chemotherapy to cure cancer. Alteration in apoptotic-associated genes are often associated with chemotherapeutic resistance (Fisher 1994). The tumor suppressor genes p53 and pRb, the key regulators in DNA damage-induced apoptosis, are frequently mutated in human tumors (Thor et al; 1992). Overexpression of anti-apoptotic Bcl-2 family members has been detected in primary tumors and tumor cell lines (Lotem et al., 1993). Recently, loss of caspase-3 owing to 47-base pair deletion within exon 3 of the *caspase-3* gene in MCF-7 cells has been reported (Reiner et al., 1998). Mutated *caspase-3* leads to a block in DNA fragmentation of MCF-7 cells (Reiner et al., 1998). Caspase-3 deficiency is believed to contribute to chemotherapeutic resistance in many cancer cell lines (Prokop et al., 2000). Correction of these alterations could result in sensitization of the defective cells to chemotherapeutic agents (Bargou et al., 1996).

6. Histone Deacetylase (HDAC)

DNA is highly organized and packaged into the nucleus in eukaryotes (Wolffe 1995). This packaging and organization are achieved by DNA and DNA binding proteins including histones which form a complex structure referred to as chromatin. An average of 150 base pairs of DNA wrapped around a histone octamer, comprised of histones H2A, H2B, H3 and H4 forms the basic structural unit of the chromatin, the nucleosome (Kornberg 1974). Each nucleosome contains two H2As, two H2Bs, two H3s, and two H4s in the core. Linker histone H1 appears to lie on the outer portion of the nucleosome. Repeating nucleosomes are assembled into a higher order chromatin structure.

Nucleosomes have both positive and negative effects on transcription (Durrin et al., 1991). The dynamic effects of nucleosomes on transcription may be due, in part, to the acetylation of lysine residues present in the hydrophilic amino-terminal portions (tails) of the core histones (Turner, 1991 and Davie et al., 1998). This acetylated state is maintained through the competing activities of histone

acetyltransferases (HAT) and deacetylases (HDAC). HAT transfers the acetate from acetyl-CoA to neutralize the positively charged- ϵ -amino group of an unacetylated lysine residue. In contrast, deacetylation is achieved through the hydrolysis of the acetyl moiety, restoring a positive charge (Jeppesen et al., 1993). There is a general correlation between core histone acetylation and gene activity. Conversely, histone hypoacetylation leads to transcriptional repression.

To date, at least nine different mammalian HDACs were described, which are classified in to class I, class II, and Sir2 family. The class I proteins (HDAC1, HDAC2 and HDAC3) are homologous to the yeast transcriptional regulator Rpd3, bind to E2F and repress transcription through an association with pRb (Taunton, et al., 1996; Brehm, et al., 1998); the class II proteins (HDAC4, HDAC5, and HDAC6) are homologous to yeast HDA1 and form a second family of deacetylases that are specifically involved in cell differentiation (Verdel, et al., 1999; Grozinger, et al., 1999). The class II proteins localize to distinct nuclear bodies within the cell nucleus and only recently have been shown to interact with SMRT (silencing mediator for retinoid and thyroid receptor)/N-CoR (nuclear receptor corepressor) as result of transcriptional repression. TSA completely disrupted these nuclear bodies (Kao, et al., 2000). The Sir2 family depends on nicotinamide-adenine dinucleotide (NAD) (Imai, et al., 2000). Specific roles for these enzymes and their target genes remain to be clarified. Inhibition of HDAC activity results in the accumulation of acetylated histones H2a, H2b, H3, and H4. As a result of HDAC inhibition, rather specific effects occur upon the transcription of a small subset of the genome (Van Lint, 1996). Evidence provided by DNA microarrays using malignant cell lines cultured with a HDAC inhibitor showed that a small (1-2%) number of gene products is altered (Van Lint et al., 1996).

In MCF-7 cells, histone deacetylase 1 (HDAC1) is the most abundant enzyme in HDAC family and is mainly located in nuclear matrix (Hendzel et al., 1991). Histone H4 is the primary target of HDAC1 enzymatic activity (Chen et al., 1999), and this histone undergoes acetylation at lysine residues at

multiple sites within the histone tails extending from the histone octamer of the nucleosome core (Davie et al., 1998).

6.1. Disruption of HAT and /or HDAC activity and development of cancer

Recent evidence suggests a relationship between alteration in chromatin structure by histone hyperacetylation or deacetylation and the development of cancer (Archer et al., 1999). Human *CBP* participates as a coactivator in cyclic-AMP-regulated gene expression. Mutations in the *CBP* gene affect HAT activity, and are associated with leukemogenesis (Yang et al., 1996; Petrij et al., 1995). One of the functions of pRb is to recruit HDAC1 and suppress E2F activity, but the pRb pathway of G1-S cell cycle control is aberrantly altered in most cancers (Archer, et al., 1999). HDAC inhibitors have therapeutic potential because of their ability to elevate histone acetylation level and perhaps restore normal growth control upon tumor cells. Pharmacological inhibitors of HDAC modulate transcription, induce differentiation, and cause cancer cell apoptosis (Marks et al., 2000).

6.2. Pharmacological HDAC Inhibitors

Histone deacetylase inhibitors such as sodium butyrate (NaB), trapoxin (TPX), and trichostatin A (TSA), increase acetylated histones in many cell types (Yoshida et al., 1995). Unlike NaB which also has nonselective effects, TSA is thought to specifically inhibit HDAC (Kijima et al. 1993). For this reason, TSA has been used as a tool to study the consequences of histone acetylation in vivo or in vitro (Ogryzko et al., 1996).

The molecular structures for TPX and TSA are shown in Figure 3. The functional group of TSA responsible for the inhibition of HDAC is the hydroxamic acid moiety (Yoshida et al., 1990), which chelates the zinc ion in the active-site pocket of HDAC. TPX contains an epoxyketone structure at the end of the side chain of a constituent amino acid. The epoxyketone function group inserts and binds to the pocket of HDAC, leading to the irreversible inhibition of HDAC (Kijima et al., 1993).

The characteristic cell response to HDAC inhibitors includes cell cycle arrest in G1, elevated p21(WAF1) expression, hypophosphorylation of pRb, hyperacetylation of histones H4, cell differentiation and apoptosis (Yoshida et al., 1995; Sambucetti et al., 1999). Histone hyperacetylation by NaB and TSA is directly linked to the activation of p21(WAF1/Cip1) transcription by Sp1 and/or Sp3 transcription factor activation, which occurs in a p53-independent manner (Xiao et al., 1999). HDAC inhibitors such as TSA also synergize with retinoic acid to enhance differentiation of myeloid leukemia HL-60 cells (Janson 1997).

TSA demonstrated antiproliferative actions in a number of tumor cell lines. TSA has been named as a transcriptional therapeutic agent for neoplasia (Kosugi et al., 1999). A big concern of transcriptional therapy with TSA is its toxicity. Svensson et al. (1998) reported that TSA treatment in vitro caused malformation in embryos. However, a systematic analysis of the toxicology of TSA in adult mice and during embryonic development showed that TSA was not toxic to mouse embryos, and did not intervene with postnatal development (Nervi et al., 2001). Therefore, TSA might be a nontoxic or minimal toxic HDAC inhibitor for the transcriptional therapy of neoplasia. Most recently, FK228, (Nakajima et al., 1998), a synthetic HDAC inhibitor with antitumor activity, is under going clinical trial (phase I) at the National Cancer Institute (Frederick, MD) for cancer therapy.

Figure 3. Structures of TPX and TSA

6.3. Drug Resistance and HDAC Inhibitors

The chemotherapeutic agents are challenged by both intrinsic drug resistance and the development of acquired drug resistance during breast cancer treatment (Drewinko, et al., 1981). At the molecular level, at least three protein systems have been reported to be involved in drug resistance: the glucose-regulated protein (grp), the P-glycoprotein (Pgp), and the heat shock protein (hsp).

Cell stress conditions, such as glucose starvation, can be associated with drug resistance. Glucose-starved stress condition is not observed in normal tissues. This tumor specific condition commonly causes the glucose-regulated stress response of cancer cells (Shen, et al., 1987). Induction of grp leads to an increase in resistance to multiple anticancer drugs, such as doxorubicin, etoposide, and vincristine (Wallner et al, 1986; Shen, et al., 1987; Hughes, et al., 1989). The mechanism by which grp induces drug resistance is not clear. However, in mammalian cells the grps represent a subset of stress-induced proteins that were not associated with heat shock and thermotolerance (Shen, et al., 1987).

The second system implicated in drug resistance is the transmembrane Pgp encoded by the *MDR* (multidrug resistance) gene family (Gottesman, et al., 1988). Composed of two members (*MDR1* and *MDR2*), only *MDR1* seems to be functionally linked to development of the MDR phenotype (Roninson, et al., 1986). The acquisition of the MDR phenotype, defined as increased resistance against cytotoxic drugs, represents one of the major obstacles for chemotherapy of tumors and malignancies. One of the mechanisms that may account for MDR is the surface accumulation of Pgp. Pgp is involved in the transport of small molecules and mediates the efflux of drugs from cells, which is called the multidrug transporter (Germann et al., 1993). The *MDR1* gene is subject to control by a variety of internal and external stimuli, including differentiation signals, hsp, cytokines, hormones and a variety of toxic insults (Ince et al., 1997). *MDR1* mRNA levels are increased by heat shock, sodium arsenite, and cadmium chloride treatment but not by stresses that activate grp expression (Chin et al.,

1990). Tumor cells that overexpress Pgp are resistant to a wide range of caspase-dependent apoptotic inducers (Smyth et al., 1998); thus, new approaches to inhibit Pgp protein expression are needed and are clinically important.

Overexpression of Pgp can be circumvented by interrupting the transcriptional activation of the MDR1 gene. The proximal promoter of *MDR1* contained several regulatory regions, including an inverted CCAAT box at -82 to -73 and a GC element at -56 to -42, both of which have been shown to be required for constitutive promoter activity (Jin, et al., 1998). Binding of Sp1 to the GC element activates the *MDR1* promoter in *Drosophila* cells (Cornwell, et al., 1993), while interaction of the trimeric transcription factor NF-Y with the CCAAT box has been implicated in constitutive regulation of the *MDR1* promoter in several cell lines (Goldsmith, et al., 1993; Jin, et al., 1998). Recent studies have shown that the MDR1 gene can be more activated by HDAC inhibitors such as sodium butyrate (NaB), TSA and differentiating agents (Jin et al., 1998; Abolhoda et al., 1999). These agents stimulated the histone acetyltransferase PCAF to bind with the *MDR1* promoter as result of acetylation of promoter-proximal histones following transcriptional activation (Mickley, et al, 1989; Jin, et al., 1998; Hu et al., 2000). The net effect needs to be determined; it is possible that Pgp protein function is blocked by TSA and NaB treatment.

The third stress-responsive system implicated in drug resistance is the heat shock protein system. Resistance to doxorubicin has been observed in human breast cancer cells with elevated hsp27. MDA-MB-231 human breast cancer cells transfected with a plasmid containing the structural gene for human hsp27 expressed both thermoresistance and drug resistance (Oesterreich, et al., 1993). Hsp27 could be a very important mediator of chemoresistance.

Program Aims

Quinidine is used as an anti-malarial and also as an anti-arrhythmic agent. Previous studies from our laboratory demonstrated that quinidine arrested cells in early G1 phase, and induced apoptosis in MCF-7 cells (Wang et al., 1998), but the biochemical mechanisms for the anti-proliferative effects of quinidine are not well understood. To clarify the molecular mechanisms of the anti-proliferative activity of quinidine, we investigated the effects of quinidine on cell cycle regulatory proteins and histone acetylation. Based on effects of quinidine, a prototype quinoline derivative, on HDAC activity, histone H4 and apoptosis, we begin to explore whether all quinoline-based antimalarials have a similar profile of antitumor activities in human breast cancer cells.

II. MATERIALS AND METHODS

1. Chemicals

Amodiaquin, chloroquine, etoposide, Oil Red O, quinidine, quinine, quinoline, quinolinic acid, and trichostatin A (TSA) were purchased from Sigma Chemical Company (St. Louis, MO). Calcium acetate was purchased from Fisher (Pittsburgh, PA). Mefloquine and Halofantrine Methylsulfate were provided by the National Cancer Institute (Bethesda, MD). Recombinant histone H4 protein (expression in *E.coli*, cat #14-412) was purchased from Upstate Biotechnology (Lake Placid, NY).

2. Tissue Culture

Permanent cell lines derived from patients with breast carcinomas were used in these studies. MCF-7 cells, passage numbers 30 – 50, were maintained in Dulbecco's Modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah), and 40 ug/ml gentamicin. MCF-7-YAMA cells were obtained from Dr. Lahti (St. Jude's Children's Research Hospital, Memphis, TN). The cells were stably transformed with a plasmid expressing caspase-3. MCF-7 –YAMA cells were grown in DMEM supplemented with 10% FBS, and 500ug/ml geneticin. MDA-MB-468 cells were from the American Type Culture Collection (Rockville, MD). MDA-MB-468 cells lines were maintained in medium consisting of DMEM/F12 (1:1) (Life Technologies, Rockville, MD) supplemented with 10% FBS, and 50units/ml of both penicillin and streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 93% air/7% CO₂. After 4 or 5 days, cells became about 70-80% confluent and were passaged at a 1:5 ratio.

3. Western Blotting

Confluent cells were subcultured (1×10^6) in 100mm² dishes. Twelve hours after plating, cells were treated with drug. At specific time points, cell lysates were prepared according to Dr. J.R Davie's

protocol (personal communication). The cells were harvested at the times indicated by scraping into harvesting buffer (1% SDS and 10mM Tris-HCl, pH 7.4). Cell lysates were boiled for 5 min, centrifuged in an Eppendorf microcentrifuge (14,000 rpm, 5 minutes) at 4°C, and then the supernatants were collected. One tablet CompleteTM (protease inhibitor cocktail, cat. # 1836153, Roche Boehringer Mannheim, Indianapolis, IN) was dissolved in 1.5 ml distilled H₂O; 2.5% (v/v) of reconstituted protease inhibitor solution was added immediately to the supernatant. The protein concentration of the supernatant was determined using a BCA protein assay (Pierce, Rockford, IL) and bovine serum albumin as a standard unless otherwise noted. For Western blots, 5x SDS (sodium dodecyl sulfate)-loading buffer (3.15mM Tris-HCl, pH 7.0, 50%(v/v) glycerol, 0.1% SDS (w/v), and 1% (v/v) bromphenol blue) plus 2-mercaptoethanol (12.5ul of 2-mercaptoethanol per 100 ul 5x SDS-loading buffer) was added to the protein samples. The protein samples were heated in a sand bath for 5 min at 100°C. Equal amounts of protein were loaded onto 10% SDS-polyacrylamide mini-gels. Colored molecular weight protein markers (RPN756, Amersham Pharmacia Biotech Inc., Piscataway, NJ) were used to indicate different protein molecular weight. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Novex, San Diego, CA) and blocked overnight at 4 °C using 3% nonfat milk blocking buffer (3g non-fat dry milk in 100 ml of TBS (20 mM Tris-HCl, pH 7.5, 0.5M NaCl) and 0.05% (v/v) Tween 20). Membranes were incubated for 3 h at room temperature with the following primary antibodies: mouse monoclonal anti-p21 (WAF1) (Ap-1, cat. # OP64-100UG) from Calbiochem (Cambridge, MA), mouse monoclonal anti-epithelial glycoprotein (cat. # MAB-4043) from Chemicon International (Temecula, CA) and anti-acetylated histone H4 antibody (rabbit polyclonal, cat.# 06-598, Upstate Biotechnology, NY). The primary antibodies were diluted 1:500 in Western washing solution (0.1% non-fat dry milk, 0.1% albumin (chicken egg), 1% (v/v) FBS, 10%(v/v)10xPBS (1.4 M NaCl, 0.02 M KCl, 0.08 M Na₂HPO₄.7H₂O and 15 mM KH₂PO₄, pH 7.0) and 0.2%(v/v) Tween-20). After washing three times with Western washing solution and then one time

with TBS, the antigen-antibody complexes were incubated 1h at room temperature with HRP (horseradish peroxidase)-conjugated secondary antibodies (rabbit IgG-HRP (SC-2004), and mouse IgG-HRP (SC-2005) from Santa Cruz Biotechnology) diluted 1:3000 in Western washing solution. After washing three times with TBS, antibody binding was visualized using enhanced chemiluminescence (SuperSignal West Pico, cat. #1856136, Pierce, Rockford, IL) and autoradiography. The experiments were repeated at least three times.

4. Histone extraction

Confluent MCF-7 cells were plated at 6×10^6 (maximal cell number: 1×10^7) /T-162 flask in 20 ml of 5% FBS/DMEM. The cells then were grown in a humidified incubator chamber in 7% CO₂ at 37⁰C for 12 h. MTS IC50 of each antimalarial was added into each flask and incubated for the times indicated. After that, the growth medium was removed. Following a quick rinse with ice-cold PBS, cells were scraped into 4 ml of ice-cold lysis buffer (10mM Tris HCl, 50 mM sodium bisulfite, 1% Triton X-100 (v/v), 10 mM MgCl₂, 8.6% sucrose, pH 6.5) and nuclei released by Dounce homogenization (glass T type, five strokes). The nuclei were collected by centrifugation (3,000 rpm, 10 minutes, SS-34 rotor) at 4⁰C and washed three times with the lysis buffer. Histones were extracted from the crude nuclear pellets using the procedure of Nakajima et al. (1998). The pellets were resuspended in 0.1 ml of ice-cold sterile water using a vortex and concentrated H₂SO₄ to 0.2 M was added. The preparation was incubated at 4⁰C for 1 h, then centrifuged (17,000 rpm, Sorvall SS-34 rotor) for 10 minutes at 4⁰C. The supernatant containing the extracted histones was mixed with 10 ml of acetone, and the precipitate obtained after an overnight incubation at -20⁰C was collected and air-dried. The acid-soluble histone fraction was dissolved in 50 ul of H₂O, and stored at -70⁰C.

5. Cell Death ELISA (enzyme-linked immunosorbent assay)

Apoptotic death cells were measured with the use of the cell death ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN). The assay is based on quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes that are released into the cytoplasmic fraction of cell lysates during the early stage of apoptosis.

Cells (4.0×10^3) were plated in each well of 96-well plates and treated with either drug in 5%FBS/DMEM or 5% FBS/DMEM alone (control cells) for 48 or 72 h. Each treatment was performed in triplicate. To prepare cell cytoplasmic fractions, the supernatant which contains necrotic cell bodies was carefully removed by fast inversion of the plate. The attached cells were resuspended in 200 μ l lysis buffer, incubated for 30 min at room temperature. The lysate was centrifuged using a microtiter plate centrifuge (Sorvall GLC-2B General Laboratory centrifuge) at 200x g for 10 min, and then 20 μ l of the supernatant (cytoplasmic fraction) was transferred into the streptavidin coated cell culture grade microtiter plate (MTP) for analysis. A mixture of anti-histone-biotin and anti-DNA-POD (peroxidase) was added and incubated for 2 hours at room temperature. During the incubation periods, the anti-histone antibody bound to the histone-component of the nucleosomes present in the cell lysate, and simultaneously fixed the immunocomplex to the streptavidin-coated MTP via its biotinylation. Additionally, the anti-DNA-POD antibody reacted with the DNA-component of the nucleosomes. After removal of unbound antibodies by a washing step, the amount of the nucleosomes was quantified by the POD retained in the immunocomplex. POD was determined spectrophotometrically using ABTS (2,2-Azino-di-3-ethylbenzthiazolin-sulfonat) as the substrate. The sample absorbances were measured at 405 nm in the microtiter plate reader (EL340 BIOKINETICS READER, Bio-Tek Instruments, Winooski, Vermont).

6. MTS Assay

The MTS assay is based on the reduction of the yellow-colored-tetrazolium compound (MTS) by mitochondrial dehydrogenases of metabolically active cells to a blue formazan product which can be measured spectrophotometrically. It is a measurement of cell viability.

MCF-7 cells were plated at 4.0×10^3 cells/well in 96-well plates in 225 μ l of 5% FBS/DMEM. The cells then were grown in a humidified incubator chamber in 7% CO₂ at 37⁰ C for 12 h. Stock solution (10mM) of quinidine and other compounds were diluted in the appropriate medium immediately prior to use. Test agents were added at different concentrations into each well of the plate and incubated for 48 h. Cell proliferation was measured using a MTS assay kit (CellTiter 96 AQueous one solution assay, Promega, Madison, WI) according to the manufacturer's instructions. Briefly, the culture medium was removed from each well of the plate; 100 μ l of DMEM plus 5% FBS were added into all wells of the plate; 20 μ l of CellTiter 96 AQueous One Solution were immediately pipetted into each well. The plate was incubated for 2 h at 37⁰ C in a humidified 5% CO₂ atmosphere, and the absorbance was read at 490nm using the microtiter plate reader (Spectra Max 340PC). This assay is linear for 2.5 h.

Assays run in triplicate were repeated at least two times. Means $\pm$ SE were given. The concentration of each agent that inhibited cell growth by 50% (IC₅₀) was determined in each case using nonlinear regression analysis to fit inhibition data to the appropriate dose-response curve (GraphPad Prism version 3.0; GraphPad Software, Inc., San Diego, CA).

7. Ki67 Immunohistochemical Assay

An immunohistochemical assay for Ki67 antigen was performed according to the protocol described by Wang et al (1998). MCF-7 cells were plated on 5x5 mm² 95% ethanol-washed glass coverslips in 35 mm² dishes at 2×10^5 cells per dish in 5% FBS/DMEM culture medium. After 12 h,

cells were treated with different antimalarials at the IC₅₀ determined in the MTS assay for 48 h. At that time point, cells were fixed in acetone:ethanol (50:50) on ice for 10 min, and then washed with Dulbecco's phosphate-buffered saline (PBS (140 mM NaCl, 2 mM KCl, 80 mM Na₂HPO₄·7H₂O, 1.5 mM KH₂PO₄, pH 7.0))-0.15% bovine serum albumin (BSA), fraction V (A-9647, Sigma) (PBS-BSA). Coverslips were incubated with peroxidase blocking solution (0.3% hydrogen peroxide in methanol) for 10 min. Cells then were rinsed with PBS-BSA, and incubated with 10% horse serum in PBS-BSA for 30 min. After incubation, primary antibody for Ki67 (MIB-1, cat # M7240, Dako Corporation, Glostrup, Denmark) was diluted in PBS-BSA at 1:77 and added to the coverslips for 60 min. After rinsing with PBS-BSA, the coverslips were immediately incubated with second antibody (1:250 in PBS-BSA, biotinylated horse anti-mouse IgG, Vector Laboratories, Burlingame, CA) for 30 min. Following washing with PBS-BSA, the coverslips were incubated with the avidin-biotin-peroxidase reagent (Vector Laboratories) for 30 min. The coverslips were washed in PBS, and the antigen-antibody complex was visualized using diaminobenzidine (Stable DAB, Research Genetics, Huntsville, AL), and then counterstained with Mayer's hematoxylin (Fisher Scientific, Pittsburgh, PA). Finally, coverslips were mounted using Permount (Fisher Scientific, Pittsburgh, PA). Ki67 negative cells were visualized by light microscopy (400x objective, Ortholux microscope, Ernst Leitz, Wetzlar, Germany).

Cells were individually assigned with negative or positive staining, and the Ki67 negative percentage of the cell population was determined by counting. In each coverslip, at least 500 cells were counted.

8. Oil Red Assay

Lipid droplet accumulation, a classic differentiation marker, was measured in breast cancer lines using Oil Red O staining (Graham et al, 1988). Oil Red O stock solution (0.5% Oil Red O in 98% isopropanol) was prepared. MCF-7 cells were plated on 5x5 mm 95% ethanol-washed glass coverslips in 35 mm² dishes at 1×10^5 cells per dish. After cells were treated with drug or vehicle for 48 and 72 h respectively, cells were fixed in 10% formaldehyde and 0.2% calcium acetate in PBS for 3 minutes and then transferred to an Oil Red O staining solution for 10 minutes (Oil Red O stock solution diluted 6:4 in distilled water, and filtered through No.42 Whatman filter paper (Bancroft et al., 1984)). The slides were washed briefly in distilled water and then counterstained with Mayer's Hematoxylin solution (Fischer, Pittsburgh, PA) for 1 min at room temperature. After incubation, slides were washed in 0.4 % NH₄OH, rinsed in distilled water, and then mounted using 50% glycerol (50:50 glycerol/water (v/v)). The lipid accumulation was visualized by light microscopy (400x objective, Ortholux microscope, Ernst Leitz, Wetzlar, Germany).

Cells were individually assigned either as negative ORO denoting no lipid or less than 10 lipid droplets within a cell or positive ORO cells that had at least 10 lipid droplets per cell. The percentage of positive ORO cells was determined; at least 300 cells were counted .

9. p21(WAF1) ELISA

Cells were plated at the density 1×10^6 cells/100mm² dish in 5% FBS/DMEM culture medium. After 12 h, cells were exposed to each antimalarial at the MTS IC₅₀ for 24 h. The total cell p21(WAF1) protein levels were measured using a colorimetric assay (WAF1 ELISA, cat.# QIA18, Oncogen, Boston, MA) according to the manufacturer's instructions. Briefly, the cells were harvested at the times indicated by scraping into harvesting buffer (1% SDS and 10mM Tris-HCl, pH 7.4). Cell lysates were boiled for 5 min, and then centrifuged in an Eppendorf microcentrifuge (14,000 rpm, 5 minutes)

at 4 °C. The supernatants were collected. One tablet CompleteTM (protease inhibitor cocktail, cat. # 1836153, Roche Boehringer Mannheim, Indianapolis, IN) was dissolved in 1.5 ml distilled H₂O; 2.5% (v/v) of reconstituted protease inhibitor solution was added immediately to the supernatant. The protein concentration of the supernatant was determined using a BCA protein assay (Pierce, Rockford, IL) and bovine serum albumin as a standard. Fifty ug of cell lysate was transferred into the streptavidin-coated cell culture grade microtiter plate (MTP) for analysis. One hundred ul of p21 (WAF1) antibody was added to each well, and incubated for 2 h at room temperature. Additionally, 100ul of 1X Streptavidin Conjugate (horseradish peroxidase) were pipetted into each well. After removal of unbound antibodies by a washing step, the amount of p21(WAF1) was determined spectrophotometrically with substrate solution. The samples were measured at 490nm in the microtiter plate reader (Spectra Max 340PC). Quantitation is achieved by the construction of a standard curve using known concentrations of WAF1. In each experiment, a p21(WAF1) antibody-antigen standard curve was performed (the samples were incubated for 20 min). By comparing the absorbance obtained from a sample containing as unknown amount of WAF1 with that obtained from the standards, the concentration of WAF1 in the sample can be determined.

10. Histone Deacetylase (HDAC) Fluorescent Activity Assay

The HDAC Fluorescent Activity Assay (Drug Discovery kit cat # AK-500, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA) was used to screen drugs for the ability to directly inhibit HDAC activity. This assay system measures histone deacetylase activity present in a commercial HeLa (human cervical cancer cell line) cell nuclear extract, which is rich in HDAC activity. The potent HDAC inhibitor, trichostatin A (0.4 uM), was used as a positive control.

The principle of this assay is follows. The HDAC substrate contains an acetylated lysine side chain. After incubation with the HeLa nuclear extract, HDAC-mediated deacetylation of the substrate

sensitizes the substrate so that, upon addition of a developer agent, the deacetylated lysine becomes a fluorophore. Briefly, reactions were performed in 50 μ l. Fifteen μ l of diluted HeLa extract and 10 μ l of different concentrations of antimalarials were added into appropriate wells of the microtiter plate at room temperature; HDAC reactions were initiated by adding diluted substrate (25 μ l) to each well and mixing thoroughly; the reactions were performed for 10 min, and then the developer (50 μ l) was added into each well of the microtiter plate to stop the HDAC reactions. The plate was incubated at room temperature for 10 min, and read in a microtiter-plate reading fluorimeter using an excitation at a wavelength in the range 350-380nm and emission in the range 440-460nm (CytoFluor, Multi-well Plate Read Series 4000, PerSeptive Biosystems, Farmingham, MA).

11. Densitometric Analysis

Autoradiograms of the Western immunoblots were scanned using ChemiImager software (Alpha Innotech Corporation, San Leandro, CA). The blots were adjusted for brightness and contrast, and the mean density for each band was analyzed using ChemiImager analysis program. In this program, a rectangle which was called as a object was drawn around the detected spot, the AUTO BACKGRD (the program function name) determined the average of the 10 lowest pixel values in each individual object and assigned that value to the background. This background value was unique for each object, and was subtracted from all the pixels in the object. Unless specified otherwise, the results shown in each case are representative of three independent experiments with similar findings.

12. Statistical Evaluation

Data are expressed as the mean $\pm$ SER (standard error), with the number of replicates indicated in the figure legends. One-way ANOVA (analysis of variance) followed by Bonferroni's t-tests were used to assess statistical significance of difference between control- and drug-treated samples. Differences among the means were considered different if p values less than 0.05 were obtained using the GraphPad InStat computer program (Intuitive Software for Science, San Diego, CA).

III. RESULTS

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

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1.1. Introduction

Histone deacetylase (HDAC) 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 co-repressors (Davie et al., 1998). The spectrum of genes that show alterations in gene transcription rates in response to decreased HDAC activity is quite restricted (Van Lint et al., 1996). Yet, small molecule inhibitors of the enzyme histone deacetylase (HDAC) such as trichostatin A (TSA), superoylanilide hydroxamic acid (SAHA), trapoxin, and phenylbutyrate cause major alterations in cellular activity including the induction of cellular differentiation and apoptosis (Medina et al., 1997; Richon et al., 1998; Sambucetti et al., 1999). Trichostatin A, SAHA and trapoxin stimulate histone acetylation by acting as direct inhibitors of HDAC enzyme activity (Yoshida et al., 1999). 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 (Finnin et al., 1999). Trapoxin is an irreversible HDAC enzyme inhibitor (Kajima et al., 1993).

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 G1, 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 (Sambucetti et al., 1999). This observation provides an important link between HDAC inhibition and cell cycle arrest because p21/WAF1 plays a critical role in causing G1 cell cycle arrest via inhibition of the G1 cyclin-dependent kinase family (Xiong et al., 1993). Overexpression of p21/WAF1 has also been associated with apoptosis, but the mechanism of p21/WAF1-induction of apoptosis requires further investigation (Sheikh et al., 1995).

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 overlapping 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 phenylbutyrate was recently shown to have clinical anti-tumor activity (Warrel et al., 1998).

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 μ M) is an anti-proliferative agent as well. Quinidine arrested cells in early G1 phase, and induced apoptosis by 72-96 hr in MCF-7 cells (Wang et al., 1998), 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, downregulation 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.

2.2. 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 (Kasid et al., 1985), 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), 2mM glutamine, and 40 ug/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% CO₂. After six 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 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 Chemical Company (St. Louis, MO). The cell permeant proteasome inhibitors, MG-132 and lactacystin, were purchased from Calbiochem-Novabiochem (San Diego, CA).

Growth inhibition assays

Growth inhibition by cell numbers was assayed by plating cells in 35 mm² dishes (1- 1.5 X 10⁵) containing DMEM/ 5% FBS plus quinidine (90 uM). Viable cells were counted using a hemacytometer 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), that is based upon metabolic bio-reduction 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 mm² dishes, and grown for 96 h in DMEM / 5% FBS supplemented with either 10 uM all-trans retinoic acid (in 0.01% ethanol) or 90 uM quinidine (in H₂O). 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 to cells grown in DMEM / 5% FBS. Cells were fixed in 3.7% formaldehyde – PBS, rinsed in PBS, 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 (5 ug/0.1ml, 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 1mg/ml acetone solution, Sigma) (Greenspan et al., 1985 and Toscani et al., 1990). 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 X objective).

Immunoblotting:

Cells were harvested from confluent T-75 flasks and subcultured (1×10^6) in 100mm^2 dishes. On subcultivation, this confluent population of cells (85% in G1) 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, 1mM EDTA, 50 mM NaF and 0.1 mM Na_3VO_4 , pH 7.4). Protease inhibitors (protease inhibitor cocktail, Roche Molecular Biochemicals, Indianapolis, IN) were added immediately. Cell lysates were centrifuged in an Eppendorf microcentrifuge (14,000 rpm, 5 min) at 4°C , and the supernatants 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 removed. Following a quick rinse with ice-cold PBS, cells were scraped into 1ml of ice-cold lysis buffer (10mM 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. (1998). 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, 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 obtained after an overnight incubation at -20°C , was 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, Rockford, IL) 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 Life Science Inc., Arlington Heights, IL). Proteins were transferred to polyvinylidene difluoride membranes (PVDF) (Novex, San Diego, CA) and blocked at 4⁰C using 5% nonfat milk blocking buffer (1M 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), and mouse monoclonal anti-p16 (Ap-1), p21 (WAF1,Ap-1), p53 (Ap-6) from Calbiochem (Cambridge, MA) and anti-acetylated histone H4 antibody (1:500, rabbit polyclonal, Upstate Biotechnology, NY). 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 (TBS: 10 mM Tris-HCl, pH 7.5, 0.5M 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 uM (Saunders et al., 1999). HDAC assays were performed as described in Hendzel et al. (1991). Briefly, the cellular extract was incubated with 500 ug acid-soluble

histones isolated from [^3H] acetate-labeled chicken erythrocytes for 60 min at 37 $^{\circ}\text{C}$. Reactions were terminated by addition of acetic acid/HCl to a final concentration of 0.12N/0.72N. Released [^3H]-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.

1.3. Results

1.3.1. Hyperacetylation of histone H4

Antibodies that recognize acetylated forms of histone H4 have been used as a probe for agents that cause histone hyperacetylation. (Schmodt et al., 1999). In Western blot experiments, we compared the ability of quinidine to cause hyperacetylation of H4 in MCF-7 cells with that of TSA, an established HDAC inhibitor, known to inhibit proliferation in MCF-7 cells (Sun et al., 1999). H4 acetylation in response to TSA was rapid (within 0.5 h) (Figure 4B) 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 (Figure 4A) 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 (Saunders et al., 1999). Quinidine caused the rapid disappearance of HDAC1 from MCF-7 cells. HDAC1 protein levels in quinidine treated cells were reduced after 15-20 minutes compared with control cells, and HDAC1 protein was undetectable between 30 minutes and 6 hours

Figure. 4. Histone Hyperacetylation in MCF-7 Cells. (A) Histones were extracted from cells grown in the presence of 90 μ M quinidine for 0.5 , 1, 2, 6, 12 , or 24 h; histones (20 μ g/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 μ g of histone/lane were electrophoresed and analysed for acetylated histone H4 by immunoblotting. (C,D) HDAC1 protein in cell lysates was prepared from control MCF-7 cells at 0.5, 9, 12, 24 or 48 h, control groups were treated with distilled H₂O (C) or cells treated with 300 nM TSA 0.5, 12, 24 or 48 h, control groups were treated with 0.1% DMSO (D); 50 μ g protein/lane were electrophoresed in 12% polyacrylamide gels containing 1% SDS, and assayed for HDAC1 protein by immunoblotting. (E,F) HDAC1 protein in whole cell extracts from cells grown in the presence of 90 μ M quinidine for (E) 15,20, 30 min or 9,12, 24 or 48 h or (F) 0.5,1,2, or 6 h; extracts were electrophoresed (50 μ g protein/lane) and assayed for HDAC1 protein by immunoblotting.

A

Quinidine

0.5	1	2	6	12	24	48 h
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B

TSA

0.5	6	12	24	48 h
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C

Control

0.5	9	12	24	48 h
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D

Control

TSA

0.5	0.5	12	24	48 h
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E

Control

Quinidine

h		min			h			
48	15	20	30	9	12	24	48

F

Control

Quinidine

Control				Quinidine			
0.5	1	2	6	0.5	1	2	6 h

(Figure 4E and 4F). 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 (Figure 4C). TSA-treated MCF-7 cells also showed reduced levels of HDAC1 protein as early as 30 minutes after drug addition, and the reduced HDAC1 protein level was maintained through 48 h (Figure 4D). 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 26S proteasome pathway, MCF-7 cells were treated simultaneously for 30 min with quinidine and MG-132 (30 μ M), an inhibitor of the 26S proteasome. Cells treated with 90 μ M quinidine showed a complete loss of HDAC1 protein which was prevented when MG-132 and quinidine were added simultaneously (Figure 5A). Treatment with the solvent, DMSO, 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 DMSO, 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 (Wang et al., 1998), 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, Figure 6). Additional

Figure 5. Protection of HDAC1 protein by proteasome inhibitors. (A) MCF-7 cells were released from confluency and subcultured in normal growth medium supplemented with 30 uM MG-132 in 0.1% DMSO, 90 uM quinidine plus 0.1%DMSO or 0.1% DMSO 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 uM in 0.1% DMSO) except were harvested after 24 h, and analyzed for the presence of immunoreactive acetylated histone H4.

studies are required to define the spectrum of proteins affected by quinidine in a proteasome-sensitive manner. Quinidine (90 or 250 μ M) 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 (Figure 5B). These results support the idea that quinidine-induced loss of HDAC1 protein is involved in the H4 acetylation response via a proteasomal-sensitive pathway.

1.3.2. 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 p21/WAF1 protein are numerous (Kim et al., 1999; Saito et al., 1999; Sowa et al., 1999). It was of interest to determine whether p21/WAF1 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 p21/WAF1 were increased in response to quinidine treatment approximately 11-fold, and this elevated level of protein expression persisted through 48 h. A small, less than 2-fold increase in p27 levels was observed in cells exposed to quinidine for 24–48 h, while levels of p16 were not increased (Figure 6). Quinidine treatment decreased cyclinD1 and CDK4 protein levels by 24 h of treatment (Figure 7), indicating that the CKI inhibitor, p21/WAF, 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

Figure 6. G1 cell cycle proteins in MCF-7 cells. Cells released from confluency were plated into control medium or medium containing 90 uM quinidine. Whole cell lysates were prepared 12, 24 or 48 h after plating and assayed by immunoblotting for the CKIs , p21/WAF1 (n=3), p27 (n=1) , p16 (n=3) and p53 (n =3) after electrophoresis of 50 ug protein/lane through 12% SDS-polyacrylamide gels. Rb protein was immunoprecipitated from 500 ug of whole cell lysate protein using an antibody that recognizes phosphorylated and non-phosphorylated pRb (Liu, et al., 1999). 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.

Figure 7. Cyclin D – CDK4 in MCF-7 cells. Confluent MCF-7 cells were subcultured in control medium or medium containing 90 μ M quinidine. Whole cell lysates were prepared 0.5, 12, 24 and 48 h after subculture. Equal protein aliquots (50 μ g/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.

with the observed cell cycle arrest of quinidine-treated MCF-7 cells in mid-G1 phase (Wang et al., 1998).

In MCF-7 cell extracts probed using anti-Rb antibodies, two separate but closely migrating bands were distinguishable. The upper band contained more highly phosphorylated pRb and the lower band, unphosphorylated or hypophosphorylated pRb. Control cells showed a faint pRb signal at 12 h, typical of cells in early G1 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 Rb protein failed to increase with progression through G1 phase as seen in the control, proliferating cells (Figure 6). The decrease in Rb phosphorylation level was predictable based on the increase in p21/WAF1 and decreased levels of both cyclin D1 and CDK4 (Figure 7). In addition, Nakanishi et al. (Nakanishi et al., 1999) showed that p21/WAF1 can bind Rb protein and block its phosphorylation. However, the actions of quinidine upon p21/WAF1 and cyclinD/CDK4 activity do not explain why the levels of total Rb protein were so low. Reductions in the cellular content of phosphorylated Rb protein in MCF-7 cells by quinidine is an important additional level of cell cycle control that effectively attenuates progression of cells out of G1 phase, and has been reported in other tumor cell lines in response to HDAC inhibition (Levine et al., 1997). In Figure 8 we show data suggesting that the 26S 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.

Figure 8. Proteasome inhibitor modulates retinoblastoma protein levels. Confluent MCF-7 cells were subcultured in the presence of 90 uM quinidine, 30 uM MG-132, or quinidine + MG-132 for 24 h, then harvested, and whole cell extracts (100ug/lane) were analyzed for pRb. A Coomassie blue stained protein is shown as the loading control.

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, G1 cell-cycle arrest and apoptosis (Levine et al., 1997). Wild-type p53 down-regulates pRb levels in MCF-7 cells (Ameyar et al., 1997). Although Saito et al. (Saito et al., 1999) showed that p53 is not required for pRb downregulation by HDAC inhibitors in all cell lines, quinidine-treated MCF-7 cells have elevated p53 levels (5-7-fold) (Figure 6). 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.

1.3.3. 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 (Nieves-Neira et al., 1999). 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 (Figure 9). 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 μ M quinidine, and maximal growth inhibition was observed at $\sim$ 90 μ M 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 (Stampfer et al., 1993).

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 (Stingl et al., 1998) were used to probe the organization of the cytoskeleton (Figure 10). In these studies, all-trans retinoic acid (10 μ M) was used to compare the differentiation response (Jing et al., 1996). Control

Figure 9. Growth of human breast cell lines in quinidine. MCF-7, MDA-MB-231, T47D, MCF-7ras tumor cells and normal HMEC were replica plated in 96-well plates in control medium (open symbols) or medium containing 90 uM 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-7ras cell line (data not shown). Viable cell counts/dish of replica plated MCF-7, MDA-MB-231, T47D and MCF-7ras tumor cells and normal HMEC cells in 35 mm² dishes in control medium (open symbols) and medium containing 90 uM quinidine (solid symbols) is indicated with the dashed lines. The MTS data are the result of single experiments conducted in quadruplicate for all the cell 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, T47D) or single dishes (MDA-231) for each experiment. MCF-7ras data are the mean ($\pm$ range) of two experiments performed in duplicate dishes. HMEC data are from one experiment performed in single dishes. Data taken from Zhou, et al., 2000 as performed by Dr. Zaroui Melkounian.

MCF-7 cells showed expression of cytoplasmic cytokeratin 18 in a disorganized fashion. 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 (Halm et al., 1990), 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 (Douglas et al., 1998), the HER-2/neu kinase inhibitor, emodin (Zhang et al., 1995), overexpression of c-erbB-2 (Giani, et al., 1998) the vitamin D analog, 1-alpha-hydroxyvitamin D₃ (Mehta et al., 2000), the HDAC inhibitor, sodium butyrate (Bacus et al., 1990) and retinoic acid (Bacus et al., 1990) 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 (Figure 11). 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, while 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 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.

Figure 10. Cytokeratin 18 in MCF-7 cells. Cells were replica plated (2×10^5) on sterile coverslips in 35 mm² dishes in medium containing 0.01% ethanol (control), 10 uM retinoic acid , or 90 uM quinidine and grown for 96 hs. 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. Data taken from Zhou, et al., 2000 as performed by Dr. Zaroui Melkounian.

MCF-7

Cytokeratin 18

Control

Retinoic Acid

Quinidine

Figure 11. 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 mm² dishes in medium containing 0.01% ethanol (control), 10 uM retinoic acid (0.01% ethanol) or 90 uM 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. Data taken from Zhou, et al., 2000 as performed by Ann Lucktong.

1.3.4. 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 MCF-7, MCF-7ras, T47D and MDA-MB-231. All four cell lines were incubated for 24 h in the presence or absence of quinidine, and then histones were extracted for immunoblotting. Figure 12 shows that histone H4 was hyperacetylated in all cell lines treated with quinidine. Control cells contained no hyperacetylated histone H4.

Figure 12. Histone H4 hyperacetylation in human breast tumor cell lines. MCF-7, MDA-MB-231, T47D and MCF-7ras tumor cells were replica plated (1×10^7 /T-162 flask) in control medium (C) or medium containing 90 μ M quinidine (Q) . Histones were extracted from the cells after 24 h, and 20 μ g/lane of histone proteins were electrophoresed in 15% SDS-polyacrylamide gels. Immunoblotting was performed to detect acetylated histone H4.

Table 1. Summary of effects of quinidine on cell cycle proteins in MCF-7 cells. Protein signals were analyzed from autoradiographic signals on X-ray film using Molecular Dynamic Research Densitometer. These signals were estimated as fold changes due to the limitation of the x-ray film sensitivity. Quantitation of signal density for each the cell cycle regulatory protein expression is shown as fold induction of the protein by quinidine treatment over the control group. “+” indicates increase in protein level; “-” indicates decrease in protein level. Data are presented as the mean $\pm$ SER of values from typical experiment.

Table 1. Summary of Effects of Quinidine on Cell cycle Regulatory Proteins in MCF-7 Cells

Proteins	12h	24h	48h
P21(n=4)	11.2±1.1	9.2±1.8	9.3±1.2
P53(n=3)	6±0.2	5±0.3	7.4±0.5
P27(n=1)	1.2	1.8	3.2
Cyclin D1(n=3)	-5.6±0.3	-2±0.4	-4.0±0.7
CDK4(n=3)	-1.1±0.5	-3.5±0.4	-4.0±0.2
pRb(n=2)	3.0±0.8	-17±0.5	-15±0.4

1.4. 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 fully explain 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 G0, a quiescent state marked by the absence of Ki67 antigen immunoreactivity (Wang et al., 1998). 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-alpha, ER-beta), T47D (ER-alpha and ER-beta), and MDA-MB-231 (ER-beta). MDA-MB-435 cells expressed very low levels of ER-beta (Fuqua et al., 1999; Vladusic et al., 2000). MCF-7 and T47D cells display an epithelial morphology and show similarities with mammary ductal and luminal epithelial cells, respectively (Jin et al., 1996; Soule et al., 1973). MDA-MB-231 exhibit an elongated cellular morphology which 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 (Jones et al., 1997; Sweeney et al., 1998). 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 H-*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 (Sambucetti et al., 1999; Saito et al., 1999). Growing MCF-7 and T47D cells express p21/WAF1 protein in moderate to low levels (Sheikh et al., 1994), 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 (Sheikh et al., 1994; Futamura et al., 1995), 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 (Sambucetti et al., 1999).

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 p21/WAF1 protein and G1 cell cycle arrest occurred prior to differentiation (Itazaki et al., 1990; Chang et al., 1999; DiCunto et al., 1998; Evers et al., 1996; Freemerman et al. 1997; Missero et al., 1996; Matsumura et al., 1997), 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 (Yeola et al., 1996). 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 (Watson et al., 1996).

In the presence of quinidine, MCF-7 cells accumulate at a position 12 h into G1 phase (Wang et al., 1998). 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 (Pardee et al., 1974) near the G1/S transition. The present work showed that quinidine treatment caused elevated levels of p53 and p21/WAF1 protein by 12 h (Figure 6), the point within G1 where MCF-7 cells arrest in response to quinidine (Wang et al., 1998). When p53 and p21/WAF1 proteins were assayed before

12 h, p53 was undetectable, and p21 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 Rb 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 Rb 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 (Wang et al. 1998; Woodfork et al., 1995; klimatcheva et al., 1999). 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 (Wang et al., 1998).

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.

2. Effects of Antimalarials on Cell Growth, Differentiation and Apoptosis in Human Breast Cancer Cells

2.1. Introduction

Quinidine, a quinoline antimalarial, is a growth inhibitor of breast cancer cells, which may be useful for adjunct therapy of human breast cancer. We have shown that inhibition of MCF-7 cell growth in response to quinidine involves cell cycle arrest in G1/G0 phase followed by apoptotic cell death (Wang et al., 1998). Quinidine induces a variety of changes within the nucleus, including elevated p21(WAF1) protein and hyperacetylation of histone H4 (Zhou et al., 2000). Overexpression of the cyclin-dependent kinase inhibitor p21(WAF1) by quinidine might be through a process that involves activation of tumor suppressor gene p53. We also reported that antiproliferative actions of quinidine might involve activation of proteasome pathway, which, in part, degrades HDAC1 protein without directly inhibiting HDAC activity (Zhou et al., 2000). Quinidine is a novel HDAC inhibitor, and bears potential as new antitumor drug because of its ability to induce p21(WAF1) protein and to trigger differentiation and apoptosis. Quinidine also caused a decrease in cyclin D1 protein expression (Zhou et al., 2000). This is relevant to the potential use of quinidine in cancer therapy, because cyclin D1 has been strongly associated with proliferation and carcinogenesis in the mammary gland (Buckley et al, 1993).

We hypothesized that effects of quinidine on HDAC and apoptotic response were generalizable to quinoline antimalarials. To test this hypothesis, we screened quinoline antimalarials, including amodiaquin, chloroquine, halofantrine, mefloquine, primaquine, quinine, quinoline and quinolinic acid, for the ability to inhibit tumor cell growth. In this study, the main work is to determine if quinoline antimalarials exert their growth regulatory effects on MCF-7 cells via common mechanisms which involve cell cycle arrest, differentiation, apoptosis, and induction of hyperacetylated histone H4 without directly inhibiting HDAC activity. The selection of these molecular targets is based on the fact that they are the major contributors, possibly causative, to the mechanisms of breast cancer cell death.

The long-term goal of these studies is to obtain structure-activity information that will enable us to predict unique chemical structures with greater potency.

The molecular mechanisms by which quinidine induces apoptosis have been postulated including inhibition of anti-apoptotic Bcl-2 protein, and increase in cytochrome c release, which are a central coordinating events in apoptosis (Zhou and Strobl, 2000). However, the mechanism of quinidine-induced apoptosis still remains to be clarified.

One cell cycle-related gene implicated in quinidine action is pRb. The histone deacetylase HDAC1 physically interacts and cooperates with pRb, and there is evidence that this complex is a key element in the control of proliferation and differentiation (Magnaghi-Jaulin et al., 1998). Quinidine treatment leads to pRb hypophosphorylation, and this is likely to be involved in the mechanisms underpinning quinidine inhibition of cell proliferation. We probed whether pRb status modulates quinidine-induced apoptosis. MDA-MB-468 cells, pRb deficient breast carcinoma cells were used for this work. In the current study, we also determined whether caspase-3 might influence the apoptotic responses of MCF-7 cells to quinidine treatment.

2.2. Results

2.2.1. Effects of TSA on cell growth and HDAC activity

Trichostatin A (TSA), a specific and potent inhibitor of HDAC, inhibits cell growth, blocks cell cycle progression in G1 phase, induces differentiation, and causes apoptosis in many breast cancer cell lines (Vigushin et al, 2001, Kim et al; 2000 and Furumai et al, 2001). TSA is the most well studied HDAC inhibitor, and its effects on cell cycle, HDAC activity, and p21(WAF1) reporter gene expression have been published (Kosugi et al., 1999 and Furumai et al., 2001). Yet, interpretation of these data is confusing because the concentration of TSA in published reports varies from low nanomolar to micromolar concentrations. We have reported that 300 nM TSA causes hyperacetylation of histone H4 by 12 h in MCF-7 cells (Zhou et al., 2000). The TSA concentration-response in MCF-7 cells for several end-points are now reported in the following study; we compared the concentration-response effects of TSA on cell viability and HDAC activity in MCF-7 cells.

The MTS assay at 48 h of TSA exposure shows that TSA induced a concentration-dependent decrease in MCF-7 cell growth (Figure 13A). The MTS IC₅₀ value of TSA is 35nM in MCF-7 cells. The IC₅₀ value of TSA for HeLa HDAC activity is 8.4 nM (Figure 13B).

2.2.2. Effects of TSA on Ki67 antigen and p21(WAF1) protein in MCF-7 cells

Ki67 is a nuclear protein that is expressed in all phases of the cell cycle (Van et al., 1989). The absence of Ki67 protein from cells is a marker that cells have exited the cell cycle (Van et al., 1989). The percentage of Ki67 negative cells in the TSA-treated cell population is an indication that TSA causes cell cycle exit. About a 5-fold increase in Ki67 negative cells was observed after 48 h TSA treatment compared to control group (Figure 14A).

Transcription of p21(WAF1) and p21(WAF1) protein levels have been shown to be increased by TSA (Yoshida et al., 1999 and Kim et al., 2000). p21(WAF1) has also been shown to play an

Figure 13. Effects of TSA on cell growth and HDAC activity in human carcinoma cells. (A) TSA inhibited MCF-7 cells growth. MCF-7 cells (4000 cells/well) were plated in 96-well plates; 12 h after plating, cultures were treated with indicated concentrations of TSA. Total cells were determined after 48 h of treatment using the MTS assay. Cell growth was presented as percentage of vehicle control (0.01% DMSO). Data are mean $\pm$ SER of two independent assays performed in quadruplicate. (B) Effect of TSA on HDAC activity of HeLa cell nuclear extracts. The assay is described in *Materials and Methods*. The enzyme activity was expressed as a percentage of the control. Data represent mean $\pm$ SER of three independent experiments.

Figure 14. Effects of TSA on Ki67 negative cell population and p21(WAF1) in MCF-7 cells. (A) MCF-7 cells were plated (2×10^5) in 35-mm² dishes on sterile glass coverslips and grown in DMEM plus 5%FBS. Cells were treated with solvent (0.01% DMSO), or TSA 35nM for 48 h. Cells were stained for Ki67 immunoreactivity. The bar (the mean $\pm$ SER) represents the percentage of Ki67 negative cells in each treatment group. Data are from two independent experiments in which 500 cells per experiment condition were counted. (B) MCF-7 cells were plated at a density of 1×10^6 cells/100 mm² dish. Cells were cultured in DMEM with 5% FBS and at 80% confluency were treated with solvent (0.01% DMSO), or TSA 35nM for 24 h. Cell lysate was obtained at the indicated time points. Proteins (50ug) from the cell lysates were assayed for p21(WAF1) protein using an ELISA kit. Data are the mean $\pm$ SER of two independent experiments.

important role in G1 to G0 cell cycle transition and differentiation (Xiong et al., 1993; Doglioni et al., 1996). To determine whether p21(WAF1) is regulated in MCF-7 cells by TSA, the levels of p21(WAF1) in control and TSA-treated MCF-7 cells were examined using a p21(WAF1) ELISA. p21(WAF1) protein levels increased 5-fold in MCF-7 cells exposed 35 nM TSA for 24 h (Figure 14B).

2.2.3. Induction of differentiation by TSA in MCF-7 cells

The effect of TSA on cell differentiation was determined by ORO staining. As seen in Figure 15, lipid droplets accumulated in the cytoplasm around the nucleus in MCF-7 cells exposed to 35 nM TSA for 48 h. Lipid droplets were still present up to 72 h after TSA addition (data not shown). Figure 16 shows that 20, 30, and 40 nM of TSA treatment significantly increased cytosolic accumulation of lipid droplets in MCF-7 cells compared to control cells ($p < 0.05$). Collectively, these results indicate that induction of p21(WAF1) protein, G0 transition (loss of Ki67 antigen expression), and differentiation (lipid droplet accumulation) occurred in MCF-7 cells treated with a TSA concentration that reduces MTS activity by 50%.

2.2.4. Effect of TSA on apoptosis in MCF-7 cells

Apoptotic cell death was quantitated in MCF-7 cells following treatment with TSA using a cell death ELISA that measures soluble DNA-histone complexes generated during cell apoptosis. MCF-7 cells treated with increasing concentrations of TSA for 72 h exhibited a concentration-dependent increase in the number of soluble DNA-histone complexes, but the MTS IC_{50} for TSA (35nM) did not increase DNA-histone complex formation (Figure17). We conclude that the MTS IC_{50} TSA was sufficient to cause differentiation but not apoptosis, and the induction of

Figure 15. Induction of differentiation by TSA treatment in MCF-7 cells for 48 h. (A) and (B) Cells (1×10^5 cells per 35mm^2 culture dish) were grown on coverslips treated with solvent only (0.01% DMSO), or TSA 35 nM for 48 h; cells were stained with Oil Red O and counterstained with hematoxylin as described in “*Materials and Methods*”. Cells were visualized using light microscopy (x400). (C) Data are presented as percentage of ORO positive cells in solvent and treatment groups. The data are from three experiment in which 300 cells per treatment were counted. *, $p < 0.05$ for TSA treatment versus control group using paired t-tests.

MCF-7 Cells (48 h)

A

Control

B

TSA 35nM

C

Figure 16. Induction of differentiation by TSA in MCF-7 cells. Cells (2×10^5 cells per 35mm^2 culture dish) were cultured with 0.01% DMSO, or increasing concentrations of TSA for 39 h, at which time the lipid droplets were determined as an indicator of terminal differentiation using Oil Red O staining. The data are presented as the percentage of Oil Red O positive cells for each treatment. Data are mean $\pm$ SER of three independent assays. *, $p < 0.05$ for TSA treatment versus control group using ANOVA followed by Bonferroni's t-tests.

Figure 17. Nucleosome release in the cytoplasm of MCF-7 cells treated with TSA. MCF-7 cells (4000 cells/well in a 96-well plate) were incubated for 72 h with increasing concentrations of TSA. Cells were lysed, and mono- and oligonucleosome levels were determined by cell death detection ELISA. The release of nucleosomes of each sample was monitored by the absorbance at 405 nm. Data are mean $\pm$ SER of three independent experiments, each performed in triplicate. **, $p < 0.01$ for TSA treatment versus control group using ANOVA followed by Bonferroni's t-tests.

differentiation was effected at a lower threshold concentration than that required for induction of apoptosis in the MCF-7 cell line.

2.2.5. Antimalarials inhibit MTS metabolism in MCF-7 cells

Quinidine is known to inhibit cell proliferation in MCF-7 cells (Wang et al., 1998; Zhou et al., 2000). To compare the effects of other antimalarials on cell growth of MCF-7 cells, we determined the cell growth arrest upon treatment with antimalarial drugs using the MTS assay. The IC₅₀ after 48 h was calculated for each drug (Figure 18A). Mefloquine and primaquine were the most potent cell growth arrest agents, and quinidine was least potent agent in this assay. As shown in Figure 18B, there was an initial loss of about 60% of the cell population by chloroquine treatment for 60 h. Quinoline, quinidine and quinine caused growth arrest (no change in cell number) within 60 h.

MTS reduction activity could be affected within 4 h (Berridge, et al., 1993). To study whether antimalarials effect early changes in mitochondrial dehydrogenase in MCF-7 cells, the MTS IC₅₀ of each antimalarial was added to MCF-7 cells for 2 or 6 h. Figure 19 indicates that only primaquine treatment significantly inhibited the activity of mitochondrial dehydrogenase at times up to 6 h compared to control group ($p < 0.05$). No significant changes in mitochondrial dehydrogenase were detected in MCF-7 cells treated with the other tested compounds for 2 or 6 h. MTS IC₅₀ of antimalarials did not affect cell viability within 2 h (Figure 20).

2.2.6. Hyperacetylation of histone H4 by antimalarials in MCF-7 cells

To analyze the effects of quinoline ring-containing antimalarial drugs on histone acetylation, Western blot assays were performed to detect acetylation of histone H4 in MCF-7 cells exposed to MTS IC₅₀ of each antimalarial compound. Quinidine, quinine, and chloroquine possess a quinoline base ring structure and a tertiary amine side chain. Since quinidine causes hyperacetylation of histone

Figure 18. Antimalarials inhibit MCF-7 cell growth. (A) Antimalarials Inhibit MTS Metabolism in MCF-7 cells. MCF-7 cells were grown in the presence of increasing concentrations of each compound for 48 h, and cell viability was estimated using the MTS assay. The IC₅₀ value for each agent was determined using nonlinear regression analysis to fit the inhibition data. The results are the average of at least two independent experiments carried out in quadruplicate, and are presented as the means $\pm$ SER of the percentage of untreated, control cells. The IC₅₀ value for each compound is shown in the table. Typical or a complete concentration response curve for each compound is shown in Appendix 1. (B) Effects of antimalarials on cell growth. MCF-7 cells (5×10^5) growing in 60mm² dishes in 5% FBS/DMEM were treated with concentrations of antimalarials corresponding to 50% inhibition of MTS activity as measured at 48 h. After 60 h incubation with antimalarials or solvent alone, viable cells that excluded trypan blue were counted using hemacytometer. Data are the mean of $n=3 \pm$ SER independent experiments. Abbreviations: AQ (amodiaquin), CL (chloroquine), ETOH (ethanol), HF (Halofantrine), MQ (Mefloquine), QA (quinolinic acid), QL (quinoline), QD (quinidine), PQ (primaquine), and QN (quinine).

A

**Summary of IC₅₀ (log uM) for Antimalarial Drugs on MIS
Metabolism in MCF-7 Cells**

Compound	MQ	PQ	AQ	HF	QA	CL	QN	QL	QD
IC ₅₀ (log uM) (SER)	0.48 (0.162)	0.52 (0.13)	0.84 (0.073)	1.03 (0.089)	1.46 (0.125)	1.483 (0.157)	1.6 (0.08)	1.79 (0.3)	2.04 (0.3)
N	3	4	2	2	2	5	3	2	4
uM	3.0	3.4	7.0	11.0	28.0	33.0	40.0	62.0	110.0

B

Figure 19. Effects of antimalarials and TSA on MTS metabolism in MCF-7 cells.

MCF-7 cells were grown in the presence of MTS IC₅₀ of each compound for 2 h (A) or 6 h (B), and cell mitochondria metabolism was estimated using the MTS assay. The results are the average of one experiment carried out in quadruplicate, and are presented as the means $\pm$ SD. The microtiter plates were incubated with MTS for 1 h at 37 °C, and the absorbance was read at 490 nm using the microtiter plate reader. *, $p < 0.05$ for compound treatment versus control group using ANOVA followed by Bonferroni's t-tests.

A **B**

Figure 20. Effects of antimalarials and TSA on cell viability. MCF-7 cells (5×10^5) were growing in 60mm^2 dishes in 5% FBS/DMEM, and treated with concentrations of antimalarials corresponding to 50% inhibition of MTS activity as measured at 48 h. After 2 h incubation with each compound or distilled H_2O alone (control), viable cells that excluded trypan blue were counted using hemacytometer. Data are the mean of $n=3 \pm \text{SER}$ independent experiments.

Figure 21. Hyperacetylation of histone H4 by antimalarials in MCF-7 Cells.

Histones were extracted from cells grown either in the presence of compound or solvent control (distilled H₂O or 0.1% ethanol) for 12 or 24 h. Histone proteins were electrophoresed in 10 % SDS-polyacrylamide gels, and assayed for the presence of acetylated H4 by immunoblotting. (A) Cells were treated with either MTS IC₅₀ of antimalarials or 0.1% ethanol (control) for 24 h. Recombinant histone H4 proteins (20ug) were used as the negative control for acetylated histone H4 antibody. (B) Cells were treated with either amodiaquin 10 uM or distilled H₂O (control) for 24 h. (C) Cells were treated with quinidine, primaquine, or halofantrine for 24 h. (D) Cell were treated with 0-, 10-, 50-, and 90 uM of quinine for 12 h. (E) Summary of effects of antimalarials on acetylation of histone H4 protein in MCF-7 cells. “+”, agent induced acetylated histone H4; “-”, agent did not cause induction of acetylated histone H4.

E

Antimalarials	Test 1	Test 2	Test 3	Total N
CL	+ (30uM)	+ (33uM)	+ (33uM)	3
QD	+ (90uM)	+ (110uM)	+ (110uM)	3
QN	+ (50uM)	+ (40uM)	+ (90uM)	3
PQ	+ (5uM)	+ (3.4uM)	+ (3.4uM)	3
AQ	+ (10uM)	+ (7uM)		2
MQ	- (3uM)			1
HF	+ (11uM)	+ (11uM)		2
QL	- (62uM)	- (62uM)		2
QA	- (28uM)			1

H4, we hypothesized that these antimalarials might be the same or related. Quinoline antimalarial compounds lacking a tertiary amine side chain (quinoline or quinolinic acid) were used as negative control to test this hypothesis.

As shown in Figure 21, 4-alkylamino substituted quinolines (chloroquine and amodiaquin), and an 8-amino substituted quinoline (primaquine) induced hyperacetylation of histone H4 in MCF-7 cells treated with their MTS IC₅₀. Hyperacetylation of histone H4 was also observed in cells treated with halofantrine, quinidine and its stereoisomer quinine. Those are 4-amino substituted quinoline ring compounds containing a quinuclidine ring, and have structural parallels with amodiaquin and chloroquine. Mefloquine, quinoline and quinolinic acid that lack alkylamino-substituted quinoline rings did not increase acetylated histone H4 level. The data indicates that alkylamino-substituted quinoline ring might be important for acetylation of histone H4.

2.2.7. Effect of quinoline antimalarial drugs on HDAC activity *in vitro*

The effect of quinoline antimalarials on HDAC activity *in vitro* was assessed using a fluorescent enzyme assay. Histone deacetylases came from HeLa cells. As shown in Figure 22, none of antimalarials we tested inhibited HDAC activity compared to control ($p > 0.05$). TSA was used as a positive control in these assays. IC₅₀ TSA, 8.5nM, significantly inhibited *in vitro* HDAC activity compared to control ($p < 0.05$).

2.2.8. Effects of antimalarials on Ki67 antigen in MCF-7 cells

We analyzed the effects of antimalarials on the cell cycle of the MCF-7 cells by performing Ki67 immunohistochemical staining. MCF-7 cells were treated with each antimalarial (MTS IC₅₀) for 48 h. We observed that the percentage of Ki67 negative immunoreactive cells. Chloroquine, quinidine, quinine, -treated cell populations showed highly significant increases ($p < 0.01$) (Figure 23). Moreover,

an increase in the percentage of Ki67 negative cells also was observed in amodiaquin ($p < 0.05$). This result indicates that amodiaquin, chloroquine, quinidine as well as quinine caused cells to enter G0 phase. No significant changes in the percentage of Ki67 negative cells were detected after treatment of MCF-7 cells with the other listed antimalarials ($p > 0.05$). We conclude that only a subset of quinoline antimalarials stimulates exit of MCF-7 cells from the cell cycle.

Figure 22. Effect of antimalarials on HDAC activity in HeLa cell nuclear extracts. Assay was performed using MTS IC₅₀ of each compound as described in “*Materials and Methods*”. In panel A and B, control groups were treated with distilled H₂O; in panel C, the control group was treated with 0.1% ethanol. Data represent the average of three different experiments, each performed in triplicate; bars, SER. *, $p < 0.05$ for compound treatment versus control group using ANOVA followed by Bonferroni’s t-tests.

Figure 23. Ki67 expression in MCF-7 cells after treatment with antimalarials. MCF-7 cells were plated (2×10^5) in 35-mm² dishes on sterile glass coverslips and grown in 5% FBS/DMEM. Cells were treated with vehicles (distilled H₂O, or 0.1% ethanol), or the MTS IC₅₀ of each compound for 48 h. Halofantrine and mefloquine were dissolved in 95% ethanol; other listed antimalarials were dissolved in distilled H₂O. Cells were stained for Ki67 immunoreactivity as previously described by Wang et al. 1998. The bars represent the percentage of Ki67 negative cells in each treatment group. Data are the mean of $n=3 \pm \text{SER}$ independent experiments in which 300 cells per experiment condition were counted. *, $p < 0.05$; **, $p < 0.01$ for compound versus control group using ANOVA followed Bonferroni's t-tests.

2.2.9. Effects of antimalarials on p21(WAF1) protein in MCF-7 cells

Because p21(WAF1) is rarely mutated in human cancer cells, induction of p21(WAF1) by drugs might be an important molecular target for anticancer therapy. We examined the effect of antimalarials on changes in p21(WAF1) protein. As shown in Figure 24, a p21(WAF1) ELISA was used to test if p21(WAF1) protein expression increased in MCF-7 cells exposed to quinoline antimalarial drugs for 24 h. In our experiments, each drug was used at the MTS IC₅₀. Protein p21(WAF1) was increased in chloroquine and quinidine treated cells, but not either in quinoline or other tested antimalarials. Trichostatin A (TSA) was used as a positive control for these experiments. Transcription of p21(WAF1) and p21(WAF1) protein levels have been shown to be increased by TSA (Kim et al., 2000). Western blot analysis was performed on MCF-7 cell extracts to confirm the changes in p21(WAF1) protein in response to the antimalarials (comparison shown in Figure 25A). The results from Western blot analysis are consistent with the results by ELISA (Figure 25C). Amodiaquin, halofantrine, mefloquine, primaquine, quinolinic acid and quinoline did not increase p21(WAF1) protein levels (Western blot data not shown). Chloroquine caused a 10-15 fold elevation in p21(WAF1) levels in MCF-7 cells at 24 h. The p21(WAF1) response to chloroquine exceeded that of the potent histone deacetylase inhibitor, TSA. Quinidine elevated p21(WAF1) levels 4-5 fold, approximately the same as TSA, but neither quinine nor amodiaquin raised p21(WAF1) protein.

2.2.10. Effect of antimalarials on lipid droplet accumulation in MCF-7 cells

The effects of quinidine and other listed quinoline compounds on lipid accumulation were compared using ORO staining. The percentage of MCF-7 cells with lipid droplets in cells exposed to MTS IC₅₀ of compounds for 48 h is shown in Figure 26. Quinidine (110uM) caused positive ORO staining in 65% of MCF-7 cells by 48 h. Fifty percent ORO positive cells was achieved by and quinidine (110uM) and quinine (40uM),

Figure 24. Effect of antimalarials and TSA on expression of p21(WAF1) protein in MCF-7 cells. Cells were plated at a density of 1×10^6 cells/100-mm² dish. Twelve hours after plating, cells were treated with solvent (distilled H₂O, or 0.01%DMSO) or MTS IC₅₀ of each compound for 24 h. p21(WAF1) protein levels were measured by p21(WAF1) ELISA kit according to the manufacturer's protocol (Oncogen). Each bar represents the mean $\pm$ SEM of two independent experiments.

Figure 25. Effect of antimalarials on p21(WAF1) protein expression in MCF-7 cells.

(A) Proteins (50ug) from the cell lysates (same lysate as p21(WAF1) ELISA) were separated by 12% SDS-polyacrylamide gel electrophoresis and analyzed with Western blotting using mouse monoclonal antibody for p21(WAF1) as detailed in "*Materials and Methods*". (B) The signal density for each band in Western blot analysis was quantitated by densitometric analysis. (C) Comparison the results from p21(WAF1) ELISA and Western blot analysis. The 'W' bar represents the fold increase which is the signal density in drug treatment group divided by the signal density in solvent control using Western blot; the 'E' bar represents the fold increase which is the absorbance obtained from drug treatment group divided by the absorbance obtained from solvent control using p21(WAF1) ELISA .

Figure 26. Effect of antimalarials on lipid droplet accumulation in MCF-7 cells. Cells were plated (2×10^5) in 35-mm² dishes on sterile glass coverslips and grown in DMEM plus 5% FBS. Cells were treated with solvent (distilled-H₂O, or 0.1% ethanol), or MTS IC₅₀ of each compound for 48 h; halofantrine (HF) and mefloquine (MQ) were dissolved in 95% ethanol; other listed antimalarials were dissolved in distilled H₂O. Cells were stained with Oil-Red O; and counterstained with hematoxylin. The data were presented as percentage of ORO positive cells in each treatment group. Data are the mean of $n=3 \pm \text{SER}$ independent experiments in which 300 cells per treatment were counted. *, $p < 0.05$; **, $p < 0.01$ for compound versus control group using ANOVA followed by Bonferroni's t-tests.

primaquine (4uM) and chloroquine (33uM) also stimulated lipid accumulation (40% and 30% of ORO-positive cells respectively). Quinidine (90uM), quinine (90uM) and primaquine (10uM) induced positive ORO staining in 79-89% of MCF-7 cells by 72 h (Johnson and Strobl et al., 1999). Quinidine (110uM) treated MCF-7 cells for 72 h show a more intense increase in accumulation of lipid droplets compared with 48 h treatment (data not shown). These data suggest that quinidine was the most effective compound stimulating lipid droplet accumulation, followed by quinine, primaquine and chloroquine. Other quinolines tested were inactive as differentiating agents in this assay (<10% ORO-positive).

2.2.11. Effect of quinoline antimalarials on apoptosis in MCF-7 cells

To determine whether apoptotic cell death may be a contributing factor for antimalarial-induced decreases in MTS metabolism, we quantified the release of nucleosomes into the cytoplasm of MCF-7 cells using a photometric enzyme immunoassay. Etoposide, a DNA-damaging agent, was used as the positive control. Etoposide causes a double-strand breakage of DNA by forming a complex with the enzyme, topoisomerase II. Quinolinic acid and quinoline, compounds that contain only quinoline ring structure, did not change nucleosome release as compared with controls ($p>0.05$). Similarly, amodiaquin, mefloquine and primaquine treatments failed to induce apoptosis in MCF-7 cells (Figure 27). Clearly, quinoline-induced apoptosis was observed with chloroquine, quinidine and halofantrine treatment. Induction of apoptosis by these three agents was dependent on their concentrations. A statistically significant increase in apoptotic activity was observed after 48 or 72 h of treatment with 90, 110, 200 and 250uM quinidine in MCF-7 cells compared with control ($p<0.05$). These data are consistent with previous reports that quinidine induced apoptosis as evidenced by nuclear morphological changes (nuclear collapse) of the MCF-7 cells by 72 h using Hoechst 33342 staining of DNA (Wang et al., 1998). Chloroquine-treated cells (33uM) for 48 h exhibited an increase

Figure 27. The apoptotic response to antimalarias in MCF-7 cells. MCF-7 cells (4000 cells/well in 96-well plate) were grown for 48 h in the presence of increasing concentrations of agents. Cells were evaluated for apoptosis by the histone-DNA cell death detection kit as described in “*Materials and Methods*”. Data are the mean $\pm$ SER of three independent experiments. One asterisk indicates a p value of <0.05 for compound vs. control using ANOVA followed by Bonferroni’s t-tests.

Table 2. Enrichment of nucleosome release in the cytoplasm of MCF-7 cells treated with MTS IC50 antimalarials, etoposide, and TSA for 48 or 72 h. Cells were lysed, and mono- and oligonucleosome levels were determined by photometric enzyme immunoassay using cell death detection ELISA. The released nucleosome level of each sample was indicated as the absorbance at 405 nm. Each experiment was performed in triplicate. The apoptosis enrichment factor of nucleosomes released into the cytoplasm for each sample is calculated using the ratio: units of absorbance of sample divided by units of absorbance of control group. Data are mean $\pm$ SER of three experiments, each performed in triplicate. *, $p < 0.05$ for compound vs. control using ANOVA followed by Bonferroni's t-tests.

Compounds (MTS IC50)	Apoptosis Enrichment Factor (Mean± SER, N=3)	
	48 h	72h
Amodiaquin (AQ)	0.71 ±0.09	0.86±0.03
Quinolinic Acid (QA)	0.98 ± 0.05	1.01±0.02
Quinoline (QL)	1.00±0.06	1.09±0.03
Primaquine (PQ)	0.92±0.07	1.17±0.07
Mefloquine (MQ)	1.09±0.11	1.19±0.06
Quinine (QN)	1.13±0.09	1.19±0.07
Quinidine (QD)	1.50±0.10 *	1.73±0.09 *
Halofantrine (HL)	1.2±0.02	1.1±0.2
Chloroquine (CL)	1.75 ±0.14 *	1.82±0.07 *
Etoposide (ET 30uM)	2.74±0.13 *	1.23±0.20
Trichostatin A (35nM)	0.96 ±0.06	0.91±0.05

Table 3. A comparison of Ki67, ORO, p21(WAF1) protein, acetylated histone H4 and apoptotic response in MCF-7 human breast cancer cells treated with MTS IC50 antimalarials. Cells were treated with MTS IC50 of each antimalarial for 48 h. The Ki67 immunohistochemical assay is index of cell exit to G0. “-”, Ki67 negative cell population is less than 10%; “+”, Ki67 negative cell population is more than 20%. Cells accumulating lipid droplets were determined by Oil-Red O (ORO) assay. “-”, ORO positive cells are less than 10% or 19%; “+”, ORO positive cells are more than 20% and less than 50%; “++”, ORO positive cells are more than 50%. P21(WAF1) protein was measured by p21(WAF1) ELISA. “-”, induction of p21(WAF1) protein by antimalarial agent treatment for 24 h was not increased compared with control cells; “++”, induction of p21(WAF1) protein in treated cells was increased compared with control cells. Acetylated histone H4 was analyzed in MCF-7 cells exposed antimalarials for 24 h by Western blot. “+”, agent induced acetylated histone H4; “-”, agent did not cause induction of acetylated H4. Apoptotic response for 72 h treatment in MCF-7 cells was examined using cell death ELISA. *, $p < 0.05$, treated versus control group as evaluated using ANOVA followed by Bonferroni’s t-tests.

**Table 3. Summary of Antimalarials on G1/G0 Transition, p21(WAF1),
Differentiation, Hyperacetylated Histone H4 and Apoptosis in MCF-7 Cells**

Name	Alkylamino side chain	Ki67 negative cells	Lipid droplet accumulation	p21(WAF1)	Acetylated histone H4	Apoptosis
Quinolin	NO	-	-	-	-	-
Quinolinic Acid	NO	-	-	-	-	-
Amodiaquin	YES	+	-	-	+	-
Chloroquine	YES	+	+	++	+	*
Halofantrine	YES	-	-	-	+	-
Mefloquine	NO	-	-	-	-	-
Quinidine	YES	+	++	++	+	*
Quinine	YES	+	+	-	+	-
Primaquine	YES	-	+	-	+	-

in the number of soluble nucleosome complexes released ($p < 0.01$) compared with control cells (Figure 27). However, neither quinine, a quinidine stereoisomer, nor amodiaquin, an analog of chloroquine, enhanced the apoptotic response ($p > 0.05$) after 48 or 72 h of treatment compared with control cells.

When MCF-7 cells were treated with halofantrine for either 48 or 72 h, nucleosome release displayed an increase as the drug concentration was increased from 40 to 100 μM (Figure 27).

The enrichment of apoptotic response in MCF-7 cells treated with MTS IC_{50} concentrations of antimalarials for 48 and 72 h are summarized in Table 2. Quinidine (110 μM) and chloroquine (33 μM) induced apoptosis in MCF-7 cells ($p < 0.05$).

2.2.12. Role of caspase-3 in quinidine-induced apoptosis

MCF-7 human breast cancer cells are relatively resistant to apoptosis and lack the effector caspase-3 (Prokop et al., 2000). The apoptotic response to quinidine was compared in caspase 3^{-/-} MCF-7 cells and caspase-3^{+/+} reconstituted MCF-7 cells. Apoptosis was evaluated on the basis of nucleosome release, a characteristic of apoptosis. Figure 28 shows that the nucleosome release (DNA-histone fragmentation) profiles of caspase 3^{-/-} MCF-7 cells as compared with caspase-3^{+/+} MCF-7 cells after treatment with increasing concentrations of quinidine. Both cell lines were incubated with quinidine for 48 or 72 h. Caspase 3^{-/-} MCF-7 cells, as well as caspase-3^{+/+} MCF-7 cells, exhibited increased DNA-histone fragmentation in response to quinidine treatment. Caspase 3^{-/-} MCF-7 cells were less sensitive to quinidine in the DNA-histone fragmentation assay, induction of DNA-histone fragmentation by quinidine did not exceed 170% compared to control cells, even in cells treated with high concentrations of quinidine for 48 or 72 h. In contrast, caspase-3^{+/+} MCF-7 cells displayed high sensitivity towards quinidine-induced apoptosis, and incubation with 200 and 250 μM quinidine led to a 2-3-fold increase in apoptotic response by 72 h compared with control group. The 48 h apoptosis response in the quinidine-treated caspase-3^{-/-} MCF-7 cells and quinidine-treated caspase-3^{+/+} MCF-7

cells was not different statistically ($p>0.05$). However, the apoptosis response to quinidine-treated caspase-3^{+/+} MCF-7 cells was significantly increased as compared with caspase-3^{-/-} MCF-7 cells ($p<0.05$) at 72 h. The results indicated that caspase-3 reconstitution sensitized MCF-7 cells to quinidine-induced apoptosis.

2.2.13. Role of Rb on quinidine-induced apoptosis

Inactivating mutations in the tumor suppressor gene, pRb, have been found not only in 100% of retinoblastomas but also in many other adult cancers, including breast cancers (Lee et al., 1988). Rb is necessary to initiate and maintain the cell cycle exit in the differentiation pathway; pRb-mutations in cells prevent cell cycle exit and block differentiation (Maione et al., 1997). MDA-MB-468 human breast cancer cells contain mutated pRb (Munster et al., 2001). The growth of MDA-MB-468 cells treated with quinidine was inhibited over a concentration range similar to that for MCF-7 cells. MDA-MB-468 cells exposed to 110uM quinidine are not as sensitive as MCF-7 cells in the MTS assay (Figure 29A). There was however no statistically significant difference in DNA-histone fragmentation even in MDA-MB-468 cells exposed to high concentrations of quinidine for 48 or 72 h (Figure 29B). MDA-MB-468 cells were resistant to quinidine-induced apoptosis. In order to examine the hypothesis that wild-type pRb is required for quinidine-induced apoptosis, further studies are necessary to test whether pRb reconstituted MDA-MB-468 cells are more sensitive to quinidine-induced apoptosis compared to parental MDA-MB-468 cells.

Figure 28. Quinidine induces apoptotic response in caspase-3 (-/-) MCF-7 and YAMA (MCF-7, caspase-3 (+/+)) cells. Cells (4000 cells/well in 96-well plate) were treated with increasing concentrations of quinidine for 48 or 72 h. The nucleosome levels were determined by photometric enzyme immunoassay using a cell death detection ELISA as described in “*Materials and Methods*”. The release of nucleosomes into cytoplasm of each sample was indicated as the absorbance at 405 nm. Data represent the mean of three different experiments, each performed in triplicate; bars, SER. * $P < 0.05$, quinidine-treated caspase-3(+/+) cells vs. quinidine-treated caspase-3(-/-) cells as evaluated using ANOVA followed by Bonferroni’s t-tests.

A. 48 h Treatment

B. 72 h Treatment

Figure 29. Effects of quinidine on cell proliferation and apoptosis in MCF-7 and MDA-MB-468 human breast cancer cells. (A) MCF-7 and MDA-MB-468 cell viability were assessed by MTS assay. 4000 cells/well in 96 well plates were treated with increasing concentrations of quinidine for 48 h. The percentage of living cells was calculated as percentage of control by dividing average absorbances of triplicated drug-treated wells by that of control ones. Data are presented as the mean of three independent experiments, each performed in quintuplicate; bars, SER. (B) Effect of quinidine on apoptotic response in MDA-MB-468 cells. Cells (4000 cells/well in 96-well plate) were treated with increasing concentrations of quinidine either for 48 or for 72 h. The released nucleosome levels were determined by photometric enzyme immunoassay using cell death detection ELISA as described in “*Materials and Methods*”. The released nucleosome levels of each sample was indicated as the absorbance at 405 nm. Data represent the mean of three different experiments, each performed in triplicate.

A.

B. MDA-MB-468 cells

2.3. Discussion

The enzyme histone deacetylase (HDAC) is a new target for anticancer drug development. TSA is an irreversible inhibitor of HDAC activity in cultured mammalian cells and in fractionated cell nuclear extracts at low nanomolar concentrations (Kijima et al., 1993). TSA has been shown to arrest cells in G1 and G2 phases of the cell cycle, to induce differentiation and apoptosis in cultured cells (Yoshida et al., 1995). G1 arrest by TSA in HeLa cells was accompanied by induction of p21(WAF1) (Kim et al., 2000). p21(WAF1) plays a critical role in TSA-induced growth inhibition because HCT116 (p21^{-/-}) cells were resistant to TSA treatment compared with the parental line (p21^{+/+}) (Kim et al., 2000). TSA caused a rapid decrease in c-myc gene expression in human lymphoid cell line (Van Lint et al., 1996). Consistent with these literature reports, we observed that TSA affects cell growth, p21(WAF1), differentiation, cell cycle progression and apoptosis in MCF-7 cells. The TSA IC₅₀ for HDAC activity in HeLa nuclear extract was 8.5 nM. The MTS IC₅₀ of TSA (35nM) increased expression of p21(WAF1) protein in MCF-7 cells. One interesting finding was that MTS IC₅₀ of TSA did not trigger apoptosis but caused cell differentiation in MCF-7 cells. Higher concentrations of TSA (at least 100 nM) were required for induction of apoptosis in MCF-7 cells. These results demonstrate that cellular differentiation could be dissociated from apoptotic response in MCF-7 cells treated with TSA.

Although a variety of structurally unrelated natural inhibitors of HDAC such as butyrate, trapoxin, FR901228, and TSA have been isolated (Kijima et al., 1993 and Jung, 2001), the major limitation for natural HDAC inhibitors is that the efficacies are very limited because of its low antiproliferative activity and short half life (rapidly metabolized and excreted *in vivo*) in blood. On the other hand, these inhibitors such as butyrate (a natural fatty acid derived from a high-fiber diet) are weak and non-specific inhibition of HDAC activity (Jung, 2001). Therefore, several derivatives of butyrate have been studied to improve the rapid metabolism in the body (Pouillart et al., 1991). TSA, trapoxin and

FR901228 were reported to exhibit strong HDAC inhibition *in vitro*, no *in vivo* antitumor efficacy has been reported, presumably because of instability, low retention, or nonspecific toxicity of the compounds in the body. These limitations might be circumvented by development of antimalarials as new HDAC inhibitors, which have more favorable pharmacological properties. In this study, we summarized a series of experiments that addressed antimalarial induced changes in cell proliferation, cell cycle regulation, HDAC activity, histone acetylation and apoptosis in MCF-7 cells. These studies also advance the fundamental understanding of antitumor activity by antimalarials, and provide knowledge that is essential to developing appropriate anticancer strategies.

We demonstrated that antimalarials inhibited cell growth. Inhibition of cell growth by antimalarials was examined using the MTS assay and cell counting in MCF-7 cells. All antimalarials tested inhibited MCF-7 cell growth after 48 h treatment. Inhibition of cell growth by these compounds was concentration dependent. A comparison of the IC₅₀ values for the nine tested antimalarials indicated that mefloquine and primaquine are the most potent agents in MCF-7 cells. In contrast, quinidine is the least potent MTS inhibitor in MCF-7 cells. The variety of MTS IC₅₀ might involve difference in composition and orientation of the side-chain structure attached to quinoline rings in each compound. Such experimental observations implicate a mitochondria contribution to cell proliferation control.

Mitochondria are vital in controlling cell life by at least three general mechanisms, including (1) disruption of electron transport, oxidative phosphorylation, and adenosine triphosphate (ATP) production; (2) change of the permeability transition pore in the mitochondrial membrane and release of proteins that induce cell apoptosis; (3) alteration of mitochondria membrane potential via potassium channels. MTS assay measures the activity of mitochondrial dehydrogenase in cells alive. MCF-7 cells were exposed to MTS IC₅₀ of each antimalarial for 2h or 6 h, we only found that primaquine-treated-cells for 6 h significantly inhibited the activity of dehydrogenase compared with control cells ($p < 0.05$), this result is consistent with previous report that primaquine targeted succinate

dehydrogenase in mitochondria of malarial parasites (Kokaze, et al., 2000). Cell viability measured by trypan blue assay showed that those treatments did not inhibit MCF-7 cell growth within 2 h. These results suggest that except for primaquine, antimalarial effects on mitochondrial dehydrogenase reflect cell numbers. In the future, it is necessary to test if antimalarials regulate mitochondrial pore transportation and mitochondrial membrane potential, which might help us to understand the mechanisms by which antimalarials inhibit cell growth in MCF-7 cells.

Theoretically, apoptosis could contribute to the observed antimalarial inhibition of MTS metabolism in MCF-7 cells at 48 h. However, in time course experiments, apoptotic cell death was detected not earlier than 48 h after quinidine treatment, and maximal yields of apoptosis were induced as late as 120 h after treatment (Wang et al., 1998). Therefore, to determine whether antimalarials cause apoptosis and whether apoptosis could contribute to breast cancer cell growth inhibition, the yield of nucleosomes released was measured after 48 h treatment of MCF-7 cells with increasing concentrations of antimalarials. Apoptosis occurred in MCF-7 cells treated with MTS IC₅₀ of quinidine and chloroquine ($p < 0.05$). Enhanced apoptosis in MCF-7 cells treated with halofantrine only occurred above 40 μ M, but its MTS IC₅₀ (11 μ M) treatment did not increase the rate of apoptosis. These data indicate that induction of apoptosis by chloroquine, halofantrine and quinidine was concentration dependent and might be a contributor to cell killing (cell death). However, other tested antimalarials could not trigger apoptotic cell death pathways to inhibit cell growth.

A more intriguing mechanism of quinidine-induced cell growth inhibition was explored. This study was performed to gain insight into the molecular mechanism of quinidine-induced cell killing by the apoptotic pathway in breast cancer cell lines. Damage to mitochondria can result in release of cytochrome c to the cytosol. Cytochrome c causes caspase activation by forming a complex with Apaf-1 and procaspase-9, leading to activation of caspase-9, which in turn activates procaspase-3, leading to DNA cleavage. In this process, Bcl-2 is thought to be involved by preventing the release of

cytochrome c from mitochondria thereby inhibition of apoptotic pathway. We previously demonstrated that decline of Bcl-2 triggered by quinidine was accompanied by accumulation of cytosolic cytochrome c (Zhou and Strobl 2000). It has been reported that MCF-7 cells lack expression of caspase-3 mRNA and protein because of a mutation in the caspase-3 (CPP/YAMA) gene (Kurokawa et al., 1999). Abrogation of caspase-3 has been associated with acquired multidrug resistance (Kojima et al., 1998). High concentration of quinidine induced apoptosis in a caspase-3 independent fashion. We tested whether caspase-3 would enhance quinidine-induced apoptosis.

MCF-7 (caspase-3^{-/-}) and MCF-7/YAMA (caspase-3^{+/+}) cells were useful for studying the specific role of caspase-3 and caspase-3-dependent signaling in response to quinidine. We characterized the apoptotic responses of the MCF-7 cells to quinidine in comparison with MCF-7/YAMA cells. As demonstrated by the cell death ELISA, caspase-3 reconstituted MCF-7/YAMA cells showed sensitization to apoptosis. Treatment with quinidine between 110 and 250 μ M for 72 h exerted more dramatic effects on growth inhibition and apoptosis in caspase-3^{+/+} MCF-7/YAMA cells than did quinidine in MCF-7 cells, suggesting caspase-3 defects are one mechanism for chemoresistance to quinidine.

HDACs regulate histone acetylation by catalyzing the removal of acetyl groups on the NH₂-terminal lysine residues of the core nucleosomal histones. Modulation of the acetylation status of core histones is involved in the regulation of the transcriptional activity of certain genes. HDAC activity is associated with transcriptional repression. Aberrant recruitment of HDAC activity has been associated with the development of certain cancers (Lin, et al., 1998), because the cells are unable to undergo differentiation and lead to excess proliferation. Inhibition of HDAC activity results in the accumulation of acetylated histones H2a, H2b, H3, and H4. The result of hyperacetylation of histones by HDAC inhibitors is believed to activate a small subset of the genes, which involve cell differentiation and apoptosis in cancer cells (Jung, 2001). The HDAC inhibitor sodium butyrate

induces hyperacetylation of histone H4, and activates the four genes including actin, adipsin, lipoprotein, and adipocyte P2, which mainly modulate differentiation of the preadipocytes into mature adipocytes (Toscani, et al., 1990). Sodium butyrate as well as TSA enhanced p21(WAF1) transcriptional activation and apoptosis (Lee, et al., 1996; Archer, et al., 2001). Recently, the p21(WAF1) has attracted more attention, because the p21 gene appears to be rarely mutated in common human cancer cells, whereas the p53 gene is frequently mutated.

Previously we showed that quinidine induced hyperacetylation of histone H4 without inhibition of HDAC activity (Zhou et al., 2000). HDAC1 protein levels were reduced by quinidine treatment, and co-treatment with quinidine plus MG-132, a proteasome inhibitor, restored HDAC1 in MCF-7 cells within 12 h (Zhou et al., 2000). We hypothesize that other quinoline antimalarials might share these properties. The present study proved that the tested antimalarials do not inhibit HDAC activity *in vitro*. These data are consistent with previous observations that chloroquine, quinidine and quinine did not directly inhibit HDAC activity in a cell free assay (Zhou, et al., 2000). Moreover, amodiaquin, halofantrine, chloroquine, quinidine, quinine and primaquine stimulated acetylation of histone H4 in MCF-7 cells. These results indicate that alkylamino-substituted antimalarial ring might play a role for acetylation of histone H4. Further studies are necessary to examine if HDAC1 protein degradation is stimulated by all antimalarials that induce H4 acetylation in MCF-7 cells.

The manner of activation of p21(WAF1) gene expression by antimalarials might contribute to understanding the mechanisms that mediate the anti-proliferative effects of antimalarials. p21(WAF1) expression is usually controlled at the transcriptional level by both p53-dependent and p53-independent mechanisms. An up-regulation of p21(WAF1) strongly correlates with cell growth inhibition, and can force cellular decision between differentiation and/or cell death. Inhibition of p21(WAF1/Cip1) expression through transfection of p21(WAF1/Cip1) antisense oligonucleotides has been shown to block growth factor-induced apoptosis of SH-SY5Y neuroblastoma cells (Guo et al.,

1995). Conversely, transfection of p21(WAF1/Cip1) promotes a variety of cell lines into differentiation and /or apoptosis (Jiang et al., 1995, Pagliacci et al., 1995; Sheikh et al., 1995). These lines of evidence indicate that antiproliferative actions of chemotherapeutic agents may be mediated by up-regulation of p21(WAF1). We hypothesize that p21(WAF1) might play a determining role in apoptosis and /or differentiation in MCF-7 cells treated with antimalarials.

The different effects of quinidine and chloroquine in comparison with quinine and amodiaquin may be related to differences in p21(WAF1) gene expression. Amodiaquin, primaquine and quinine did not induce p21(WAF1) expression and apoptosis in MCF-7 cells, but chloroquine and quinidine both elevated p21(WAF1) protein levels and increased apoptosis compared to control cells. At the chloroquine concentration used in this study, chloroquine exerted a more dramatic effect on MCF-7 cell cycle arrest and apoptosis than did quinidine. Our data suggest that p21(WAF1) protein induction might be used to predict the ability of antimalarials to cause apoptosis.

Halofantrine (11uM) was not as effective as chloroquine in inducing p21(WAF1) expression and apoptosis, but exhibited comparable apoptotic responses at 40uM in MCF-7 cells. Halofantrine is not a quinoline antimalarial, and we concluded that halofantrine-induced apoptosis might be mediated via a different pathway. It is clear that the growth inhibitory effects of antimalarials in breast cancer cells as reflected a 50% inhibition of MTS metabolism can be dissociated from apoptosis. Individual agents, while all are able to inhibit MTS metabolism, vary with respect to induction of p21(WAF1) and apoptosis.

The cell cycle transition into G0 is a prerequisite step for differentiation. Only amodiaquin, chloroquine, quinidine, and quinine at their MTS IC50 led to an increase in the number of cells in the G0 phase in MCF-7 cells (Ki67 assay). These data indicate that up-regulation of p21(WAF1) by chloroquine and quinidine must contribute to cell cycle arrest at G0 phase. However, amodiaquin as well as quinine-induced cells into G0 phase are independent of P21(WAF1), which might involve

other cyclin-dependent kinase inhibitors. Using lipid droplet accumulation measured by ORO staining as a marker of mammary cell differentiation, only MTS IC₅₀ of chloroquine, quinidine, quinine and primaquine were observed to induce differentiation in MCF-7 cells. We found that p21(WAF1) is not determined factor to contribute to antimalarial-mediated differentiation. Furthermore, quinine and primaquine did not exhibit signs of cell apoptosis during the following 48 or 72 h treatment. Therefore, induction of differentiation by quinine and primaquine was dissociated from apoptosis, suggesting that apoptosis is not the only mechanism for inhibition of cell growth by antimalarials. The data also suggested that lipid droplet accumulation is not always a marker of cell differentiation, because there was dissociation between the ORO and Ki67 assay, which was observed in either amodiaquin or primaquine treatment. In an effort to confirm our conclusion, we need to test the milk fat protein expression, another cellular differentiation marker (Munster, et al., 2001), in MCF-7 cells exposed to amodiaquin and primaquine.

In summary, our major conclusion is that quinidine and chloroquine induce growth arrest, cell cycle exit, differentiation, hyperacetylation of histone H4, p21(WAF1) and apoptosis in MCF-7 cells. Our data is the first report of induction of apoptosis in breast cancer cell lines by the antimalarial chloroquine. Quinine (a steric configuration of quinidine) as well as amodiaquin (an analogue of chloroquin) induced cell arrest without apoptosis. We conclude that tertiary amine side chain and its configuration may be fundamental requirements for triggering apoptosis or histone hyperacetylation in MCF-7 cells.

Although quinidine and chloroquine exerted similar effects on the cell cycle regulatory protein p21(WAF1), distinct differences in the kinetics and magnitude of p21(WAF1) protein regulation were observed in response to these agents. The activity of chloroquine was significantly more potent than that of quinidine. Induction of apoptosis following treatment with halofantrine was not correlated with induction of differentiation. These data suggest that differentiation is not obligatory response for

apoptosis in MCF-7 cells, implying that antimalarials might trigger multiple cell death pathways. Chloroquine is the most potent p21(WAF1) gene regulatory therapy agent and may be of use as a lead compound in the design of breast cancer cell apoptotic agents.

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Appendix 1. Antimalarials inhibit MTS metabolism in MCF-7 cells. MCF-7 cells were grown in the presence of increasing concentrations of each compound for 48 h, and cell viability was estimated using the MTS assay. These assays were performed after 2 h incubation, the MTS activity was then measured at 490 nm using the microtiter plate reader. A complete concentration response curve for each compound is shown.