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Pharmacological regulation of c-myc gene expression in human breast cancer cells

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Pharmacological Regulation of *c-myc* Gene Expression in Human Breast Cancer Cells

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ABSTRACT

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Previous studies demonstrated that quinidine causes G1/G0 cell cycle arrest and inhibition of proliferation in MCF-7 human breast cancer cell line (Woodfork, K. et al, 1995). The goal of studies reported here was to understand the molecular mechanisms of *c-myc* gene regulation by quinidine. *c-myc* is one of the most common oncogene aberrations in breast cancers (Deming, S.L. et al, 2000). *c-myc* functions include regulation of cell cycle, proliferation, differentiation, and apoptosis. The results of these studies demonstrated that quinidine causes rapid (within 1 hour) suppression of Myc protein and mRNA levels that precedes the quinidine-induced G1 cell cycle arrest point (D point) in MCF-7 cells. Additionally, the activity of *c-myc* promoter was suppressed by quinidine over the same range of concentrations that suppress levels of *c-myc* mRNA and protein, suggesting that changes in Myc protein and mRNA levels by quinidine may be attributed to its effect on *c-myc* promoter. A 168 bp region of *c-myc* promoter (-100 to +68 in respect to P1) was identified as a quinidine responsive region (QRR). Suppression of Myc by quinidine was consistent with inhibition of growth and induction of more differentiated phenotype in four different breast tumor cell lines. In contrast, quinidine had minimal effect on Myc levels or proliferation in normal mammary epithelial cell lines. Furthermore, MCF-7 cells treated with *c-myc* antisense oligonucleotides exhibited cytoplasmic lipid droplets, similarly to the quinidine-treated cells, suggesting that suppression of Myc may play a causative role in the induction of more differentiated phenotype by quinidine in human breast cancer cells.

DEDICATION

I would like to dedicate this work to my son Mark

ACKNOWLEDGMENTS

I would like to acknowledge a number of people, without whose support the completion of this dissertation would not be possible. First, I would like to thank my research advisor, Dr. Jeannine Strobl, for revealing to me a beauty of science and teaching me many valuable scientific techniques; for her great guidance, support, and endless optimism and smile, which have been a constant source of inspiration and encouragement. Special thanks to my graduate committee: Drs. Mike Mawhinney, Mike Miller, Bill Wonderlin, and David Yelton for their critical remarks and guidance. Thanks to Dr. Mawhinney for long, invaluable discussions of my project. Thanks to my friends and colleagues: Meredith McCracken, Anna Martirosyan, Rayhana Bata, and Qun Zhou for their unconditional help, support, and friendship throughout all these years. Thanks to everyone on the department for organizing unforgettable baby shower for my son Mark.

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CDK	Cyclin dependent kinase
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
E2	Estradiol
EDTA	Ethelenediamine Tetraacetic Acid
EMSA	Electrophoretic Mobility Shift Assay
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HMEC	Human Mammary Epithelial Cells
K_{ATP}	ATP –sensitive potassium channels
MAPK	Mitogen Activated Protein Kinase
MOPS	3-[N-Morpholino] Propanesulfonic acid
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl fluoride
PRF-DMEM	Phenol Red Free Dulbecco's Modified Eagle's Medium
PVDF	Polyvinylidene Difluoride
QRE	Quinidine Response Element
QRR	Quinidine Response Region
SERM	Selective Estrogen Receptor Modulator
SSC	Standard Saline Citrate
TCE	TGF β Control Element
TGF β	Transforming growth factor β

I. INTRODUCTION

1. Breast Cancer

1.1. Current methods of breast cancer therapy

Worldwide, breast cancer remains the most common malignancy affecting women, accounting for 18 % of all female cancers (McPherson, K. et al, 2000). In the United States each year more than 180,000 women are diagnosed with breast cancer and 44,000 will die from this disease (NIH, 2000) making it the second leading cause of cancer death among the females after lung cancer. Risk factors for the development of breast cancer include older age, immediate family history of breast cancer at a young age or bilateral breast cancer, early menarche, late menopause, nulliparity, use of hormone replacement therapy, exposure to ionizing radiation at a young age, lifestyle, and past history of atypical hyperplasia, in situ or invasive carcinoma (McPherson, K., et al, 2000).

Current methods of breast cancer therapy include surgery, radiation, cytotoxic and hormonal chemotherapy, and preventive chemotherapy. The clinical management of breast cancer patients is guided by the number of parameters, including tumor size, histological type or grade, the presence of steroid receptors, involvement of the axillary lymph nodes, and presence of metastasis (NIH, 2000). Carcinoma in situ is a preinvasive lesion within breast without penetration of the basement membrane and the potential for metastasis. *Intralobular* and *intraductal carcinomas in situ* are treated with the local surgical excision and wide local excision following by irradiation, respectively (Harris, J.R. et al, 1992). *Primary breast cancers* are treated with modified radical mastectomy or partial mastectomy following by external beam irradiation (Fisher, B. et al, 1989). Axillary lymph node dissection during the surgery is necessary to determine the adjuvant therapy of choice, since axillary nodal

involvement is predictive of relapse in operable breast cancer. Because adjuvant polychemotherapy (≥ 2 agents) has been shown to substantially improve the long-term, relapse-free, and overall survival, it should be recommended to the majority of women with localized breast cancer regardless of nodal, menopausal, or hormone receptor status (NIH, 2000). The currently recommended adjuvant therapy includes tamoxifen treatment for five years among women aged over 50 years and standard oral CMF (Cyclophosphamide, Methotrexate, 5-Fluorouracil) for six months or ovarian ablation in women younger than 50 years (NIH, 2000; Fisher, B. et al, 1990; Wood, W.C. et al, 1994). The benefit from tamoxifen therapy is nearly doubled for patients with Estrogen Receptor (ER) rich tumors compared to ER poor ones.

Virtually all of the deaths in the United States due to breast cancer are associated with *progressive metastatic breast cancer*. Unfortunately, there is no cure for this stage of breast cancer, although the length and quality of patients' lives with metastatic cancer varies greatly. Clinical studies showed that about one third of patients with metastatic breast cancer will respond to endocrine therapy (Glauber, J.G. and Kiang, D.T., 1992). Endocrine agents are best used as initial therapy in older patients with ER-positive tumors. First-line endocrine therapy for postmenopausal patients generally consists of tamoxifen, followed by a progestin (Megestrol acetate), high dose estrogen, an aromatase inhibitor (Anastrozole), or androgen (Fluoxymesterone) when tumor progression occurs. For premenopausal patients, oophorectomy or tamoxifen treatment may be considered first, followed by progestin or androgen treatment in the event of progression (Glauber, J.G. and Kiang, D.T., 1992). The median duration of response to any of these endocrine therapies is between one and two years. Although chemohormonal treatment may lead to higher response rates compared to

hormonal therapy alone, a survival benefit has not been demonstrated. About two-thirds of all patients with metastatic breast cancer will have a response to some form of cytotoxic chemotherapy. The median duration of response is six to twelve months (Henderson, I.C. and Harris, J.R., 1991). The most widely used combinations in the treatment of metastatic breast cancer are CAF (Cyclophosphamide, Adriamycin, 5-Fluorouracil) and CMF (Cyclophosphamide, Methotrexate, 5-Fluorouracil) (Abramowicz, M., 2000). Second-line therapy for refractory patients results in lower response rate and duration of response compared to those for front-line therapy. Paclitaxel (Taxol) is recommended for second-line therapy of metastatic breast cancer (Seidman, A.D., 1994). Other second-line agents include mitomycin C and vinblastine, yielding response rates of 25 to 30% (Henderson, I.C. and Harris, J.R., 1991).

The major limitation associated with the use of cytotoxic chemotherapeutic agents is their lack of selectivity for tumor cells and associated with it toxicity in normal tissues. In general, anticancer drugs affect rapidly dividing cells. These include tumor cells, hematopoetic cells, cells that line the digestive tract, and hair follicle cells. Accordingly, the most common side effects of cancer chemotherapy are immunosuppression, GI disturbances, and alopecia (hair loss). There are also other toxicities associated with some anticancer drugs. For example, high doses of methotrexate can cause acute renal failure and neurotoxicity. Doxorubicin at cumulative doses exceeding 450 mg/m^2 causes significant cardiotoxicity (Von Hoff D.D. et al, 1979). Also mutagenic potential of some chemotherapeutic agents can cause development of secondary tumors, such as leukemia. There are several side effects associated with endocrine therapy as well. Recent study by Fisher, B. and coworkers (Fisher, B. et al, 1998) showed that tamoxifen increases the women's risk for three rare but life-threatening health

problems: endometrial cancer (cancer of the lining of the uterus), pulmonary embolism (blood clot in the lung), and deep vein thrombosis (blood clots in major veins). Another important problem in cancer therapy is a development of resistance to antineoplastic drugs. Were it not for drug resistance, control and cure of most cancers would be possible.

Some of the most exciting developments in cancer research were the discovery of oncogenes and tumor suppressor genes, as well as the better understanding of the internal and external stimuli that result in malignant transformation, tumor progression, invasion, and metastasis. The advances in our understanding of the basic biology of breast carcinoma contributed to the development of novel approaches to cancer treatment to supplement traditional chemotherapy. One such example is a development of monoclonal antibody, trastuzumab (Herceptin) to epidermal growth factor receptor HER-2/neu. This antibody binds with great affinity to the extracellular domain of HER-2 and inhibits the transmission of growth stimulatory signal. In clinical trials Herceptin as a single agent has been shown to produce antitumor responses in 15-20 % of patients with metastatic breast carcinoma (Baselga, J. et al, 1998; Cobleigh, M.A. et al, 1998). Another area of intensive research is the development of Selective Estrogen Receptor Modulators (SERM). Based on our growing knowledge about estrogen receptor subtypes, their distribution and functions in different tissues, researchers are searching for compounds, that would function as a potent anti-estrogens in the breast and uterus to prevent estrogen-driven cell proliferation and, at the same time, have strong estrogenic effects in bone, the cardiovascular system, and the central nervous system, where the hormones have beneficial effects. To date, the only three SERM available on the market are tamoxifen (Nolvadex), raloxifene (Evistar), and toremifene, although there are more second and third generation SERM showing promising results in

preclinical data (McNeil, C., 1998, Williams, G.M., 1998; Labrie, F. et al, 1999). Selective targeting of deregulated oncogenes in cancer cells with antisense oligonucleotides or delivery of disrupted tumor suppressor genes using viral vectors are other advancing areas in cancer research.

Due to the tremendous progress in breast cancer research, for the first time the annual mortality rates are decreasing in the United States (NIH, 2000). There is still more to learn about breast cancer genetics, breast cancer susceptibility genes, such as BRCA1 and BRCA2, molecular mechanisms regulating mitogenic stimulation, and malignant transformation in breast cancer cells. This knowledge may reveal new pharmacological targets for the development of better therapeutic approaches to treat breast cancer.

The next section of the introduction provides a general overview of basic cell cycle regulatory processes, as well as more detailed discussion of G1 to S phase transition in MCF-7 human breast cancer cell line. The purpose of these background materials is to facilitate understanding of the subsequent sections of the introduction, disusing functional role of *c-myc* gene in cell cycle regulation.

2. Cell Cycle

2.1. General overview

The cell cycle is a sequence of highly ordered and controlled processes that results in a duplication of a cell. The cell cycle consists of 4 distinct phases: G1, S, G2 and M. DNA replication and cell division (cytokinesis) occur during the S and M phases, respectively. Gap phases (G1 and G2) precede S and M phases and are responsible for the synthesis of essential proteins for DNA replication and mitosis. Certain conditions, for example starvation, differentiation cause cells to exit from the cell cycle into a non-proliferative state, called G0 phase. Some non-dividing cells, such as skeletal muscle fibers, are unable to re-enter the cell cycle. Other, such as lymphocytes, hepatocytes and fibroblasts are usually in G0 but can be activated by external agents (mitogens) to enter cell cycle. Each phase of the cell cycle is regulated by three families of key cell cycle regulatory proteins: *cyclins*, *Cyclin Dependent Kinases* (CDKs), and *Cyclin-dependent Kinase Inhibitors* (CKIs). In response to mitogenic stimuli, such as growth factors, there is a transient increase in levels of cell cycle phase-specific cyclins. Each cyclin binds to the corresponding CDK and activates its catalytic subunit. Activated CDK then phosphorylates another important cell cycle regulator, the retinoblastoma protein, pRb. A schematic diagram of the temporal relationship between different cyclin/CDK complexes and cell cycle phases are shown in Figure 1. Cyclin D1 is expressed throughout G1 phase but its peak occurs during mid G1. The main function of cyclin D1 is to activate CDK4/6 catalytic subunits to induce initial phosphorylation of pRb. Cyclins E and A begin to rise later in G1. They are responsible for activation of CDK2 to maintain pRb in its hyperphosphorylated state during the late G1 and ensure G1 to S progression. In its hypophosphorylated form pRb is bound to the family of heterodimeric

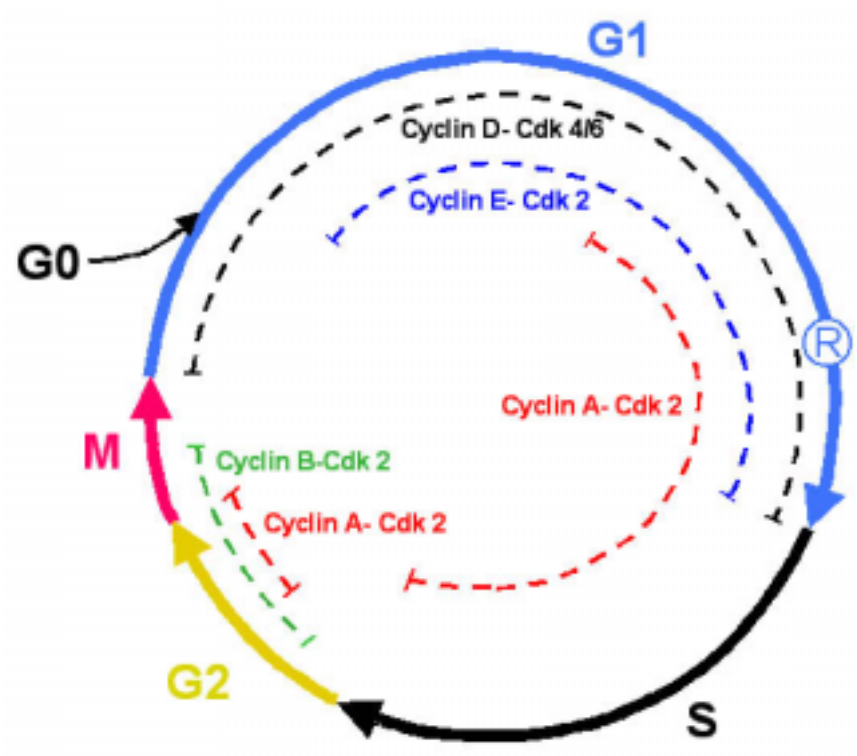


Figure 1. Temporal relationship between expression of Cyclin-Cdk complexes (dashed lines) and different phases of cell cycle (solid lines).

R – late G1 restriction site

transcription regulators, collectively called the E2Fs, which can transactivate genes whose products are essential for S phase entry. Some of these genes are dihydrofolate reductase, thymidylate synthase, DNA polymerase α, and DNA polymerase δ subunit PCNA.

In the complex with pRb, E2Fs are unable to perform their function as transcription factors. Phosphorylation of pRb by CDKs removes its inhibitory capacity, causing release of E2Fs, which then transactivate the expression of genes needed for entry into S phase. Activation of E2F-1 also leads to transcriptional activation of cyclin E and A, reinforcing the initial activation of E2F. Figure 2 summarizes these events. Extracellular stimuli initiate progression of cells from G1 to S; other events regulate progression of cells from S phase into G2 and from G2 into M phase. These transitions are controlled by internal signals that are triggered by the completion of biosynthetic processes unique to S and G2 phases, respectively. They are insensitive to external conditions (Murray, A.W. and Kirschner, M.W., 1989). The point in G1 phase beyond which a cell is no longer dependent on the presence of extracellular factors and is committed to complete cell cycle is called the G1 "restriction point" or “R point" (Pardee, A.A, 1974). When fibroblasts are arrested in R, for example by withdrawal of growth factors for a long period of time, they will enter quiescence state or G0 (Campisi, J. and Pardee, A.B., 1984).

In addition to the positive regulation by cyclins, CDK activity is negatively regulated by another important family of cell cycle regulatory proteins: *Cyclin-dependent Kinase Inhibitors*, CKIs. These inhibitors can be up-regulated when required, thus blocking the activation of CDKs by cyclins. The known CKIs are grouped into two gene families, Ink4 and Cip/Kip, according to structural similarities. The Cip/Kip family consists of p21, p27, and p57. p21 is also known as Wild-type p53 Activated Fragment 1 (WAF1) or CDK2

Interacting Protein 1 (CIP1). p27 and p57 are known as Kinase Inhibitory Protein 1 (Kip1) and 2 (Kip2), respectively. These inhibitors tend to have wide ranging roles and can potentially inhibit a number of different CDKs. The Ink4 (for inhibitor of CDK4) family is far more specific and consists of p15 ^{INK4b}, p16 ^{INK4a}, p18 ^{INK4c}, p19 ^{INK4d} (Anxo Vidal and Andrew Koff, 2000). Until recently it was thought that Ink4 family members were only involved in inhibiting CDK4. However, a second INK4A gene product has been identified recently, p19 ^{ARF}, which binds to and inactivates the p53 regulatory protein, MDM2, thus allowing increased p53 stability and subsequent cell cycle arrest (Pomerantz, J. et al, 1998).

Abnormalities in many positive and negative modulators of the cell cycle are frequent in most cancer types. As a result, cancer cells possess increased and deregulated proliferative activity. In normal cells, cell cycle regulators are subject to strict control and their activities fluctuate according to external stimuli (growth factors), whereas in tumor cells a variable degree of independence from such stimuli seems to emerge.

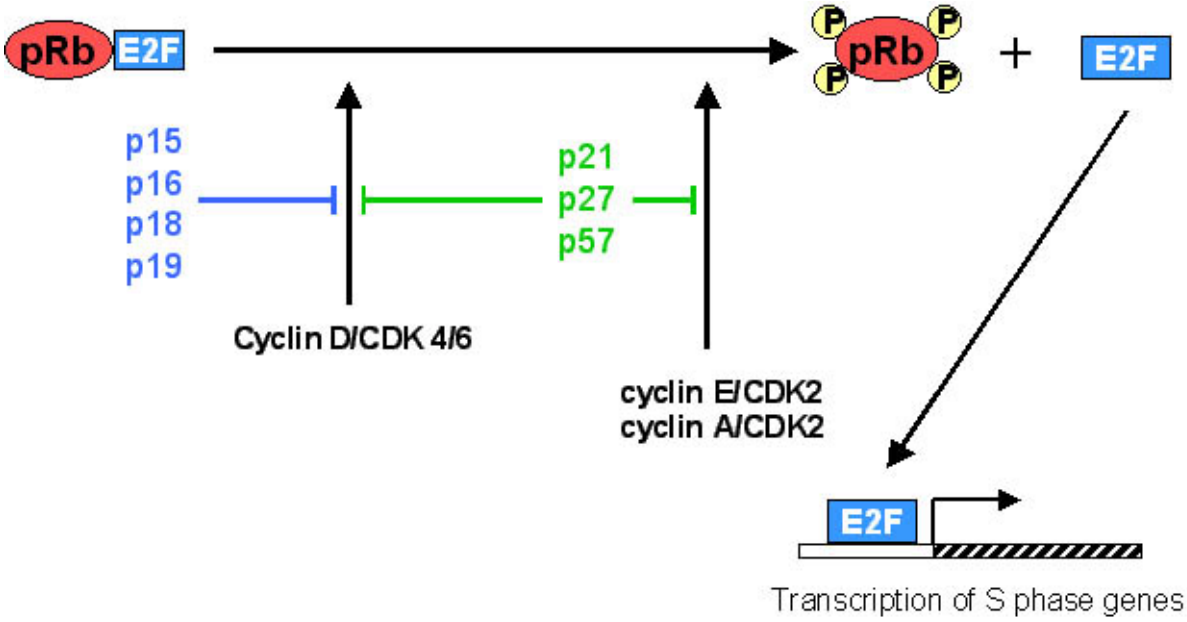


Figure 2. Schematic representation of pRb phosphorylation by cyclin/CDK complexes and E2F release during G1 to S progression.

Members of the Ink and Cip/Kip families of cyclin-dependent kinase inhibitors (CKI) are shown in blue and green, respectively.

2.2. G1 to S phase progression in MCF-7 human breast cancer cells

The goal of these studies was to understand the molecular mechanisms of *c-myc* gene regulation by quinidine in human breast cancer cells. A brief description of the cell cycle in MCF-7 cells, with the emphasis on G1-S progression in this cell line is provided. Most of the data described in this section were obtained in Dr. Strobl's and Dr. Wonderlin's laboratories.

A schematic diagram of G1 to S progression, the temporal relationship between two different G1 control points, and the levels of cell cycle regulatory proteins in MCF-7 cells is shown in Figure 3. The doubling time of MCF-7 cells was calculated by counting cell numbers in a cell population engaged in exponential growth. The doubling time was 52 hours. The fraction of cells in G1, S , and G2+M phase was calculated using flow cytometry analysis and immunohistochemical staining of Ki-67 antigen (Shiyi Wang, et al, 1998). Flow cytometry allows one to calculate the percentage of cells in G1+G0, S, and G2+M phases, based on their DNA content, but it does not discriminate between cells in G1 and G0 phase. Ki-67 is the nuclear antigen, that is present only in cycling cells (G1, S, G2, and M phases) and is absent in G0 cells, allowing for distinction of cells in G0. Based on the cell doubling time and cell fraction in each cell cycle phase, the duration of G1, S, and G2+M was estimated to be 32, 15 and 5 hours, respectively (Shiyi Wang, et al, 1998).

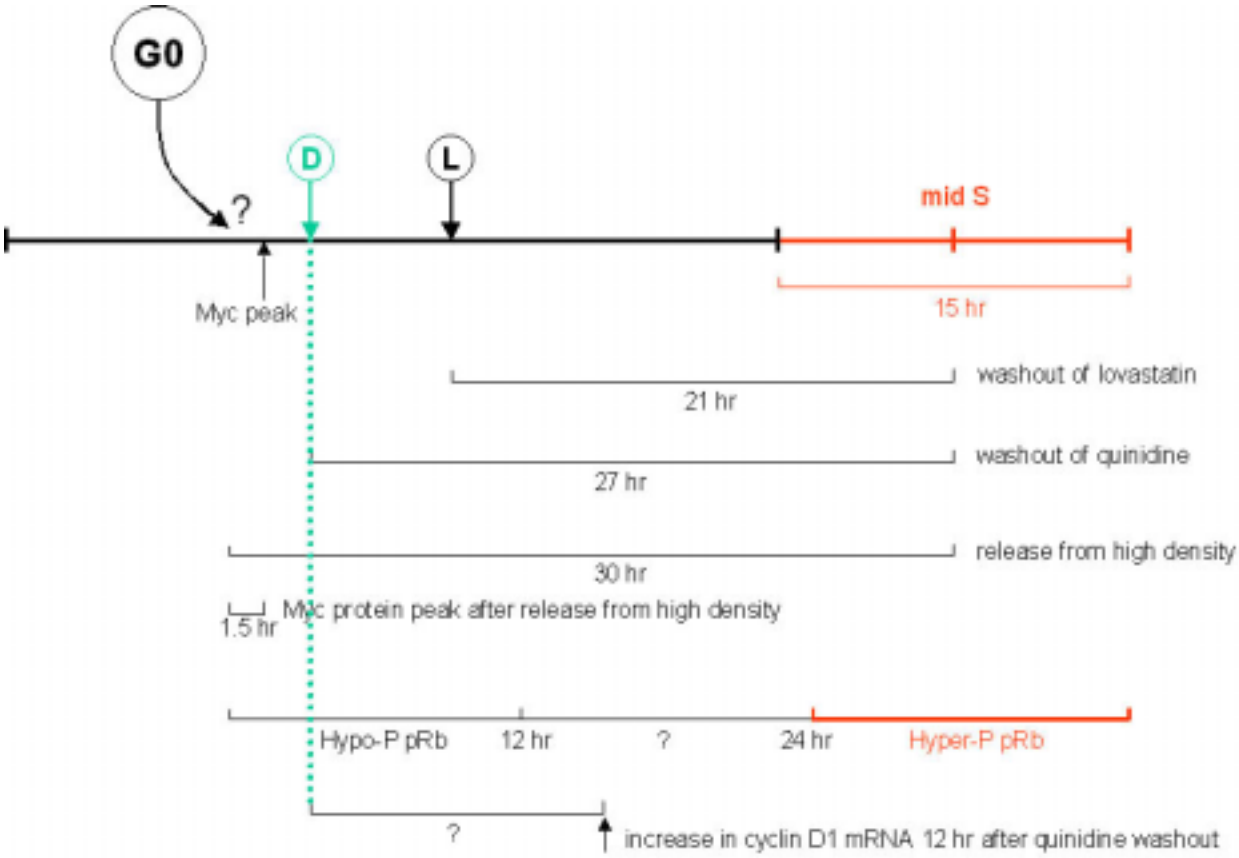


Figure 3. Temporal relationship between different regulatory events during the G1 to S phase transition in MCF-7 human breast cancer cells.

0he duration of the G1 and S phases is shown in black and red solid lines, respectively. The time required for the cells to reach mid-S phase, after washout of lovastatin (21hr) or quinidine (27 hr), or after release of cells from a high cell density condition (30 hr) is shown with black brackets. The peak of Myc protein occurs 1.5 hr after release from the high density state as shown by the arrow. The phosphorylation status of pRb is shown in black (hypophosphorylation) and red (hyperphosphorylation), respectively. D-quinidine arrest point, L - lovastatin arrest point, G0 - G0 state. Based upon data published in Woodfork, K.A. et al, 1995; Strobl, J.S. et al, 1995; Wang, S. et al, 1998; Zhou, Q. et al, 2000; Wang, S. thesis, 1996; Melkoumian, Z. and Strobl, J., 1999.

Over the past several years, our laboratory has tried to define the mechanism, by which the potassium channel blocking agent, quinidine, inhibits proliferation in human breast cancer cells. Studies by Woodfork, K. showed that quinidine caused arrest of MCF-7 cells in G1/G0 phase of the cell cycle (Woodfork, K.A. et al, 1995; Strobl, J.S. et al, 1995). This quinidine arrest point was called the depolarization control point or D point. The important role of membrane potential and its changes by quinidine in regulation of cell progression through the D point are discussed later in the introduction. Shiyi Wang estimated the location of the D point by comparing the time course of synchronous S phase re-entry after drug washout form MCF-7 cells arrested in G1/G0 phase by quinidine or known G1 arresting drug, lovastatin (Shiyi Wang et al, 1998). Cells arrested by lovastatin and quinidine peaked in mid S phase 21 and 27 hours, respectively after the drug washout. Accordingly, the D-point was designated prior to the lovastatin arrest point and 19.5 hours preceding the G1/S border. In general, MCF-7 cells growing in tissue culture reach the high density/confluency state around 5-6 days after passaging. At this state the cell monolayer covers about 90% of the growth surface and most of the cells are in G1/G0 (Wang, S., et al, 1998). The flow cytometry analysis showed that MCF-7 cells released from the high density state peak in mid S phase 30 hours later, suggesting that the high density state is located 22.5 hours away from G1/S border. At this stage we cannot clearly define the entry location of G0 into G1 phase. If most of the high density cells represent a G0 population, then the G0 to G1 entry point is located 22.5 preceding the G1/S border. In our results, a transient spike of c-Myc protein expression occurs after release of MCF-7 cells from the high density, with its peak at 90 min (Melkoumian, Z. and Strobl, J., 1999), 1.5 hours prior to the D point. *c-myc* is an early response gene, whose rapid induction is essential for the cells to progress from G0 to G1 and

progress through early G1 phase of the cell cycle. Inhibition of *c-myc* using antisense approaches causes arrest of MCF-7 cells in a G1/G0 state (Watson, P.H. et al, 1991). c-Myc protein acts as a transcription factor to activate expression of so called delayed-response genes, such as cyclin D1, CDK4, cdc25A, whose products are necessary for G1 to S progression.

In our model, quinidine causes rapid suppression of *c-myc* gene expression, which prevents progression of cells through the G1 phase, perhaps due to preclusion of Myc-dependent events later in G1. Myc-dependent events may include the synthesis of delayed-response genes, listed above, as well as others, whose presence is essential for progression of the cells through the D point. As a result, we hypothesize, cells are arrested in the D point. Washout of the quinidine would allow for Myc-dependent transcription to occur, and cell progression through G1 to S phase. In fact, Shiyi Wang showed a significant increase in cyclin D1 mRNA levels 12 hours after quinidine washout (Shiyi Wang, thesis, 1996). Unfortunately, she did not examine the earlier time points, to establish how soon after quinidine washout cyclin D1 mRNA levels begin to rise. In accordance with this increase in cyclin D1 mRNA, Zhou, Q. showed an accumulation of hyperphosphorylated form of pRb 24 hours after the release of cells from high density, although he did not test pRb phosphorylation status between 12 and 24 hours (Zhou, Q. et al, 2000). Collectively, these data are consistent with a model, where an early induction of *c-myc* gene during G0 to G1 and early G1 progression allows for the synthesis of delayed cell cycle regulatory proteins, that are essential for the progression through the D point. Inhibition of this *c-myc* induction by quinidine prevents transcription of *c-myc* regulatory genes and consequently, cells become arrested at the D point.

3. *c-myc* Gene

3.1. *c-myc* is a protooncogene

A major contribution towards understanding the mechanisms of tumorigenesis has been the discovery of protooncogenes and tumor suppressor genes. *Protooncogenes* or *c-oncogenes* (cellular homologues of oncogenes) are genes that are normally involved in the regulation of cell proliferation and can lead to the cancer when they are abnormally expressed in the cell. A mutated, cancer-causing version of a protooncogene is called an *oncogene*. Oncogenes were first isolated as part of small genomes of RNA tumor viruses, called acute transforming retroviruses (Bishop, J.M., 1983). These viruses efficiently transform cells in culture and in animals. It was then found that oncogenes carried by those viruses were responsible for their transforming capabilities. Originally, oncogenes were thought to be an innate part of the viral genome. Only later, when similar, but nonidentical, copies of the oncogenes were found in normal vertebrate cells, did it become clear that viral oncogenes arose as a consequence of genetic recombination between retroviruses and protooncogenes (Bishop, J.M., 1983). Placement of those protooncogenes in the path of strong viral promoters or their oncogene-converting mutation within the viral genome would cause continuous, uncontrolled activation of their normal functions in infected cells. Since most protooncogenes are involved in the regulation of some aspect of cell proliferation, their uncontrolled expression would lead to cellular transformation. To date, more than 70 oncogenes have been identified. *Tumor suppressor genes* are genes that negatively regulate cell proliferation and the loss of their function by mutation can contribute to the development of cancer.

The *myc* gene was originally identified as the oncogene v-*myc* (viral *myc*) of the MC29 avian myelocytomatosis virus (AMV) (Sheiness, D. et al, 1978, Sheiness, D. and Bishop, J., 1979). This retrovirus induces carcinomas, endotheliomas, sarcomas, and the leukemic disorder myelocytomatosis (hence *myc*) in susceptible birds. They transform fibroblasts and macrophages in tissue culture (Graf, T. and Beug, H., 1978). The *c-myc* gene was first isolated as the chicken cellular homologue of v-*myc* (Vennstrom, B. et al, 1982). Subsequently the human, mouse, and rat *c-myc* genes were cloned and characterized (Dalla-Favera, R. et al, 1982; Stanton, L. et al, 1984, Hayashi, K. et al, 1987). It was soon recognized that activated, oncogenic *c-myc* is a key transforming agent in the etiology of human Burkitt's lymphoma (Dalla-Favera, R. et al, 1982; Varmus, H.E., 1984). Altered *c-myc* expression has been also shown in a wide variety of human tumors including breast, colon, and cervical carcinomas, small cell lung carcinomas, osteosarcomas, glioblastomas, and myeloid leukemias (Marcu, K., et al, 1992; Spencer, C., et al, 1991). Soon after the discovery of the *c-myc* gene, two other genes N-*myc* and L-*myc* were identified as amplified *myc*-related genes in human neuroblastoma and small cell lung carcinoma, respectively. The *myc* family of oncogenes currently consists of three well-characterized members: *c-myc*, N-*myc*, L-*myc* and two additional genes, B-*myc* and S-*myc*, that have been identified only in rodents. The c-, N-, and L-*myc* genes share similar genomic organization and the corresponding proteins contain several regions of high sequence homology. The genomic structures of both S- and B-*myc* have diverged significantly from the other members of the family and their promoters are not well characterized at present.

c-Myc and cell transformation Regulated *c-myc* gene expression is critical for controlled cell proliferation, whereas deregulated expression of *c-myc* is a characteristic of tumor-

derived cells. Genetic alterations leading to oncogenic activation of *c-myc* include gene amplification (Favera, D. et al, 1982), chromosomal translocation (Magrath, I., 1990), proviral insertion (Payne, G. et al, 1982), and retroviral transduction (Fulton, R. et al, 1987). Also, changes in Myc protein levels by point mutation in *c-myc* coding sequence have been reported in several tumors (Bhatia, K et al, 1993). Alterations in *c-myc* expression have been demonstrated in many types of cancer, including Burkitt's lymphoma, myeloid and plasma cell leukemia, breast carcinoma, cervical carcinoma, small cell lung carcinoma, colon carcinoma, osteosarcoma, and glioblastoma (Cole, M.D. et al, 1986; Spencer, C. and Groudine, M., 1991; DePinho, R. et al, 1991). More direct evidence for the role of *c-myc* in abnormal cell growth comes from studies of transformed cells and transgenic animal models. Myc is capable of transforming fibroblast cell lines in vitro (Small, M. et al, 1987; Stone, J. et al, 1987) and established cells expressing activated *c-myc* form tumors in animals (Keath, E.J., et al, 1984). Transgenic animal models have demonstrated convincingly that deregulated expression of Myc induces tumorigenesis (Henriksson, M. and Luscher, B., 1996). Two groups created transgenic mouse strains harboring *c-myc* genes linked to either the mouse mammary tumor virus (MMTV) long terminal repeat (Stewart, T.A. et al, 1984) or the immunoglobulin heavy-chain enhancer (Adams, J.M. et al, 1985). In the first group, the mice developed mammary adenocarcinomas at high frequency, but only during the second or third pregnancy. In the second group, virtually all animals developed B-cell lymphomas at six-to-eight weeks of age, apparently due to the tissues-specific expression of the oncogene. These data suggest an important role of *c-myc* in tumorigenesis. However, the mechanisms by which c-Myc cause transformation remains unknown, although it has been shown that c-Myc transactivates promoters of several growth promoting genes.

The goal of these studies was to understand the molecular mechanisms of *c-myc* gene regulation by quinidine in human breast cancer cells. The following sections of the introduction focus on a review of structure, functions, and regulation of the *c-myc* gene.

3.2. *c-myc* gene organization

The human *c-myc* gene has been mapped to the 8q24 locus of the chromosome 8 (Taub, R. et al, 1982). It consists of three exons: an untranslated first exon (exon I) and two protein coding exons (exons II and III). A schematic diagram of the *c-myc* gene organization is shown in Figure 4. The *c-myc* gene has 2 major promoters P1 and P2 located 161 nucleotides apart in exon I. Transcription initiated at P1 and P2 produces 2.4 and 2.2 kb *c-myc* transcripts, respectively. These transcripts account for 95% of total *c-myc* mRNA. Two other promoters: P0, located 500 bp upstream of P1 and P3, located in the intron I give rise to less then 5% of *c-myc* mRNA each. Both P1 and P2 contain a consensus TATA box element. A strong consensus initiator element (Inr) occurs only at the P2 transcription start site. A number of different regulatory sequences have been described in the 5' upstream promoter region, in the 5' and 3' untranslated regions, and within intron I. Regulation of *c-myc* expression is highly responsive to environmental signals. Some of these regulatory domains are shown in Figure 4 and their role in *c-myc* gene regulation will be discussed in the later section of the introduction.

There is only one open reading frame (ORF) in all *c-myc* mRNA species. It starts at the canonical AUG initiation codon located at the 5' end of exon II and codes for the major 64 kDa Myc protein observed in vivo (Hann, S. and Eisenman, R. 1984; Ramsay, G. et al,

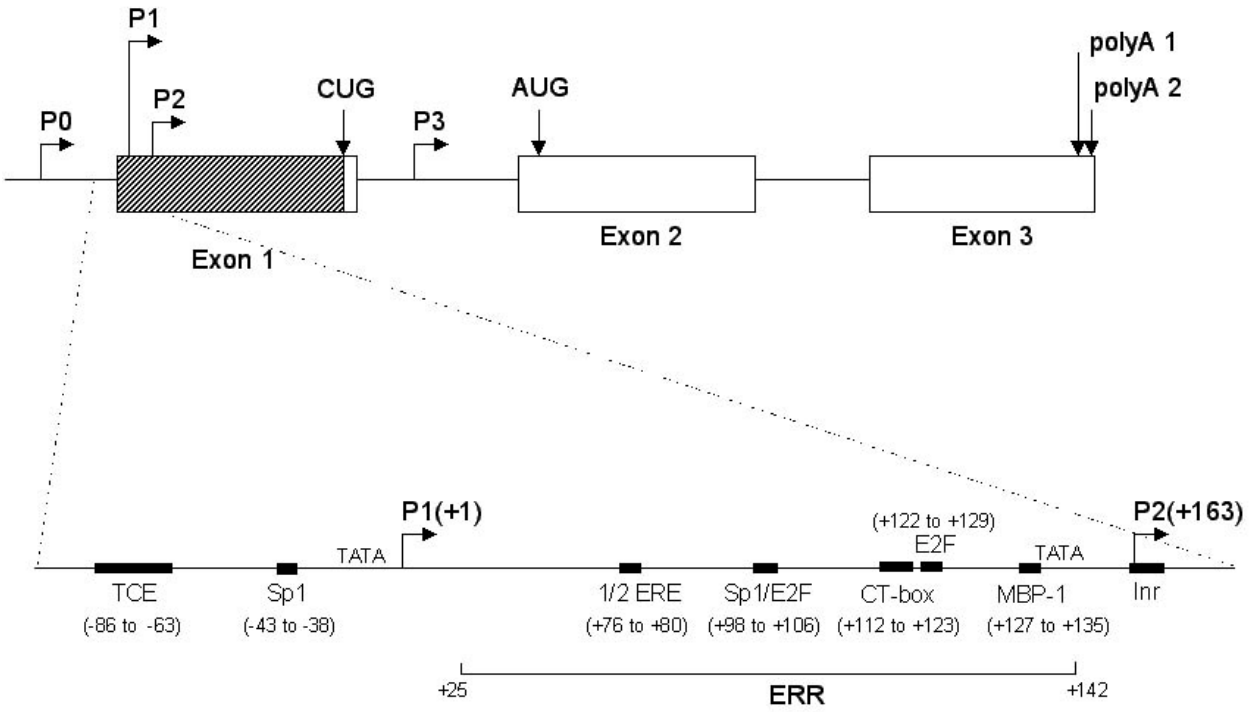


Figure 4. *c-myc* gene organization.

Exons 1,2, and 3 are represented by open rectangles. Shaded region shows the untranslated portion of the exon 1. Transcription start sites, P0, P1, P2, and P3 are shown by arrows. A more detailed diagram of the P1 and P2 promoter regions is shown in the lower portion of the figure. Positions of several regulatory regions in respect to the start site for P1 promoter are shown in black rectangles. CUG, AUG- translation initiation sites, polyA1 and 2- polyadenylation sites, TATA = TATA boxes, TCE = TGFβ1 control element, Sp1 and E2F = Sp1 and E2F binding sites, ½ ERE = half site of canonical estrogen responsive element, CT-box = CT-rich element, MBP-1 = binding site for the *c-myc* promoter binding protein-1, ERR = estrogen responsive region, Inr = initiator element.

1984). A minor protein species of 67 kDa is also observed. The translation of 67 kDa Myc protein begins at a cryptic start codon CUG located at the 3' end of exon I in frame with the previous ORF. The major translation product, p64, consists of 439 amino acids, p67 contains an additional 14 amino acids at the N-terminus of the p64 sequence (Hann, S. et al, 1988). Both products of *c-myc* gene (p64 and p67) are highly conserved nuclear phosphoproteins with a half-life of about 20-30 min (Luscher, B and Eisenman, R., 1990). The relative abundance of p67 versus p64, also known as Myc 1 and Myc 2, varies among tissues and cell lines, with the p64 species being the major isoform in most cases. A third isoform of c-Myc protein, termed Myc-S (short), has been described recently (Spotts, G.D.1997). This protein originates from the translation initiation site approximately 100 amino acids downstream of the N-terminus and lacks Myc transcription activation activity.

3.3. c-Myc protein structure

Both Myc proteins (p64 and p67) contain several structural domains characteristic of the transcription factors. The first 143 amino acids of the amino terminus comprise the transactivation domain (TAD) that contains two highly conserved Myc boxes (Mb): Mb I (amino acids 45 to 63) and Mb II (amino acids 129 to 141). The later is essential for all known Myc biological activities and is conserved among all Myc family members. Myc also contains two nuclear localization sequences (NLS): a primary motif at amino acids 320- 328 and a secondary signal from 364-374. The C-terminus of Myc contains three important domains: 1) the basic region (BR) (amino acids 355-368) implicated in specific DNA sequence recognition and binding, 2) the helix-loop-helix (HLH) (amino acids 368-410) and 3) the leucine zipper (LZ) (amino acids 411-439) responsible for specific heterodimer

formation between Myc and its binding partner Max (*c-myc* associated protein x). Contiguous BR-HLH-LZ motifs are characteristic of transcription factors that bind to specific DNA sequences.

Myc proteins can be phosphorylated at multiple sites by casein kinase II (CKII), mitogen activated protein kinase (MAPK), p34cdc2 kinase, and glycogen synthase kinase 3 (Alvarez, E. et al., 1991; Henriksson, M et al, 1993; Pulverer, B. et al, 1994; Seth, A. et al, 1992). Of these sites, Thr 58 and Ser 62 appear to be the most functionally relevant, although there are conflicting data published about the role of Thr 58/Ser62 phosphorylation in Myc transcriptional activity. More investigations are required to clarify the importance of phosphorylation in regulation of Myc functions.

3.4. Myc functions: Regulation of the cell cycle, cellular differentiation, and apoptosis

As has been discussed earlier, *c-myc* gene product belongs to the family of transcription factors that bind as dimers to the specific DNA sequences on the target genes. Our understanding of molecular functions of c-Myc has significantly advanced with the discovery of the c-Myc dimerization partner Max. Max was originally identified by screening a human cDNA expression library with a radiolabeled fusion protein containing the c-Myc carboxy terminus (Blackwood, E. and Eisenman, R., 1991). Similarly to c-Myc, Max contains contiguous BR-HLH-LZ motifs, and can form heterodimers with c-Myc, N-Myc, L-Myc, members of MAD family of proteins, as well as homodimers with itself. All these complexes bind to the same E-box sequences, CACGTG and CACATG, as well as to several low-affinity, noncanonical DNA sequences (Prendergast, G.C. et al., 1991; Blackwell, T.K. et al, 1990; Blackwell, T. K., et al, 1993). Binding of Myc-Max heterodimers to the E-box

sequences causes transactivation of Myc target genes in most cases, whereas Max-Max or Max-Mad dimers, lacking transactivating function, may block the biological effects of Myc-Max dimers by competitive occupancy of the binding sites.

c-Myc and cell cycle. In general, *c-myc* expression is correlated with the proliferative potential of the cell. Cells constitutively expressing high levels of c-Myc have reduced growth factor requirements (Daczmarek, L. et al, 1985; Sorrentino, V. et al, 1986; Stern, D. et al, 1986), spend less time in G1 phase (Karn, J. et al, 1989), and cannot become quiescent (Kohl, N. and Ruley, H. 1987). In quiescent cells, *c-myc* expression is almost undetectable. Upon mitogenic stimulation there is a rapid and transient burst in both *c-myc* mRNA and c-Myc protein expression as cells enter the G1 phase, followed by a gradual decline to low but detectable steady-state levels in proliferating cells (Campisi, J. et al, 1984; Kelly, K. et al, 1983; Moore, J. et al, 1987). Since this rapid induction of *c-myc* transcription occurs independently of de novo protein synthesis, it was suggested that *c-myc* is an immediate response gene, such as *c-fos* and *c-jun,* directly downstream of mitogenic signaling cascades. A number of studies demonstrated that sharp induction of *c-myc* expression following serum stimulation of quiescent cells is a prerequisite for normal transition from G0 to G1 and G1 to S phase in nontransformed cells (Eilers, M. et al, 1989; Heikkila, R. et al, 1987; Shichiri, M. et al, 1993). Whereas c-Myc plays a key role during the G0/G1 to S phase transition, continuous low-level *c-myc* expression throughout the cell cycle suggests its importance in other phases. Withdrawal of growth factors or treatment with differentiation-inducing agents causes *c-myc* mRNA and protein expression to decrease to almost undetectable levels. In contrast to c-Myc, expression of its partner, Max, is expressed at a constant low levels throughout the cell cycle. Max has a half-life of 24 hours (Blackwood, E., Lucher, B., 1992),

which implies that changes in Myc levels during the cell cycle play an important role in the regulation of Myc-Max heterodimers. A schematic diagram of the expression levels of c-Myc, Max, and Mad family of proteins during the cell cycle is shown in Figure 5.

c-Myc and cell differentiation c-Myc also plays an important role in cellular differentiation. It has been shown in many cells that down-regulation of c-Myc expression accompanies terminal differentiation and permanent withdrawal from the cell cycle. Blocking c-Myc activity using antisense oligonucleotides, ectopic expression of Max, or expression of dominant negative *c-myc* gene can induce differentiation of HL-60 (promyelocytic leukemia), F9 (teratocarcinoma), K562, and MEL (murine erythroleukemia) cells (Canelles, M. et al, 1997; Griep, A and Westphal, H., 1988; Holt, J. e al, 1988; Prochownik, E. et al, 1988). Conversely, independent studies showed that ecotpic c-Myc expression from a transfected gene is sufficient to block the induction of differentiation in MEL, 3T3-L1 preadipocyte, F9, PC12 neuronal, and U-937 monoblastic cell lines (Coppola, J.A., and Cole, M.D., 1986; Dmitrovsky, E. et al, 1986; Freytag, S., 1988; Larsson, L., et al, 1988; Maruyama, K. et al, 1987, Onclercq, R. et al, 1989; Prochownik, E. and Kukowska, J., 1986). These data suggest that suppression of c-Myc may be necessary for differentiation of certain cell types, perhaps because withdrawal from the cell cycle by c-Myc down-regulation is essential for terminal differentiation.

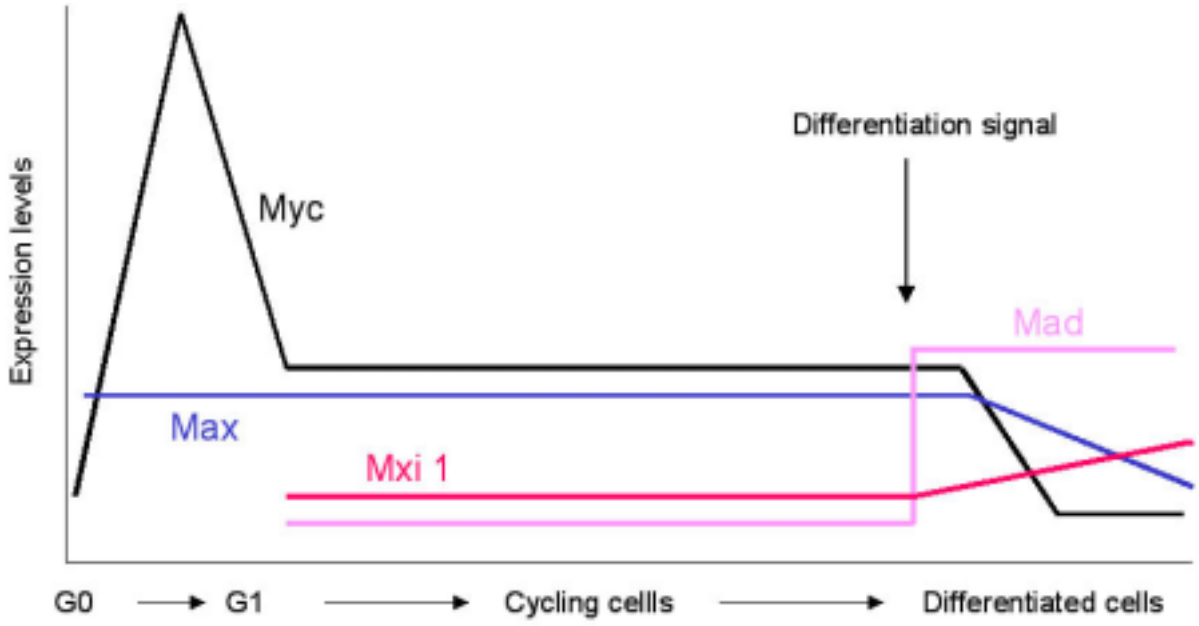


Figure 5. Expression pattern of Myc family proteins in cycling and differentiated cells.

Expression levels of proteins are shown in solid lines of different colors. Myc/Max dimers bind to the E-box sequence and transactivate Myc-target genes. Max/Max, Max/Mad, Max/Mxi1 dimers bind to the same E-box sequence, but due to the lack of transactivation domain, block the biological effects of Myc/Max dimers by competitive occupancy of their binding sites.
Henriksson, M. and Luscher, B., 1996.

c-Myc and apoptosis. A most paradoxical discovery from the work on *c-myc* was the finding that under certain circumstances c-Myc is able to induce apoptosis or programmed cell death. Separate studies in fibroblasts (Evan, G.I. et al, 1992) and in haemopoietic cells (Askew, D.S. et al, 1991), revealed that constitutive expression of the Myc required the presence of growth/survival factors in order for cells to proliferate. Withdrawal of serum from fibroblasts or removal of interleukin 3 from haemopoietic cells expression Myc caused cells to undergo apoptosis. The mechanisms of Myc-induced apoptosis are poorly understood. Several genes involved in the regulation of apoptosis, such as p53, Bax, and p21 contain c-Myc responsive regions in their promoters, but the role of these genes in Myc-induced apoptosis remains unclear. The pro-apoptotic function of c-Myc resembles that of several other oncogenes, such as E2F and E1A and might represent a safeguard mechanism important during organism development. For example, increased levels of c-Myc during development would allow for the proliferation of only those cells that receive the appropriate survival signals. Otherwise, a Myc over-expressing cell, representing a potential danger to the organism, would be eliminated by apoptosis. It has been hypothesized that *c-myc* activation may facilitate tumorigenesis in cells in which a survival pathway was already activated by mutation.

In summary, these data suggest a critical role of *c-myc* gene in the regulation of the cell cycle progression, cellular differentiation and apoptosis. Deregulated expression of *c-myc* in a variety of tumors, the ability of exogenously expressed *c-myc* to transform established cell lines in vitro and induce tumors formation in transgenic animals indicate an important role of *c-myc* gene in tumorigenesis. Although the molecular mechanisms leading to deregulated expression of *c-myc* gene in tumors are well understood, the precise cellular

function of the c-Myc protein has remained an enigma, despite continuous efforts for more then a decade to identify these functions. Some insight on these functions has been brought with the identification of *c-myc* target genes, which are discussed in the following section.

3.5. Myc target genes

The role of Myc protein as a transcription factor was proposed long before any of its target genes were identified, on the basis of certain structural features of the Myc protein. The contiguous BR-HLH-LZ motifs in Myc are characteristic of a large class of transcription factors, including AP4, the upstream stimulatory factor (USF) and the immunoglobulin-enhancer, μE3-binding proteins (Johnson, P. and McKnight, S., 1989; Beckmann, H. et al, 1990; Carr, C. and Sharp, P., 1990; Gregor, P. et al, 1990).

The following criteria have been used to identify Myc target genes:

1. Presence of functional Myc-Max binding sites within the putative target gene.
2. Pattern of gene expression correlates with that of the Myc during transition from quiescence to proliferation and/or differentiation.
3. Modulation of candidate gene's mRNA levels upon induction of Myc, or activation of conditional Myc-ER fusion protein. In the latter case, the changes in mRNA should be independent of de novo protein synthesis.
4. Functional role of the candidate target gene in c-Myc-mediated cell transformation and/or proliferation.
5. Identification of DNA sequences physically associated with c-Myc in chromatin preparations.

A partial list of Myc target genes is shown in the Table 1. Products of some of these genes function as key cell cycle regulatory proteins (cyclin D1, cyclin A, CDK4, p15Ink4b, p21WAF1, cdc25A phosphatase, gadd 45). Others are involved in the regulation of cell growth and metabolism (DHFR, LDH-A, CAD, ODC), transcription process (E2F2, eIF-2α, eIF-4E), or apoptosis (p19 ARF, p53, p21WAF1). The data on cyclin D1 is very controversial, with some reports showing up-, others down-regulation of cyclin D1 expression by c-Myc (Daksis, J.I. et al, 1994; Philipp, A. et al, 1994). Myc can also negatively regulate its own expression (autoregulation) via the Initiator element located within P2 promoter. In general, transcriptional activation of Myc-target genes by Myc-Max heterodimers occurs via the E-box elements, whereas the Initiator element is responsible for Myc-induced transcriptional repression. Both mechanisms are important for Myc-mediated cellular transformation (Dang, C.V. et al, 1999).

Table 1. c-Myc target genes

Gene	Myc effect	Technique	Cellular function
CAD	Up	E-box (5'untranslated)	Pyrimidine biosynthesis
p19 ARF	Up	Guess	Apoptosis
p15Ink4b	Down	Initiator (P2 promoter)	Cell cycle regulation
p21WAF1/CIP1	Down	Promoter	Cell cycle regulation
cdc25A	Up	Guess/E-box (intron II)	Cell cycle regulation
Cyclin A	Up	Guess	Cell cycle regulation
Cyclin D1	Up/ Down	E-box (promoter)/differ and Initiator	Cell cycle regulation
CDK4	Up	E-box (promoter)	Cell cycle regulation
DHFR	Up	E-box (promoter)	Growth and metabolism
E2F2	Up	E-box (promoter)	Transcription factor
eIF-2α	Up	Guess	Transcription initiation factor
eIF-4E	Up	Guess/ E-box (promoter)	Transcription initiation factor
p53	Up	Differ/E-box (exon I)	Tumor suppressor gene
Telomerase/ hTERT	Up	Guess/E-box (promoter)	Immortality
gadd 45	Down	Differ	Cell cycle regulation
Thrombospondin-1	Down	E-box (promoter)	Cell adhesion/metastasis
LDH-A	Up	E-box (promoter)	Metabolic enzyme

ODC	Up	E-box (intron I)	Polyamine biosynthesis
c-myc	Down	Initiator (P2 promoter)	Transcription factor

Techniques used to identify putative c-Myc genes:

Guess - expression of target gene correlates with *c-myc* expression during transition from quiescence to proliferation and/or differentiation. Functional importance for Myc-mediated phenotypes.
Differ - differentially expressed genes upon enforced expression of c-Myc in the absence of de novo protein synthesis.
E-box - presence of functional Myc/Max binding sites in the promoter or other region of the candidate gene.
Initiator - presence of functional initiator element in the promoter of the candidate gene.
Up and Down indicate effect of c-Myc on the expression levels of the gene. CAD-carbamoyl phosphate synthase, ODC - ornithine decarboxylase, LDH- lactate dehydrogenase, DGFR - dihydrofolate reductase, eIF-4E and eIF-2α - eukaryotic initiation factors.
Reviewed by Dang, C.V. et al, 1999; Galaktionov, K. et al, 1996; Philipp, A. et al, 1994; Jones, R.M. et al, 1996; Reisman, D. et al, 1993; Shim, H. et al, 1997; Belo-Fernandez, C. et al, 1993; Facchini, L.M. et al, 1997; Jansen-Durr et al, 1993; Daksis et al, 1994; Hermeking, H. et al, 2000; Gartel, A.L. et al, 2001; Staller, P. et al, 2001.

3.6. Role of c-Myc in breast tumorigenesis

c-Myc is one of the most common oncogene aberrations in breast cancer (Spaventi, R. et al, 1993; Berns, E. et al, 1992; Garcia, I. et al, 1989; Escot, C. et al, 1986). According to one study, *c-myc* amplification is a better prognostic marker than is c-erbB-2 (Berns, E. et al, 1992). In human breast cancers, *c-myc* is amplified in approximately 16%, rearranged in approximately 5%, and over-expressed in the absence of gross locus alteration in nearly 70 % of all cases, suggesting its importance in the genesis and/or progression of breast cancer (Nass, S.J. and Dickson, R.B, 1997; Deming, S.L. et al, 1999).

3.6.1. Experimental evidence for the role of c-myc in breast tumorigenesis in breast cell lines models

Constitutive expression of *c-myc* has been shown to partially transform both mouse and human mammary epithelial cells (MECs), such that they grow in soft agar (anchorage-independent growth) in response to epidermal growth factor (EGF) and transforming growth factor α (TGFα), and are no longer as dependent upon these growth factors for anchorage-dependent growth as are the parental, non-transformed cells (Telang, N.T. et al, 1990; Valverius, E.M. et al, 1990). Manipulation of the expression of *c-myc* gene in cell lines has confirmed that *c-myc* is critical for the growth of breast cancer cells (Watson, P.H. et al, 1991). In estrogen receptor positive, hormone-dependent breast cancer cells (MCF-7 and T47D), *c-myc* gene is directly regulated by estrogen (Dubik, D. and Shiu. R.P, 1988; Dubik, D. and Shiu, R.P., 1992). In vitro nuclear run-on transcription assays demonstrated estradiol-stimulation of *c-myc* gene transcription in MCF-7 cells, increasing *c-myc* mRNA transcription more than 10-fold within 20 min. Estradiol had no effect on the *c-myc* mRNA

half-life of about 20 min (Dubik, D. and Shiu, R.P., et al, 1988). Northern blot analysis showed a similar pattern. The maximum *c-myc* mRNA accumulation of 12-fold, was achieved one hour after addition of estradiol to MCF-7 cells. In a search for the mechanism of estrogen-mediated activation of *c-myc*, a 116-bp estrogen responsive region (ERR) was identified within the *c-myc* promoter (Dubik, D. and Shiu, R.P., 1992). This region contains half-site of consensus ERE as well as Sp1 transcription factor binding site (GC-rich element) (Figure 4). The presence of the GC-rich element may be important to estrogen-mediated regulation of the *c-myc* promoter, since activation of several estrogen-responsive genes by Sp1/ER complex as that bind to GC-rich element has been reported (Duan, R. et al, 1998; Sun, G. et al, 1998; Qin, C. et al, 1999). In ER-negative MDA-MB-231 human breast cancer cell line, *c-myc* is expressed at high constitutive levels and its expression is independent of estrogens (Dubik, D. et al, 1987). Antisense *c-myc* oligonucleotides inhibit both estrogen-dependent and estrogen-independent growth of MCF-7 and MDA-231 cells lines, respectively (Watson, P.H. et al, 1991). These studies in human breast cancer cell lines indicate that Myc is important for cell growth (Shiu, R.P. et al, 1993).

3.6.2. Experimental evidence for the role of c-myc in breast tumorigenesis in transgenic animal models

Three groups have independently developed transgenic mice that express the *c-myc* oncogene in a mammary associated (mouse mammary tumor virus, MMTV-*c-myc*) or mammary specific (whey acid protein gene promoter, WAP-*c-myc*) context (Stewart, T. et al, 1984; Schoenenberger, C. et al, 1988; Sandgren, E et al, 1995). WAP-*c-myc* transgenic mice, developed by both groups demonstrated a high incidence of mammary tumors:

adenocarcinomas in case of Schoenenberger's group or solid carcinomas in case of Sandgren's group. In the both laboratories, tumor incidence approached 100% in multiparous animals. In contrast, all virgin animals remained tumor-free over the fourteen months observation period. These findings are as expected, on the basis of the temporal window for the hormone-driven activity of the whey acid protein (WAP) gene promoter. The group, which developed MMTV-*c-myc* transgenic mice reported 100% incidence of mammary adenocarcinomas in multiparous animals (Stewart et al, 1984). Interestingly, WAP-*c-myc* and MMTV-*c-myc* transgenic mice displayed a lengthy tumor latency, and strong dependency upon pregnancy for tumor development. These data suggest that *c-myc* contributes, but in not sufficient to induce mammary tumorigenesis. Two bitransgenic mice models: MMTV-*c-myc*/MMTV-v-Ha-ras developed by Sinn's group (Sinn, E. et al, 1987) and MMTV-*c-myc*/MT-tgfα developed by Dickson's group (Amundadottir, L. et al, 1995; Sandgren, E. et al, 1995) demonstrated that deregulated *c-myc* expression synergized with deregulated v-Ha-ras or tgfα expression to both accelerate mammary tumorigenesis and abrogate the requirement for pregnancy in this process. Bitransgenic animals from the second group showed the complete absence of normal mammary tissue and the ability of bitransgenic mammary tissue from three-week-old mice to form tumors in athymic mice. These results suggest that *c-myc* and tgf α, two important mammary gland relevant genes, are capable of synergistically transforming the mammary epithelium, apparently requiring minimal, if any, additional genetic alterations (Amundadottir, L. et al, 1995 and 1996).

3.7. Regulation of *c-myc* gene expression

Expression of the *c-myc* gene is controlled by transcriptional initiation, transcriptional elongation and posttranscriptional processes.

3.7.1. Regulation of transcriptional initiation

c-Myc is transcribed from multiple, independently regulated transcription initiation sites. Two major promoters, P1 and P2, positioned 161 bp apart, give rise to 75-90% of *c-myc* mRNA. Two minor promoters, P0 and P3, lacking canonical TATA boxes give rise to less than 5% of *c-myc* mRNA each.

Studies of *c-myc* chromatin have revealed multiple DNase I hypersensitive sites (DH), some of which overlap with cis-acting *c-myc* regulatory elements. DNase I hypersensitive sites mark perturbations in the regular chromatin structure that are usually caused by the binding of regulatory proteins to DNA. The locations of DH sites, relative to P1 promoter and their putative roles in *c-myc* regulation are listed in the Table 2 (Spencer, C. and Groudine, M., 1991; Siebenlist, U. et al, 1984).

The DNA *cis-acting elements* are nucleotide sequences, usually located upstream of the gene coding region, which are recognized and bound by specific regulatory transcription

Table 2. Locations of DNase I hypersensitive (DH) sites in the *c-myc* promoter

DH site	**Location**	**Putative role**
DH I	-1851	negative repressor binding site
DH II_1	-1380	unknown function
DH II_2	-751	unknown function
DH III_1	-124	associated with P1 promoter activity
DH III_2	+91	associated with P2 promoter activity
DH IV	+800	correlates with the appearance of the block of transcriptional elongation in differentiated HL60 cells
DH V	+1800	unknown function

Locations are given in respect to P1 promoter.

factors (*trans-acting proteins*), thereby causing the gene to respond to various regulatory agents. A large body of work has been done to identify cis-acting elements of the *c-myc* promoter that are involved in regulation of transcriptional initiation. Most of what we know about *c-myc* regulatory elements has been identified by assaying the effects of promoter sequence deletions on promoter activity within transfected cells. In this deletion/transfection assay a series of *c-myc* deletion mutants linked to reporter genes, e.g. luciferase or chloramphenicol acetyl transferase (CAT), were transiently transfected into mouse or human cell lines and the relative promoter activities in different mutant constructs were analyzed. The nature of protein-DNA interactions in a putative responsive region were assayed by electrophoretic mobility shift assay (EMSA). Some of cis-acting elements identified within *c-myc* promoter employing the described methods are listed in the Table 3. The locations of the elements are given relative to the P1 promoter.

3.7.2. Regulation of transcriptional elongation

Regulation of gene expression at the level of transcriptional elongation occurs in both prokaryotic and eukaryotic systems. Transcriptional elongation mechanism plays an important role in regulation of a number of protooncogenes, including *c-myc*, L-*myc*, c-*myb*, c-fos, and c-mos. The region necessary for transcriptional blockage within c-*myc* has been defined by a number of studies using the in vitro run-on assay. Bentley and Groudine were the first to describe the *c-myc* transcriptional block phenomenon with their work on the human *c-myc* gene (Bentley, D. and Groudine, M., 1986). A 95 bp sequence ending 35 bp 5' of the exon1/intron1 boundary of the human *c-myc* gene was sufficient to effect premature transcriptional termination in Xenopus oocytes when cloned downstream of the HSV *tk*

promoter (Bentley, D and Groudine, M., 1988). In wild-type *c-myc* templates, transcripts terminate prematurely at two thymine-rich sequences: a T_7 stretch 20 bp upstream and a T_4/T_7 stretch 30 bp downstream of the exon I/intron I boundary, respectively. c-Myc transcription has been shown to be extremely pliable to various growth factors, mitogens and differentiation agents that may act by altering elongation. For example, a rapid and dramatic increase in *c-myc* transcriptional blockade was observed upon exposure of HL60 cells to retinoic acid (RA) (Asselin, C. et al, 1989), DMSO (Eick, D. and Bornkamm, G., 1986), or vitamin D (Simpson, R. et al, 1987). The intensity of DH IV site within intron 1 was shown to increase with block of transcriptional elongation. The 10-fold increase in the block of transcriptional elongation in HL60 cells treated with DMSO occurred within 30 min of treatment (Eick, D. and Bornkamm, G., 1986). Between 24 and 48 hours after treatment, the level of transcriptional initiation in exon I also declined. These experiments suggest that the block of transcriptional elongation can control *c-myc* mRNA levels during cellular differentiation. Also, an increase in *c-myc* steady-state mRNA, partially due to a release of the elongation block, occurs in quiescent normal peripheral blood T lymphocytes stimulated by phorbol myristate acetate (PMA) (Lindsten, T. et al, 1988).

In summary, these data suggest that regulation of *c-myc* transcription can be also achieved at the levels of transcriptional elongation in addition to initiation. It is possible that the abnormal regulation of *c-myc* expression that occurs in tumors may, in some cases, result from disruption of the block of transcriptional elongation process.

Table 3. Cis-acting elements or regions identified within *c-myc* gene promoter

Cis-acting element	**Description**
region from -60 to -37	Essential for P1 transcription (Nishikura, K., 1986). It contains a GC-rich sequence CCGCCC (-38 to -43) that binds Sp1 and Sp3 transcription factors (Majello, B. et al, 1995). Sp1 and Sp3 proteins belong to a multigene family whose members have similar, if not identical DNA binding activities (Hagen, G. et al, 1992). Both Sp1 and Sp3 bind to GC-rich and CT-rich boxes with comparable affinities (Majello, B. et al, 1995). In vivo transfection experiments have shown that Sp1-mediated transactivation of Sp1-responsive promoters are repressed by Sp3.
five tandem CT-boxes (CCCTCCCC) located 101 bp upstream P1	Required to promote transcription from P1 and for maximal activity of P2 (DesJardins, E. and Hay, N., 1993). Mutations of these CT-boxes decreases the absolute and relative activities of P1 and P2 promoters. CT-boxes were shown to bind Sp1/Sp3 (DesJardins, E. and Hay, N., 1993) as well as ZF87/MAZ transcription factors.
a single copy of the CT-box (CCTCCCTCCCT) in an inverted orientation located 53 bp upstream P2	This element has an inhibitory effect on P1 and is required for P2 transcription, respectively. It can bind both Sp1/Sp3 and MAZ transcription factors (DesJardins, E. and Hay, N., 1993).
two E2F binding sites, GATCGCGC (+122 to +129) and GCGGGAAA (+99 to +106)	Cooperate with Sp1 in transactivating *c-myc* P2 promoter (DesJardins, E. and Hay, N., 1993; Majello, B. et al, 1995).
a 116 bp estrogen responsive region (ERR) located between +25 and +142	Important for estradiol-induced activation of *c-myc* promoter in MCF-7 and HeLa cells (Dubik, D. and Shiu, R., 1992). This region contains half site of consensus estrogen responsive element (ERE) (+76 to +80) as well as Sp1/E2F (+98 to +106) binding sites, which can cooperate in transactivating estrogen-responsive promoters (Duan, R. et al, 1998; Sun, G. et al, 1998; Qin, C. et al, 1999).

transforming growth factor β1 (tgf β1) control element (TCE) located upstream P1 between -63 and -83	Inhibits *c-myc* P1 transcription initiation, possibly via the pRb gene product (Pietenpol, J. et al, 1991).
26 bp region (-343 to - 318)	Negative regulatory element. Containing binding site for Ap1 transcription factor (Takimoto, M. et al, 1989).
CGCTGAGTA region from +127 to +135, just 5'upstream of the P2 TATA box	Binds MBP-1 (*myc* promoter binding protein 1), and appears to be a negative regulator of *c-myc* transcription (Ray, R. and Miller, D., 1991).

The locations of the elements are given in respect to P1 promoter unless otherwise specified.

4. Role of *c-myc* in TGF-β Signaling

Transforming growth factor β (TGF-β) is a prototypic member of a large family of cytokines that plays an important role in a variety of biological processes, including cell division, differentiation, adhesion, movement, and death (Derynck, R. and Feng, X., 1997). TGF-β has a strong growth inhibitory effect on epithelial and other cell types. It is not surprising, that TGF-β signaling is lost in some cancers by inactivation of TGF-β signal transduction components (Massague, J. et al, 2000). There are three families of TGF-β receptors, type I (RI), II (RII), and type III (RIII) receptors. The RI and RII are transmembrane serine/threonine kinases with a single transmembrane domain. Free receptors exist in cell membrane as homodimers. Binding of the ligand (TGFβ-1, -2, or -3) to the type II receptor triggers formation of heteromeric type I-type II receptor complex, that propagates the TGF-β responses (reviewed in Derynck, R. and Feng, X., 1997). In the resulting complex, the type II receptor phosphorylates and activates the type I receptor. Activated type I receptor then phosphorylates the intracellular mediators of TGF-β signaling Smad2 or Smad3 proteins at SSXS motif present in their C-terminal domains. This, in turn, triggers the oligomerization of Smad2 or –3 with their common partner, Smad4 (Nakao, A. et al, 1997; Lagna, G. et al, 1996). The resulting Smad protein complexes then migrate to the nucleus, where they regulate the expression of TGF-β responsive genes. The RIII, also called proteoglycan, is the most abundant TGFβ-1 binding molecule on the cell surface. Type III receptor does not contain a consensus signaling motif, therefore the biological importance of RIII in vivo has not been defined. It has been proposed that RIII enhances TGFβ-1 binding to RII by directly presenting the ligand to RII (Lopez-Casillas, F. et al, 1993; Moustakas, A. et al, 1993). Expression of RIII restores autocrine TGFβ-1 activity in MCF-7 human breast

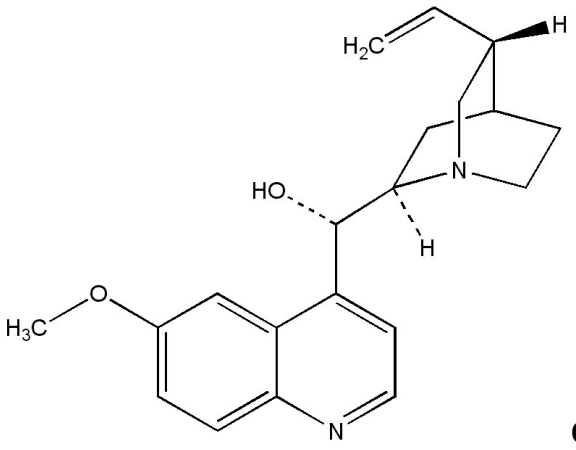
cancer cells (Chen, C. et al, 1997). In epithelial cells derived from the breast, skin, and lung, TGF-β rapidly elevates expression of the cell cycle inhibitory proteins p15Ink4b and $p21^{WAF1}$ (discussed in the section 2.1) (Sandhu, C. et al, 1997; Hannon, G. and Beach, D., 1994; Reynisdottir, J. et al, 1995; Claassen, G.F. and Hann, S.R., 2000). Interestingly, this response is suppressed by *c-myc* (Warner, B. et al, 1999; Massague, J. et al, 2000). *c-myc* itself is a target gene of TGF-β signaling. In response to TGF-β, *c-myc* is rapidly down-regulated in MCF-10A human mammary epithelial cells, HaCaT keratinocytes, and other epithelial cell types (Chen, C. et al, 2001). Two recent papers provided an important insight into the complex regulation of p15Ink4b by TGF-β and Myc (Seoane, J. et al, 2001; Staller, P. et al, 2001). Zinc-finger protein Miz-1, originally identified as a Myc-binding protein, plays the key role in that regulation. In the complex with Miz-1, Myc can bind to the initiator element of the p15Ink4b promoter and inhibit its transcriptional activation by preventing recruitment of the ubiquitous co-activator p300. Upon TGF-β treatment, Myc is rapidly down-regulated. This, in turn, allows for the interactions of Miz-1 with other transcription factors, such as Sp1, Smad3/4 on p15Ink4b promoter, which is proposed to generate a nucleoprotein complex that constitute a platform for the recruitment of co-activators, basal transcription factors, and RNA polymerase II. Hence, down-regulation of *c-myc* is required for TGF-β mediated induction of p15Ink4b. In fact, suppression of the *c-myc* promoter by TGF-β treatment through TGF-β control element (TCE) has been characterized (Pietenpol, J. et al, 1991). In accordance with these results, it was shown that the loss of *c-myc* down-regulation in mammary epithelial cells, MCF-10A, transformed with Ras/ErbB2 oncogenes and MDA-MB-231 human breast cancer cell line coincides with a loss of TGF-β

responsiveness of *c-myc* promoter (Chen, C. et al, 2000). In summary, these data suggest that down-regulation of *c-myc* is a necessary event for the induction of p15Ink4b gene by TGF-β.

5. Quinidine

Quinidine is a natural alkaloid found in the bark of the South American cinchona tree. Of the more than 20 alkaloids found in the cinchona bark only quinidine and its stereo-isomer quinine are now in use. The structure of quinidine is shown below. Quinidine is used as an antiarrhythmic drug that acts to suppress abnormal heart rhythm, particularly atrial fibrillation and flutter, paroxysmal atrial trachycardia, paroxysmal ventricular tachycardia, and premature atrial and ventricular contractions (Katzung, B.G., 1984). These actions probably result to a large extent from the quinidine's ability to block sodium channels (Craig, R.C. and Stitzel, R.E., 1994). Side effects include low blood pressure, skin rash, itching, dizziness, and blurred vision. Quinidine also has an antimalarial activity, but because of its superior absorption only quinine is used for the treatment of malaria. The exact mechanism by which quinidine and quinine exert their antimalarial activity is not known.

Our previous studies established quinidine as an antiproliferative agent in human breast cancer cell lines (Woodfork, K.A., 1995; Wang, S., 1998; Zhou, Q., 2000). The studies reported here focused on further characterization of molecular mechanisms underlying the antiproliferative effects of quinidine.



Quinidine

6. G0-G1-S Cell Cycle Regulation in MCF-7 Cells by Potassium Channel Blocking Drugs.

An important role of potassium channels in regulating cell proliferation has been reported in a number of cell lines (reviewed by Dubois, J. and Rouzaire-Dubois, B., 1993; Nilius, B. and Droogmans, G., 1994). Previous studies in Dr. Strobl's and Dr. Wonderlin's laboratories indicate that potassium channel blocking agent, quinidine causes G1/G0 cell cycle arrest and inhibition of proliferation in MCF-7 human breast cancer cell line (Woodfork, K. et al, 1995). Wodfork, K. et al, demonstrated that quinidine inhibits proliferation and causes G1/G0 arrest with the same dose dependence with which it depolarizes the membrane potential of MCF-7 cells. In addition, she identified the ATP- sensitive potassium channels (K_{ATP}) as the target of quinidine, since only the agents that inhibited K_{ATP} were able to produce G1/G0 cell cycle arrest and inhibition of proliferation (Woodfork, K., et al, 1995). The work by Klimatcheva, K. described a linear hyperpolarized (LH) potassium current, inhibited by quinidine, which is required for progression of MCF-7 cells through the G1 phase of the cell cycle. Interestingly, this current is overexpressed in MCF-7 cells transformed with Ha-ras oncogene (Klimatcheva, E. and Wonderlin, W., 1999). This notion is consistent with the reports by other investigators showing that expression or activity of K channels correlates with the degree of cell proliferation in several other cell types (Wang, L. et al, 1997; Nilius, B. and Wohlrab, W.,1992; Reichelt, W., 1989). Collectively, these studies support a model where hyperpolarization of the membrane potential by transient activation of the linear hyperpolarized current might be required for progression through the restriction point in G1 (D point) and entry into S phase. Accordingly, inhibition of LH current by blockade of K_{ATP} in response to quinidine, will prevent hyperpolarization of membrane

potential and cells will become arrested at the D point. In support to this model, a large body of evidence has shown that progression through the cell cycle is associated with changes in intracellular ionic concentrations, which can be regulated by the changes in the membrane potential (Whitaker, M. and Patel, R. 1990; Whitfield, J. et al, 1987). Intracellular ions, in turn, initiate signal transduction pathways that lead to the activation of proliferation. For example, increase in intracellular Ca^{2+} activates CREB transcription factor that stimulates transcription of growth promoting genes (Sheng, M. et al, 1991). Several immediate-response genes were shown to be induced by calcium agonists (reviewed in Roche, E. and Prentki, M, 1994). Treatment of human promyelocytic leukemia cells (HL-60) with the calcium ionophore A23187 caused an increase in *c-myc* mRNA levels to 170 % of control cells within an hour (Salehi, S., and Niedes, J., 1990). The putative intracellular calcium antagonist TMB-8 inhibited both *c-myc* expression and DNA synthesis in a dose-dependent manner in the Nb2 lymphoma cells (Murphy, P., 1988). It has also been shown that calcium influx is a very early signal transduction that precedes the specific induction of the *c-myc* proto-oncogene in lymphocytes (Liburdy, R., 1993).

The goal of studies presented here was to further understand the mechanism of G1/G0 cell cycle arrest and inhibition of cell proliferation in response to quinidine. The molecular mechanism of *c-myc* gene regulation by quinidine in human tumor and normal breast epithelial cell lines were studied. Effects of quinidine on c-Myc protein and mRNA levels, as well as *c-myc* promoter activity were examined. Furthermore, the long-term effects of quinidine in breast tumor cell lines were tested.

7. Research Objectives

Previous studies in this laboratory demonstrated that quinidine causes early G1/G0 arrest and inhibits proliferation in MCF-7 cells. The goal of these studies was to understand the molecular mechanisms of *c-myc* gene regulation by quinidine in human breast cancer cells.

1. In the first series of experiments I tested the hypothesis that quinidine causes rapid decrease of *c-myc* mRNA and protein levels during the G0/G1 transition in human breast cancer cells. Four different breast cancer cell lines (MCF-7, MCF-7ras, MDA-231, MDA-435) and normal breast epithelial cell line (MCF10A) were used.

2. Secondly, I tested the hypothesis that quinidine suppresses *c-myc* promoter activity. Effects of quinidine on *c-myc* promoter were examined in a transient reporter gene assay, using human *c-myc* promoter-luciferase constructs. Quinidine responsive region (QRR) of *c-myc* promoter was described. The role of TGF β1 and Sp1/Sp3 responsive elements located within QRR was examined.

3. Finally, I studied the long-term effects of quinidine. Three important questions were addressed:

3.1 Does quinidine induce differentiation in breast cancer cells

3.2 Does quinidine selectively inhibit growth in breast cancer, but not in normal breast epithelial cells

3.3 Whether a specific down-regulation of *c-myc* by antisense oligonucleotides is sufficient to cause quinidine-induced phenotype in breast cancer cells

II. MATERIALS AND METHODS

1. Materials

1.1. Cells

MCF-7 cells and MDA-MB-231 cells were provided by Dr. Marc Lippman (Lombardi Cancer Center, Georgetown University). The MCF-7 cells transformed with the H-*ras* oncogene (Kasid, A. et al, 1985) were provided by Dr. Robert Dickson (Lombardi Cancer Center, Georgetown University). MDA-468 cells were obtained from the American Type Culture Collection. MDA-MB-435 and T47D cells were provided by Dr. Mike Miller (Department of Biochemistry, West Virginia University) and Dr. Michael Moore (Department of Biochemistry, Marshall University), respectively. Normal human mammary epithelial cells HMEC and MCF10A were obtained from Clonetics (San Diego, CA) and American Type Culture Collection, respectively.

1.2. Drugs/Hormones/Oligonucleotides

Quinidine-HCl was purchased from Sigma Chemical Co. (St. Louis, MO). Concentrated stock solutions (10mM) were prepared fresh for use by dissolving in sterile water. Seven μl of 10N HCl/ml of the stock solution was used. Estradiol-17β (Steraloids, Wilton, NH) was dissolved in 95% ethanol at a concentration of 2mM and diluted $1:10^6$ in the medium to get the final concentration of 2nM. TGFβ1 (R&D Systems, Minneapolis, MN) stock solution (2.5μg/ml) was prepared by dissolving in 4mM HCl, 1mg/ml BSA and stored at -20°C. *c-myc* antisense (5'-AACGTTGAGGGGCAT-3') and sense oligonucleotides (5'-ATGCCCCTCAACGTT-3') were purchased from Sigma-Genosys (Woodlands, TX) and

diluted in sterile water to yeild concentrated stock solutions of 175 μM. The stock solutions were stored at -20°C for 2 months.

1.3. Plasmids

The human *c-myc* probe used for Northern blots was a 9 kb EcoR1-Hind III genomic fragment spanning exons I and II and intron I isolated from the plasmid pHSR-1 obtained from the American Type Culture Collection (Alitalo, K, et al, 1983). The reporter plasmids containing different regions of the human *c-myc* promoter linked to the firefly luciferase gene were kindly provided by Dr. Bert Vogelstein, Johns Hopkins University (He, T. et al, 1998). The structures of *myc*-luciferase plasmids were confirmed by DNA sequencing. The human cyclin D1- luciferase plasmid (1745CD1-Luc) was kindly provided by Dr. Richard Pestell (The Albert Einstein College of Medicine, Bronx, NI) (Albanese, C., et al, 1995)

1.4. Antibodies

The Ki-67 (MIB-1, Ab-1) antibody (Oncogene Science, Cambridge, MA) was used at a dilution of 1:50. The c-Myc antibody (9E10, sc-40), which reacts specifically with c-Myc p67 of human origin and β-catenin (E-5, sc-7963) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a dilution of 1:200 and 1:3000, respectively. A peroxidase-conjugated anti-mouse IgG (sc-2005, Santa Cruz) was used as the secondary antibody at a 1:2000 dilution for Western blots. A biotinylated anti-mouse IgG (H+L) (BA-2000, Vector Laboratories, Inc.) was used as the secondary antibody at a 1:125 dilution for Ki-67 immunohistochemstry. The fluorescein-conjugated phalloidin antibody (Sigma) was used at a 1:200 dilution of a 5 μg/0.1ml solution in the Nile Red assay.

1.5. Stains

The fluorescent lipid stain, Nile Red (Sigma) was used at a 1:10,000 dilution of a 1mg/ml acetone solution. The Oil Red O (Sigma) stock solution was prepared by dissolving 2.5 g of the Oil Red O in 500 ml of 98 % Isopropanol. The working solution was prepared fresh each time by diluting the stock solution in water at a 3:2 ratio. The working solution was filtered once through the P5 Fisherbrand Filter paper (09-801D, Fisher Scientific).

2. Methods

2.1. Tissue culture

MCF-7 cells between passages 40-50, MCF-7ras, T47D, MDA-MB-231, and MDA-MB-435 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Inc, Logan, Utah), 2 mM glutamine, and 40 μg/ml gentamicin. Experiments were performed in DMEM supplemented with 5% FBS. The cells were maintained at 37 °C in a humidified atmosphere of 94% air, 6% CO_2. Cells were passaged every 5-6 days (about 70-80% confluent) at a 1:5 (MCF-7 and T47D) or 1:10 (MCF-7ras, MDA-MB-231, and MDA-MB-435) ratio. Normal human mammary epithelial cells HMEC and MCF10A were grown in MEGM medium according to the directions from suppliers. HMEC were grown from frozen stocks and used for 1-3 passages only. All cells were counted using a hemocytometer. Cell viability was determined by trypan blue exclusion (0.02 % trypan blue).

2.2. Flow cytometry

Cell cycle phase distribution was anyalysed using the propidium iodide staining method of Vindelov and Christensen, 1994. Briefly, confluent cells were plated in DMEM + 5% FBS at the density of $6x10^5$/60 mm^2 dish. Cells were allowed to attach for 5 hours, then cell monolayers were rinsed twice with 1xHBSS (5.4 mM KCl, 108 mM NaCl, 0.34 mM Na_2HPO_4 x $7H_2O$, 4.2 mM $NaHCO_3$, 0.44 mM KH_2PO_4, pH 7.2) buffer to remove all traces of the medium and 5 ml of fresh phenol red free PRF-DMEM containing 2% of the charcoal/dextran treated FBS (2% stripped serum) was added. The medium was changed two more times 20 and 30 hours later to facilitate removal of the endogenous estrogens present in the cells. The total incubation period of the cells in the 2% stripped medium was 40 hours, similar to the conditions described in the literature (Dubik, D. and Shiu, P., 1992). Finally, at the end of the 40 hours, fresh 2% stripped medium +/- drugs was added to the cells. At the end of the treatment, for each sample, cell medium was collected in a 15 ml conical tube. Cells were harvested by trypsinization, placed into the same tube and counted by hemocytometer. For each sample, $5x10^5$ cells were pelleted, washed with 1x HBSS, pelleted again, and resuspended in 250 µl of lysis buffer (3.4 mM trisodium citrate dihydrate, 0.1% Nonidet P-40, 1.5 mM spermine-4HCl, 0.5 mM Tris base, 0.03 mg/ml trypsin, and 34 µM EDTA). Cells were incubated for 25 min at 37 °C, then 500 µl of trypsin inhibitor/RNAse solution (3.4 mM trisodium citrate dihydrate, 0.1% Nonidet P-40, 1.5 mM spermine-4HCl, 0.5 mM Tris base, 0.5 mg/ml trypsin inhibitor, 0.1mg/ml ribonuclease A) was added for 15 min at 37 °C. Finally, 500 µl of propidium iodide solution (3.4 mM trisodium citrate dihydrate, 0.1% Nonidet P-40, 4.8 mM spermine-4HCl, 0.5 mM Tris base, 74.8 µM propidium iodide) was added and cells were incubated at 37 °C for 10 min. Samples were

kept on ice up to 24 hours. Flow cytometric analysis of cell cycle distribution was done on FACScan (Becton Dickinson, San Jose, CA) using CellFIT software.

2.3. Western blots

Cells were rinsed with 1x PBS (phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 x $7H_2O$, 1.4 mM KH_2PO_4, pH 7.3) and harvested by scraping in a boiling Western lysis buffer (1% SDS, 10 mM Tris, pH 7.4). Cells lysates were transferred by 1cc syringe into 1.5 ml tubes, passed through the syringe three times, and boiled for 5 min. Supernatants were collected by spinning samples at maximal speed for 5 min in microcentrifuge at 4°C. Twenty μl aliquots were removed for determination of protein concentrations by BCA assay (bicinchoninic acid, Pierce, Rochford, IL) according to the manufacturer's protocol. Dithiothreitol (DTT) and protease inhibitors were added to the final concentrations indicated: DTT (1mM), PMSF (phenylmethylsulfonyl fluoride, 0.1 mM), aprotinin (1 μg/ml), and leupeptin (1 μg/ml). Extracts containing 60-90 μg of proteins were diluted 4:1 with 5x Western sample buffer (3.125 ml of 1M Tris-HCl, pH 7.0, 5 ml Glycerol, 1 g SDS, 100 μl of saturated Bromophenol Blue solution, q.s. 10 ml with water; 12.5 μl of 2-Mercaptoethanol/100 μl was added to the 5x buffer fresh each time). Proteins were denatured by heating at 100 °C for 3 minutes immediately prior to loading onto 10 % polyacrylamide gels. Proteins were separated and transferred to PVDF (polyvinylidene difluoride) membranes (NOVEX, San Diego, CA) To reduce non-specific binding of antibodies, membranes were incubated in Western blocking buffer (3% non-fat dry milk in Tris-buffered saline-Tween 20 (TBST): 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% (v/v) Tween 20)

overnight at 4 °C. On the next day, membranes were washed 3 times (5 minutes each wash) with Western washing buffer (0.1% non-fat milk, 0.1% chick ovalbumin, 1% FBS, 0.2% (v/v) Tween 20 in PBS, pH 7.3). All washes were performed at room temperature using an Orbital shaker (Bellco Glass, Inc., Vineland, New Jersey). After the washing, membranes were incubated inside the Kapak heat sealable pouches (Kapak Corporation, Minneapolis, MN) for 3.5 hours at room temperature on a rocker with primary antibody diluted in washing buffer. At the end of the incubation, membranes were washed 3 times (5 minutes each) with Western washing buffer and once with TBST buffer. The membranes then were incubated for 40 min on a rocker at room temperature with peroxidase-conjugated secondary antibodies diluted in TBST. After that, membranes were washed 3 times (10 minutes each wash) with TBST. The specific protein signals (Myc or β-catenin) were visualized using chemiluminescent peroxidase substrate (Super Signal, Pierce, Rockford, IL) for 7 minutes and exposing the membranes to the Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY). Autoradiographic signals were quantified by densitomery (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA) and Image QuaNT software, version 4.1. Myc signals were normalized to the 97 kDa β-catenin protein signals.

2.4. Northern blots

Total cellular RNA was purified by the method of Chomczynski and Sacchi (Chomczynski, P. and Sacchi, N., 1987). Briefly, cells were harvested by scraping in 2.5ml/T-75 flask of ice-cold RNA harvesting buffer (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0, 0.1M 2-mercaptoethanol, 0.5% N-laurylsarcosine) and transferred into pre-cooled sterile 15ml conical tubes. All of the following steps were performed on ice.

Cellular DNA was sheared by passing the cell lysates through a 22 gauge needle with a 5ml syringe. Two hundred and fifty µl of 2M sodium acetate, pH 4.0, 2.5 ml of water-saturated phenol, and 1ml of chloroform were added and mixed thoroughly by shaking. The lysates were incubated on ice for 30 minutes and centrifuged in a Sorvall H-1000B rotor at 3,500 rpm for 15 min at 4 °C. The upper aqueous phase containing the RNA was carefully transferred into a clean 15ml conical tube. The white interphase containing proteins and DNA should be avoided. RNA was then precipitated by adding 2.5ml volumes of isopropanol and incubating at -20°C overnight. Precipitated RNA was collected by centrifuging for 30 min in a Sorvall H-1000B rotor at 3,500 rpm at 4 °C. The supernatant was discarded; the RNA pellet was resuspended in ice-cold 75 % ethanol, and transferred to a clean Eppendorf tube. The RNA solution was spun in an Eppendorf microcentrifuge at maximal speed for 10 minutes at 4 °C. The supernatant was removed and the RNA pellet was dissolved in an appropriate amount of DEPC (diethyl pyrocarbonate) -treated water. Two µl of RNA from each sample was dissolved in 998 µl (1:500 dilution) of the DEPC water and their absorbency at 260 nm was measured using a spectrophotometer (DMS 80 Varian, Australia). To quantify RNA concentration (in µg/µl), the optical density reading was divided by 24 and multiplied by 500 (the dilution factor). Fifteen µg of RNA for each sample was mixed with 5x RNA sample buffer (0.75 ml deionized formamide, 0.15 ml 10x MOPS (3-[N-morpholino] propanesulfonic acid), 0.24 ml 37 % formaldehyde, 0.14 ml RNase-free water, 0.1 ml glycerol, 0.04 ml 10 % bromophenol blue) at a ratio of 4:1. RNA was separated on 1.2% agarose-1.9 % formaldehyde gels in MOPS running buffer (20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA, pH 8.0), and transferred to 0.2 mm nitrocellulose paper (Schleicher and Schuell, Keene, NH) by capillary action. Membranes were air-dried for 5

minutes and baked in a vacuum oven at 80 °C for 2 hours to fix the RNA. After that, the membranes were incubated in the pre-hybridization solution (50% formamide, 250 mM $NaHPO_4$, pH 7.2, 250 mM NaCl, 1 mM EDTA, pH 8.0, 1mg/ml of denatured salmon sperm DNA, 7% SDS) in the Micro-hybridization oven (Bellco Glass Inc., Vineland, NJ) at 42 °C for 15-20 hours to decrease non-specific binding of the probe. Next, the pre-hybridization solution was exchanged with the hybridization solution (the same pre-hybridization solution plus denatured *c-myc* DNA fragment labeled with [αP^{32}]dCTP by random priming (Rediprime DNA labeling kit, Amersham Corp., Arlington Hts., IL). About 1.5-2 x 10^6 cpm of the probe/ml of the hybridization mix was used. Hybridization reactions were performed at 42°C for 40-42 hours, then blots were washed in 2x SSC (Standart Saline Citrate)-0.1% SDS solution twice for 20 minutes each wash at 37°C. A high stringency wash of the *c-myc* probe was performed for 15 minutes at 42°C in 0.1x SSC-0.1% SDS buffer. Blots were exposed in a phosphorimager cassette. Quantitative analysis of the hybridization signals was performed using PSI-PC (Molecular Dynamics, Sunnyvale, CA) and ImageQuaNT software, version 4.1. Hybridization signals were normalized to levels of 18S or 28S RNA in the ethidium bromide stained gels.

2.5. Reporter gene assay

Transient transfection procedure. All plasmids used in the reporter gene assay were purified using Qiagen DNA purification columns, according to the manufacturer's protocols. On day one, confluent MCF-7 cells were re-plated at the density of $8x10^5$/60 mm^2 tissue culture dish in 5 ml DMEM/5%FBS medium. After 20-24 hours, when cells reached about 40-50 % confluency, cell monolayers were rinsed with 1xHBSS (Hank's Balanced Salt

Solution) buffer and 5ml of DMEM/2%FBS medium were added. One hour later, transfections were started by adding the transfection mixes to the cells. The transfection mixes were prepared in a sterile 3ml glass tubes by mixing 5 μg of plasmid DNA, 30 μl of the DOTAP liposomal transfection reagent (1811177, Roche Molecular Biochemicals), and water to the final volume of 150 μl for each sample. This volume was calculated to carry out transfections in 60 mm^2 dishes, in 5ml medium. Transfection mixes were incubated at room temperature for 15 minutes. Transfections were terminated 6-7 hours later by exchanging the medium with fresh medium +/- drugs.

Luciferase Assay. Cells were harvested 24 hours after the end of transfections by scraping into ice-cold lysis buffer (25 mM Tris-phosphate, pH 7.8, 1% Triton X-100, 2mM EDTA, 10% Glycerol). All the following steps were performed on ice. Cell lysates were transferred into 1.5 ml Eppendorf tubes and spun in the Eppendorf microcentrifuge at maximal speed for 5 minutes at 4°C. The supernatants were transferred into clean tubes. Aliquots of 20 μl were removed for determination of protein concentrations by BCA assay. To measure luciferase activity, 50 μl of cell extracts were added to 250 μl of ice-cold luciferase sample buffer (25 mM glycylglycyl, pH 7.8, 15 mM $MgSO_4$) in 12x75 mm polypropylene culture tubes (60818-430, VWR Scientific, West Chester, PA). Immediately before measuring the luciferase activity, the samples were warmed to 25 °C (the optimal temperature for luciferase enzymatic activity). Luciferase activity was measured using an Auto Luminat (LB 953, EG and G-Bertholdt) equipped with a dual injection system. The reactions were initiated by automatic injections of 100 μl of ATP solution (25 mM glycylglycyl, pH 7.8, 15 mM $MgSO_4$, 20 mM ATP) and 100 μl of the luciferin solution (25 mM glycylglycyl, pH 7.8, 1 mM luciferin) into each sample. The production of the light was

measured. The following luminometer settings were used: incubation temperature =25 °C, reading time =6 seconds, injection amount =100µl. The ATP solution was placed in the *measuring position 1*, and the luciferin solution was placed in the *measuring position* of the luminometer. The relative luciferase units obtained from 50 µl of cell extracts were normalized to 200 µg of cellular proteins. Light generation from purified luciferase (Sigma) was used as the standard in each experiment to ensure that all determinations were performed under linear assay conditions. The amount of sample buffer, lysis buffer, and purified luciferase used for the standard curve are given in the Table below.

Sample buffer (µl)	Lysis buffer (µl)	5 pg/µl luciferase solution (µL)
250	50	0
248	50	2 (10 pg)
246	50	4 (20 pg)
242	50	8 (40 pg)
234	50	16 (80 pg)
218	50	32 (160 pg)

2.6. Ki-67 immunohistochemistry.

MCF-7 cells were plated at the density of $2x10^5$/ 35 mm^2 tissue culture dish on sterile glass cover slips in 3 ml of DMEM/5%FBS +/- drugs. At the end of the treatment, the medium was removed by aspiration and cells were fixed by transferring the cover slips to a - 20°C fixing solution (50% acetone/50% ethanol) for 10 minutes on ice. All the following steps were performed at room temperature. Cells were rinsed with the working PBS (wPBS) solution (1x PBS, 0.15 % bovine serum albumin), and freshly made Blocking solution #1 (1:100 dilution of 30% H_2O_2 in methanol) was added to the cells for 15 minutes to block endogenous peroxidase activity. Cells were rinsed with wPBS, and then Blocking solution #2 (10% horse serum in wPBS) was added for 30 minutes to block non-specific binding of antibodies. The blocking solution was carefully removed, leaving a thin layer of it on the cover slips, and 500 μl/cover slip of diluted Ki-67 antibody (1:50 ratio in wPBS) were added for one hour. Cells used for the negative control remained in the blocking solution #2. Ki-67 antibody was removed by rinsing cells with wPBS for 5 minutes and biotinylated anti-mouse IgG secondary antibody (1:125 dilution in wPBS) was added for 30 minutes. The Biotin-Avidin solution (B/A) was prepared 30 minutes prior to use by adding 1 drop of each Reagent A and B (PK-6100, Peroxidase Vectastatin Elite ABC kit, Vector Laboratories, Inc.). The secondary antibody was removed by rinsing cells with wPBS for 5 minutes and B/A solution was added for 30 minutes. After that, cells were rinsed with wPBS for 5 minutes and a peroxidase substrate, DAB (750118, Research Genetics, Inc.) was added for 10 minutes. Cells were rinsed with wPBS and counterstained with Mayer's hematoxylin (Sigma) for 1-2 minutes to visualize cell nuclei. Cover slips were mounted with Permount

solution (SP15-100, Fisher Scientific). The images were obtained using an Ortholux microscope (Ernst Leitz Wetzlar, Germany) (40x objective). Cells were scored using Image-Pro Plus software (Media Cybenetics, Silver Spring, MD).

2.7. Oil-Red O assay

Cells were plated in 35 mm^2 tissue culture dishes on sterile glass cover slips in DMEM/5%FBS +/- drugs. At the end of the treatment, the cover slips were transferred into 60 mm^2 glass dishes containing 3 ml of cell fixative solution (10% formaldehyde and 0.2% calcium acetate in PBS) for 3 minutes. The fixative was removed by aspiration and 3 ml of freshly made Oil-Red O working solution (Oil-Red O stock solution [0.5 % Oil-Red O in 98% Isopropanol] diluted in water in a 3:2 ratio) were added for 10 minutes. Cells were then rinsed in water and counterstained with Mayer's hematoxylin solution (Fisher, Pittsburgh, PA) for 1 min at room temperature. Cells were briefly washed in 0.4 % NH_4OH, rinsed in distilled water, and then mounted with 50% glycerol solution (a filtered 1:1 dilution of Glycerol in water). The images were obtained using Ortholux microscope (Ernst Leitz Wetzlar, Germany) (40x objective). Cells were scored using Image-Pro Plus software (Media Cybenetics, Silver Spring, MD).

2.8. Nile Red staining

Cells were plated at the density of $1x10^5$/ 35 mm^2 dishes on sterile glass cover slips and grown for 96 hours in DMEM/5%FBS +/- drugs. At the end of the treatment, cells were fixed in 3.7% formaldehyde/PBS, rinsed in PBS, and then treated briefly with 0.4% Triton X-100 in PBS. After rinsing 3 times in PBS, the cells on cover slips were incubated with

fluorescein-phalloidin antibody (1:200 dilution of a 5μg/0.1 ml solution, Sigma) in the dark for 40 minutes at room temperature. Cells were then rinsed with PBS and incubated for 5 minutes with the fluorescent lipid stain, Nile Red (1:10,000 dilution of a 1mg/ml acetone solution, Sigma) (Greenspan, P. et al, 1985; Toscani, A. et al, 1990). The cover slips were rinsed with PBS and mounted with Fluoromount-G containing 2.5% N-propyl galate to minimize photobleaching. Images were obtained using a Zeiss Axiovert 100M confocal microscope (63x objective).

2.9. MTS assay

Cells were plated in a 96-well dishes in DMEM/5%FBS +/- drugs at the following densities, depending upon the relative growth rates of the cell lines: HMEC (2000), MCF-7 (1000), MDA-231 (500), T47D (1500). At the end of the treatment time, the medium was removed by aspiration. One hundred μl of DMEM/5%FBS and 20 μl of the MTS reagent (CellTiter 96 AQueous one solution cell proliferation assay, Promega Cor., Madison, WI) were added to each well using a multichannel pipetteman. The plated cells were incubated for 2 hours at 37 °C. Absorbency readings were obtained at a wavelength of 490 nm with a microplate spectrophotometer (Molecular Devices).

2.10. Statistics

The *SigmaPlot* software (SPSS Inc., Chicago, IL), version 5.0 and *JMP* software (SAS Institute, Inc., Cary, NC) were used for statistical analysis. One way analysis of variance (ANOVA) followed by the Dunnett's test for comparison of multiple groups with

control or the Turkey-Kramer test for comparison between the different groups was used. A significance level of 0.05 was used.

III. RESULTS

1. Quinidine Reduces Myc Protein Levels in Human Breast Cancer Cells

1.1. Rapid suppression of Myc protein levels by quinidine in MCF-7 cells

Transient induction of *c-myc* mRNA and protein levels is a prerequisite for G0-G1 and G1 phase transition. According to our hypothesis, rapid suppression of Myc by quinidine causes cell cycle arrest at the D point in the early G1 phase of the cell cycle due to the lack of Myc-dependent transcription of the genes required for cells to pass through the D point. The purpose of this experiment was to test how rapidly quinidine suppresses Myc protein.

MCF-7 cells were grown in T-75 tissue culture flasks until 95-100 % confluency (1.5-2 x 10^7 cells/T-75) in order to obtain a population of cells largely (85%) in G0/G1 phase (Wang, S., et al, 1998). Before plating, the viability of the cells was assayed using a hemocytometer to obtain cell counts and trypan blue dye to detect the dead cells. The viability ranged from 96 to 100 %. The synchronized cells were then plated at the density of 3.5 x 10^6 viable cells per 100 mm^2 tissue culture dish in 10 ml of DMEM/5% FBS medium. 90 μM quinidine or H_2O (vehicle) was added to the dishes. One dish of each, control and quinidine-treated cells was harvested in protein harvesting buffer 0, 60, 90, 120, 180 min after the plating. Equal amounts of proteins (60 to 90 μg depending on the experiment) were loaded into each well of 10 % polyacrylamide gels. As shown in Figure 6, a transient increase in Myc protein levels occurs after sub-cultivating confluent MCF-7 cells in fresh medium (release from the high cell density condition). The maximal increase in Myc protein levels was 15-fold, achieved at 90 min after plating. This rise in Myc protein was suppressed by quinidine, with the maximal level of suppression (60%) achieved at 60 min. The data indicate that suppression of Myc protein levels occurs rapidly, and precedes the quinidine-

induced cell cycle arrest (see Figure 3). This observation is a critical evidence in support to our hypothesis that quinidine acts on Myc early in the G1 phase of the cell cycle, prior to cell cycle arrest. Therefore, suppression of Myc protein by quinidine is not simply a result of cell cycle arrest, but, in contrary, may play a causative role in it.

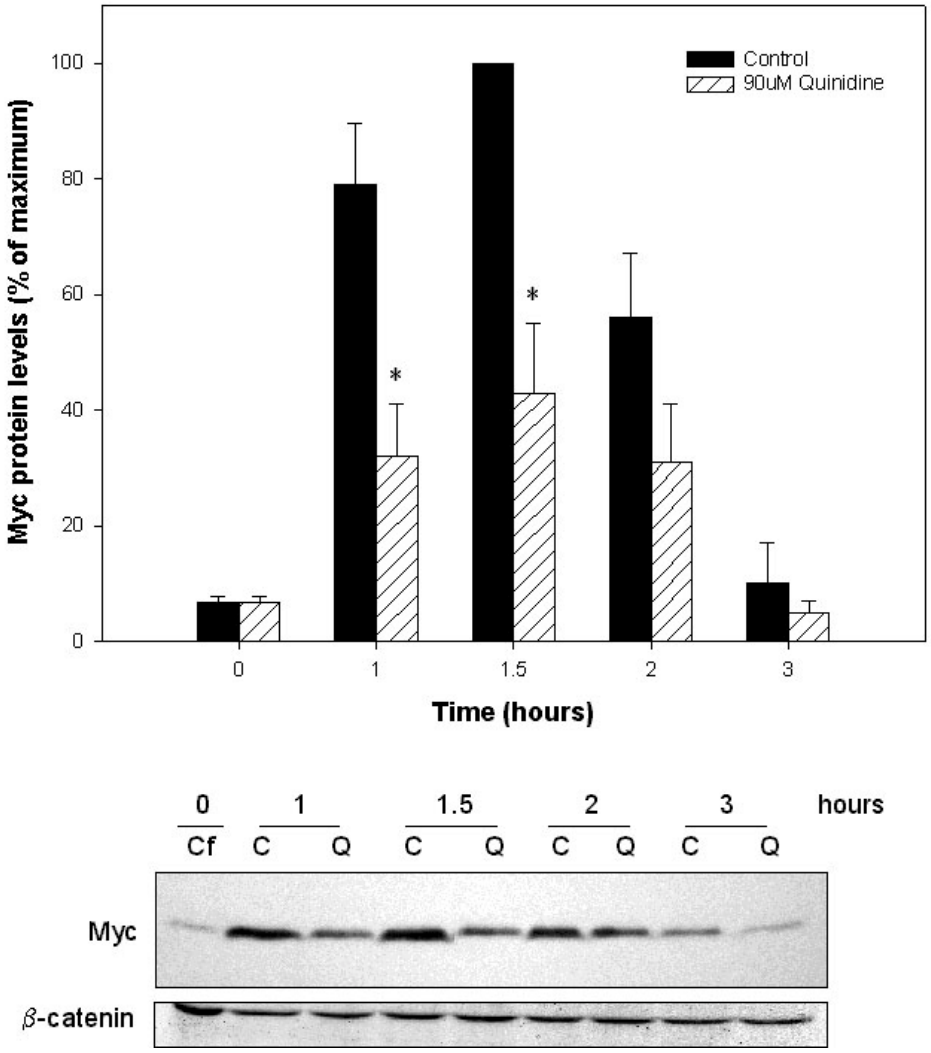


Figure 6. Quinidine suppresses Myc protein levels in MCF-7 cells.

Confluent (Cf) MCF-7 cells were sub-cultivated at 0 time in DMEM/5%FBS (solid bars) or in DMEM/5%FBS containing 90 μM quinidine (hatched bars). Cells were harvested for Western blot analysis at the times indicated. Myc protein signals were quantified by densitometry and normalized to the β-catenin signals. Data shown in the bar graph are the mean Myc protein signals +/- S.D. of n =3 experiments expressed as a percent of maximal stimulation (at 1.5 hours). The differences between the percentages of control and quinidine-treated cells were statistically significant ($p<0.05$) at 1 and 1.5 hours. The gel scans below the bar graph are a single representative experiment performed with 80 μg of cell extract protein/lane**.**

1.2. Suppression of Myc by quinidine in MCF-7 cells is not mediated by the impediment of cell attachment.

Some cell lines growing in tissue culture (epithelial cells, fibroblasts) require their attachment to the special surface for the proper cell cycling, while others (lymphocytes) can grow in a suspension. Therefore, any condition, that affects attachment of cells to the growth surface can influence cell progression through the cell cycle. In the experiment described above, quinidine was added to the cells immediately after the plating. At this point most of the cells were loosely attached to the tissue culture dishes. The purpose of current experiment was to test the influence of quinidine on the attachment of MCF-7 cells. If quinidine impedes cell attachment, then suppression of Myc by quinidine and subsequent cell cycle arrest could be simply the results of quinidine's effect on cell attachment.

Confluent MCF-7 cells were re-plated in 60 mm^2 tissue culture dishes in 5 ml DMEM-5% FBS at the density of $0.8x10^6$ cells/dish. Cells were allowed to attach for 3 hours. Then, the medium was changed to DMEM-2% FBS and 72 hours later cells were treated with: 1) DMEM-2% FBS, 2) DMEM-5% FBS, 3) DMEM-5% FBS + 90 uM quinidine. Cells were harvested 1 hour later. Seventy μg of proteins were loaded into each well of 10 % polyacrylamide gels and Western blot assay was performed as described in the Materials and Methods. As shown in Figure 7, MCF-7 cells growing in DMEM-2% FBS (low serum condition) have very low Myc protein levels. Addition of DMEM-5% FBS to these cells caused rapid (within 1 hour) induction of Myc protein. Ninety μM quinidine completely abolished this effect. These results demonstrate that quinidine suppresses Myc protein levels to the same extent in both attached (down to 36%, Figure 7) and loosely attached (down to

38%, Figure 8) MCF-7 cells, suggesting that suppression of Myc by quinidine is not mediated by quinidine's effect on cell attachment.

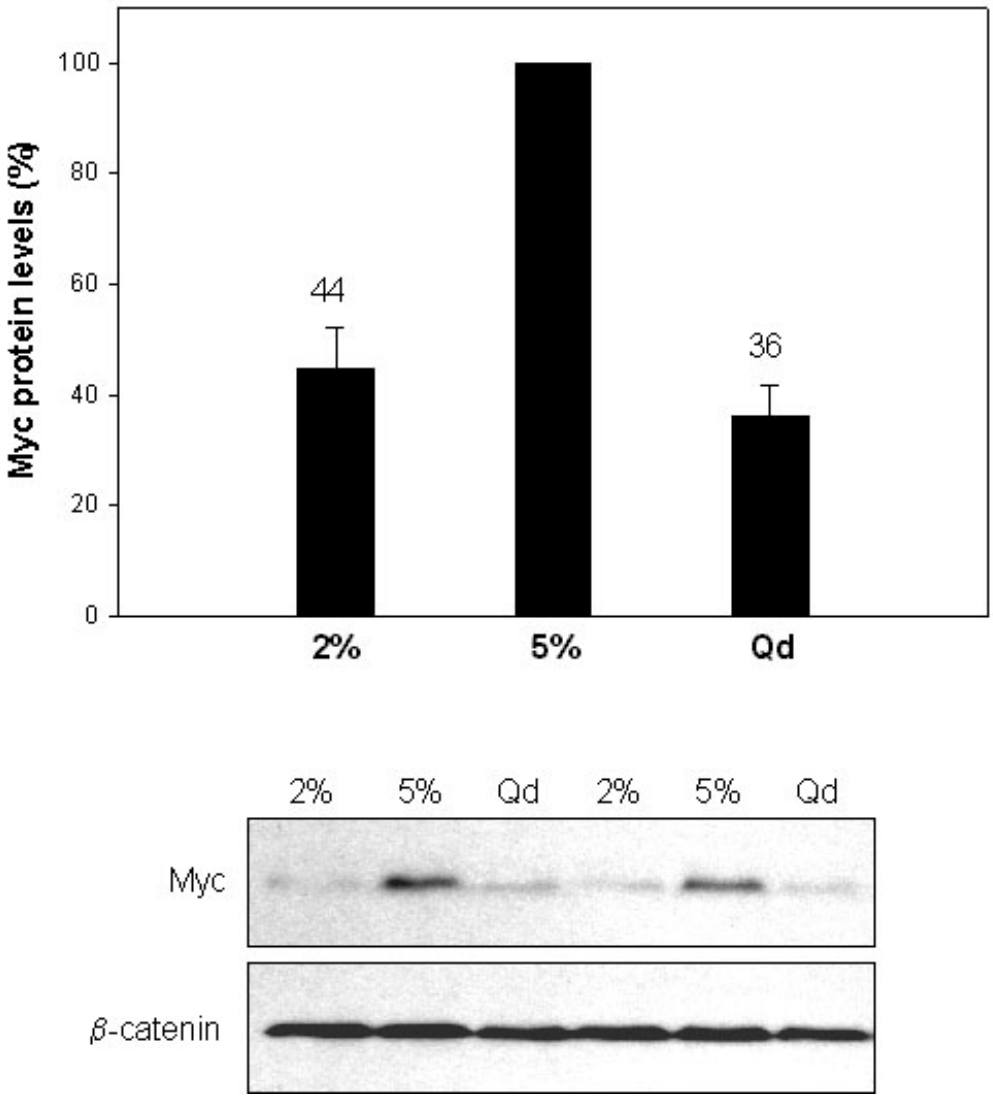


Figure 7. Suppression of Myc by quinidine in MCF-7 cells is not mediated by the impediment of cell attachment.

Confluent MCF-7 cells were sub-cultivated in DMEM-5% FBS, allowed to attach for 3 hours and then incubated in DMEM-2% FBS (low serum conditions) for 72 hours. After that, cells were treated for 1 hour with: DMEM-2% FBS (2%), DMEM-5% FBS (5%), or DMEM-5% FBS + 90 uM quinidine (Qd). Myc protein levels after Western blot assay were determined by densitometry and normalized to the β-catenin levels. Myc protein levels in DMEM-5% FBS treated cells were set to 100%. The numbers above the bars show Myc levels in DMEM-2% FBS and DMEM-5% FBS+Qnd treated cells relative to DMEM-5% FBS treated cells. The bar graph data are the mean +/- S.D. of n=2 experiments performed in duplicates. The gel scans below the bar graph are a single representative experiment.

1.3. Rapid suppression of Myc protein in other breast cancer cell lines

The purpose of this experiment was to assay whether the rapid decrease of Myc protein by quinidine is specific to MCF-7 cells or is more general response of breast tumor cells to quinidine. Three other cell lines used in this experiment were: MCF-7ras, MDA-MB-231, and MDA-MB-435. MCF-7ras cells have high, constitutive expression of Myc protein due to the activated ras-MAP kinase pathway. MDA-MB-231 and MDA-MB-435 cells are $ER\alpha^{-}/ER\beta^{+}$ and $ER\alpha^{-}/ER\beta^{-}$, respectively (Vladusic, E., et al, 2000). These two cell lines were chosen to assay if the ER status influences the Myc response to quinidine. Confluent cultures of the cells were re-plated in 100 mm^2 dishes in 10 ml DMEM-5 %FBS at the density of $3x10^6$ cells/dish and treated with 90 μM quinidine or H_2O (vehicle). Cells were harvested one (MCF-7ras, MDA-MB-231, MCF-7) or two (MDA-MB-231, MDA-MB-435) hours later. The two-hour time point for MDA-MB-435 and MDA-MB-231 cells was chosen, because in these cell lines the maximum levels of Myc protein after the release of cells from high density are reached at this time. Equal amounts of proteins (70 μg) were loaded into each well of 10 % polyacrylamide gels. As demonstrated in Figure 7, quinidine suppressed Myc protein levels by 82% (MCF-7ras), 65% (MDA-231), 62% (MCF-7), and 59% (MDA-435) in these cell lines. The variance in control Myc levels of different cell lines (Figure 7 gel scan) is in a good correlation with their proliferative rate (see Figure 18), which underlines the role of Myc in cell proliferation. These results show that rapid decrease in Myc protein levels by quinidine occurs in all four human breast cancer cell lines tested and is independent of the ERs status.

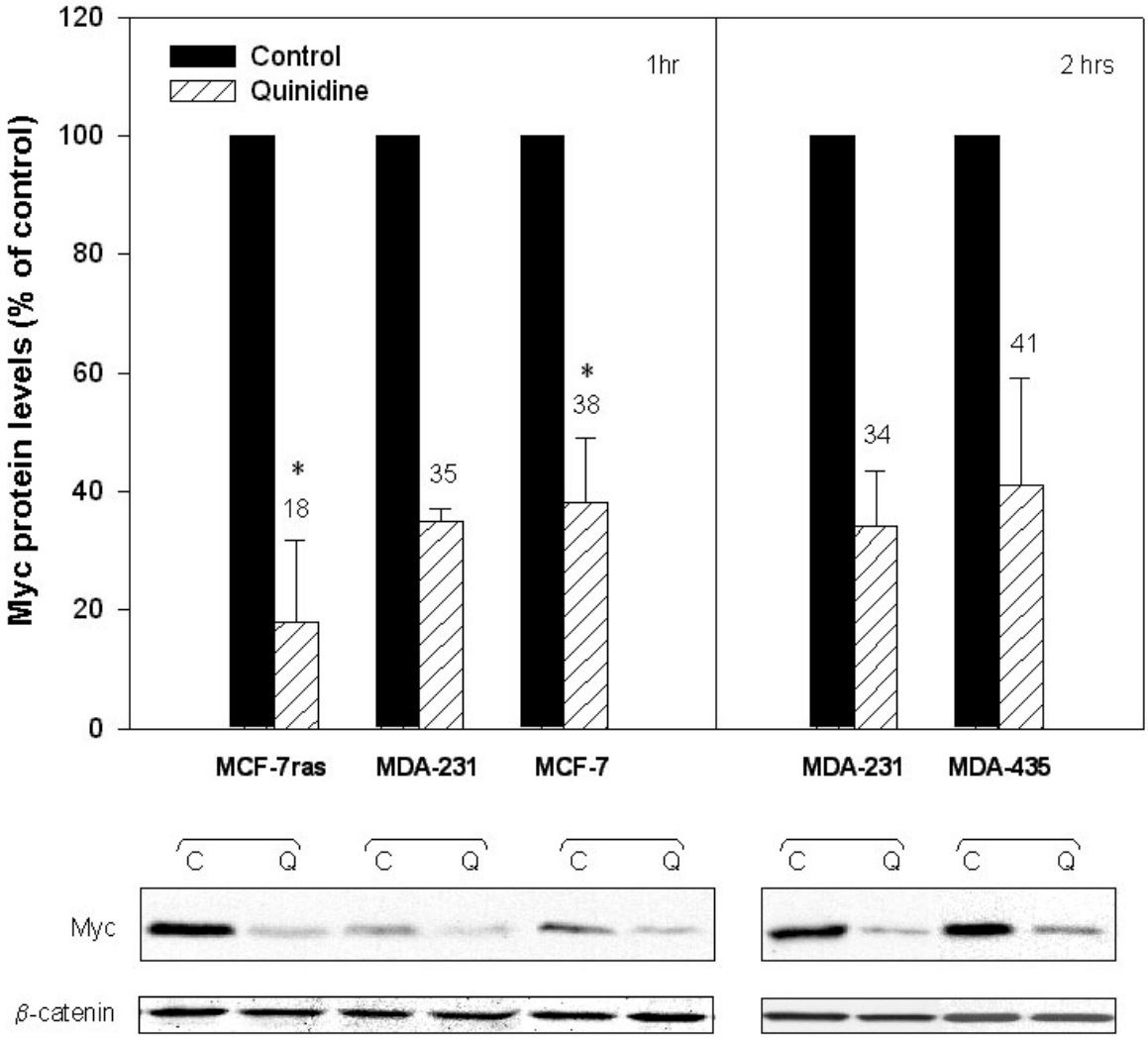


Figure 8. Quinidine suppresses Myc protein in MCF-7ras, MDA-231, and MDA-435 cells.

Confluent MCF-7, MCF-7ras, MDA-231, and MDA-435 cells were sub-cultivated in DMEM/5%FBS +/- 90 μM quinidine. Cells were harvested at indicated time points and Myc protein levels after Western blot assay were determined by densitometry in control (solid bars) or quinidine-treated (hatched bars) cells. Myc signals were normalized to the β-catenin signals. Myc protein levels in quinidine-treated cells expressed as a percent of the respective controls for each cell line are shown above the hatched bars. The bar graph data are the mean +/- S.D. of n =3 experiments with MCF-7 and MCF-7ras cells, and the mean +/- range in n =2 experiments with MDA-231 and MDA-435 cells. The gel scans below the bar graph are a single representative experiment performed with 70 μg of cell extract protein/lane.
∗ Significantly different from control values ($p<0.05$).

1.4. Suppression of Myc protein by quinidine is sustained within 24-hour tested period

The objective of this experiment was to determine the character of Myc suppression by quinidine: transient versus sustained. MCF-7 and MCF-7ras cells were re-plated from confluent T-75 flasks at the density of 3 x 10^6 cells/100 mm^2 tissue culture dish in 10 ml DMEM/5%FBS medium. Zero, 30, or 90 μM quinidine was added to the cells. Twenty-four hours later cells were harvested and 70 μg of proteins for each sample were separated on 10% polyacrylamide gels. As shown in Figure 9, quinidine caused concentration-dependent reductions in the levels of Myc protein in both cell lines after 24 hours of treatment. Thirty μM quinidine caused a more than a 50 % reduction in Myc protein levels in both cells, compared with respective untreated control cells. Ninety μM quinidine further reduced Myc protein levels by 88 % and 68 % in MCF-7ras and MCF-7 cells, respectively. The high levels of Myc protein in the control MCF-7ras cells compared to MCF-7 cells are due to the constitutive expression of the Myc in MCF-7ras cell line. In MCF-7 cells Myc is transiently induced during the G0-G1 and G1 phase transition (Figure 6) and then stays at constant low levels throughout the cell cycle (Figures 5 and 9).

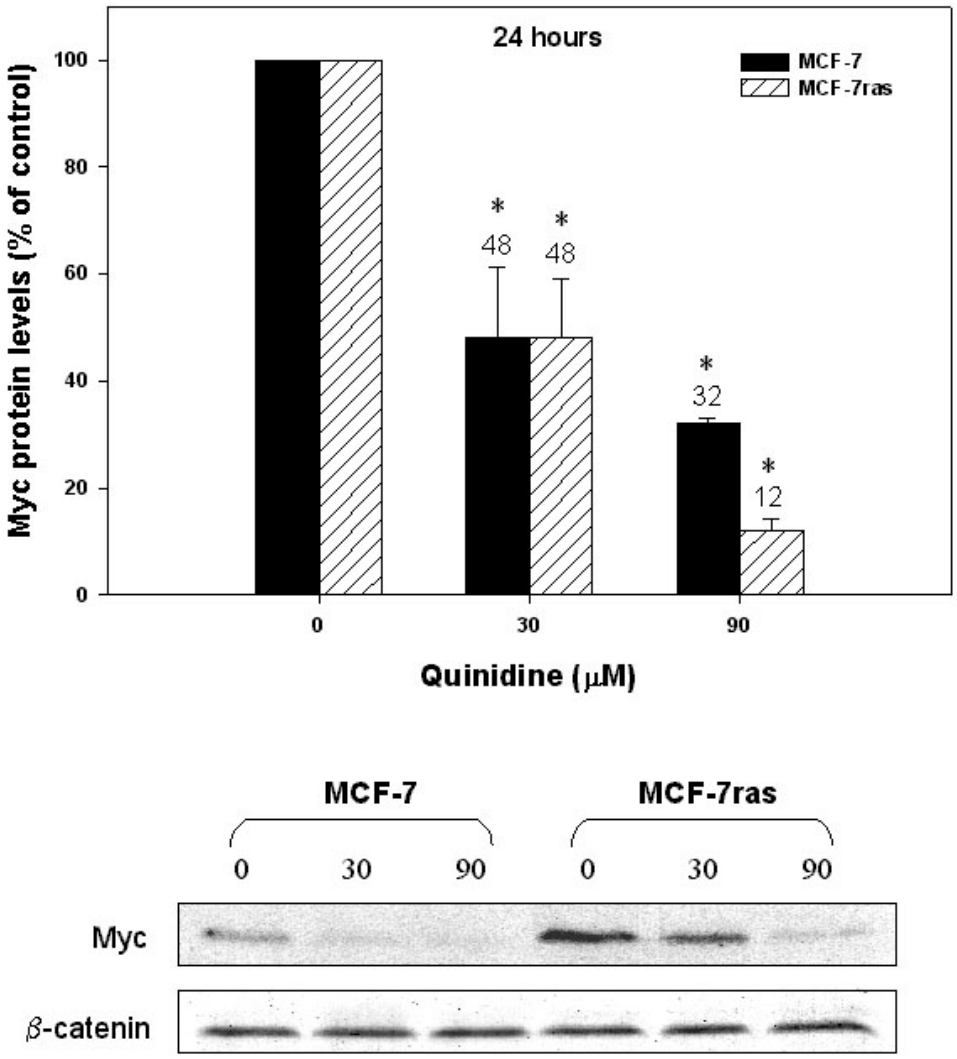


Figure 9. Suppression of Myc protein by quinidine.

Confluent MCF-7 and MCF-7ras cells were sub-cultivated in DMEM/5%FBS +/- different concentrations of quinidine. Cells were harvested 24 hours later and Myc protein levels after Western blot assay were determined by densitometry. Myc signals were normalized to the β-catenin signals. The bar graph shows Myc protein levels in MCF-7 (solid bars) and MCF-7ras cells (hatched bars) as the mean +/- S.D. of n =4 (MCF-7) and n=3 (MCF-7ras) experiments. The numbers above the bars indicate the average Myc signal (% of control). The gel scans below the bar graph are a single representative experiment performed with 70 μg of cell extract protein/lane.

∗ Significantly different from control values ($p<0.05$)

1.5. Quinidine does not affect Myc protein levels in normal mammary epithelial cell line MCF10A.

The abnormal expression of *c-myc* was reported in 32% of breast cancers, suggesting its importance in the genesis and/or progression of breast cancer. Hence, our findings that quinidine inhibits Myc protein levels in four different breast cancer cell lines by 60-80% are very promising. The goal of this experiment was to test the effect of quinidine on Myc protein in MCF10A normal mammary epithelial cell line.

MCF10A cells were re-plated from confluent T-75 flasks at the density of 6×10^5 cells/60 mm^2 tissue culture dish in 5 ml MEGM medium +/- 90 μM quinidine. Twenty-four hours later, cells were harvested and 60 or 70 μg of proteins for each sample were separated on 10% polyacrylamide gels. As shown in Figure 10, quinidine didn't cause a significant reduction in Myc protein levels in this cell line. As has been discussed earlier, one of the major problems with current chemotherapeutic drugs for breast cancer treatment is their lack of selectivity for tumor tissue and associated with it toxicity in normal tissues. That why, it is important to show, that quinidine selectively inhibits Myc protein in breast cancer but not in normal breast epithelial cell lines.

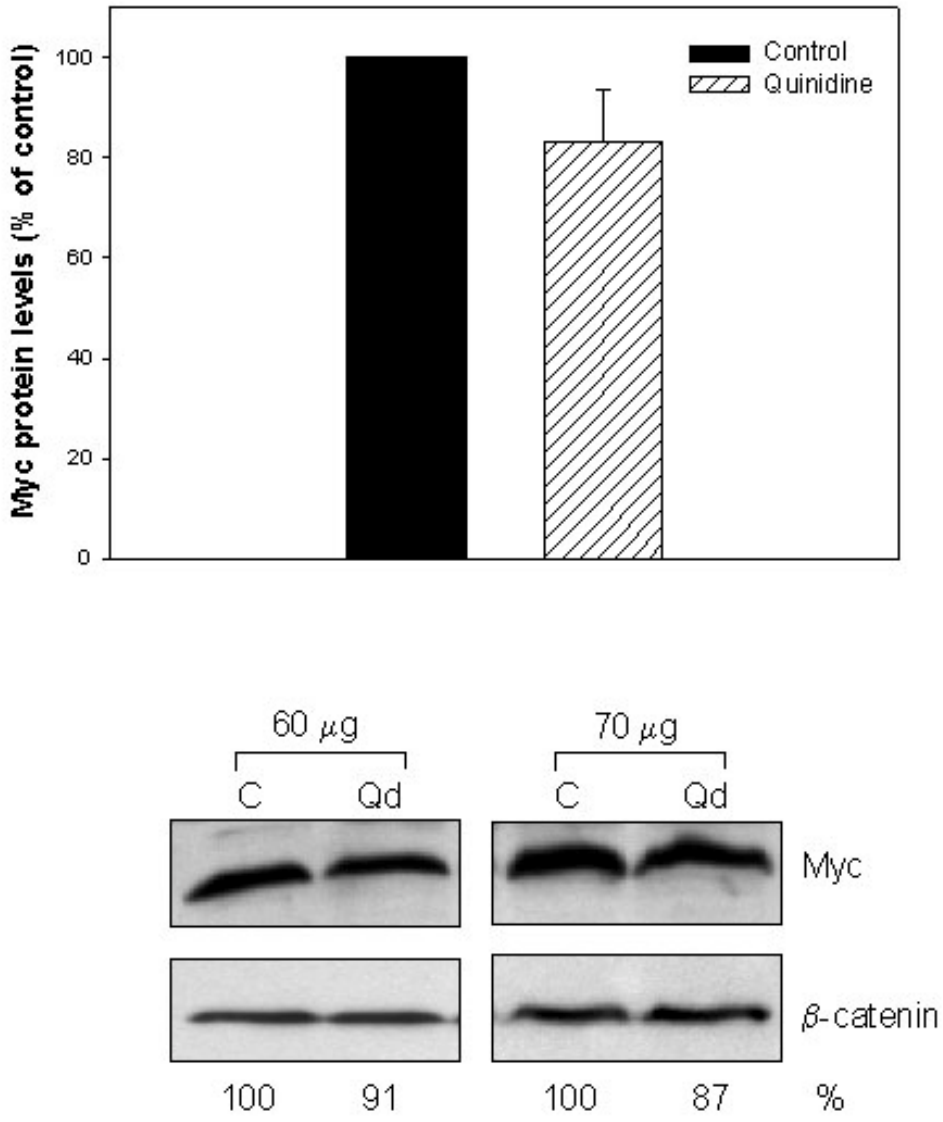


Figure 10. Quinidine does not affect Myc protein levels in MCF10A normal mammary epithelial cells.

Confluent MCF10A cells were sub-cultivated in MEGM +/- 90 uM quinidine. Cells were harvested 24 hours later and Myc protein levels after Western blot assay were determined by densitometry. Myc signals were normalized to the β-catenin signals. The bar graph shows Myc protein levels in control (solid bars) and quinidine-treated (hatched bars) cells as the mean +/- S.D. of n=3 experiments. The numbers below the gel scans indicate the Myc protein levels in two 2 independent experiments.

2. Quinidine Reduces *c-myc* mRNA Levels in Breast Cancer Cells.

2.1. Suppression of *c-myc* mRNA levels by quinidine in MCF-7 and MCF-7ras cells.

MCF-7 and MCF-7ras cells were re-plated from the confluent state into the T-75 tissue culture flasks at the density of 3.5 x 10^6/flask in 10 ml of DMEM/5%FBS. Cells were treated with 0, 30, and 90 μM quinidine. Twenty-four hours later, total cellular RNA was purified, separated on 1.2% agarose-1.9% formaldehyde gels, transferred to nitrocellulose paper, and hybridized to the radiolabeled *c-myc* probe, as described in the Materials and Methods. Fifteen μg of total RNA were loaded into each well. Quantitative analysis of the specific *c-myc* hybridization signals was performed using a PSI-PC (Molecular Dynamics, Sunnyvale, CA) and ImageQuaNT software, version 4.1. The *c-myc* mRNA signals were normalized to 18S ribosomal RNA signals on the ethidium bromide stained gel. The results are shown in Figure 11. Quinidine caused a concentration-dependent suppression of *c-myc* mRNA levels in both cell lines. Thirty μM quinidine reduced *c-myc* mRNA levels by 53% and 31% in MCF-7 and MCF-7ras cells, respectively. Ninety μM quinidine further reduced *c-myc* mRNA levels by 78% in MCF-7 and 63% in MCF-7ras cells. These results suggest that the quinidine-induced changes in Myc protein levels observed at 24 hours (Figure 9) may be due to the quinidine-induced suppression in *c-myc* mRNA levels (Figure 11).

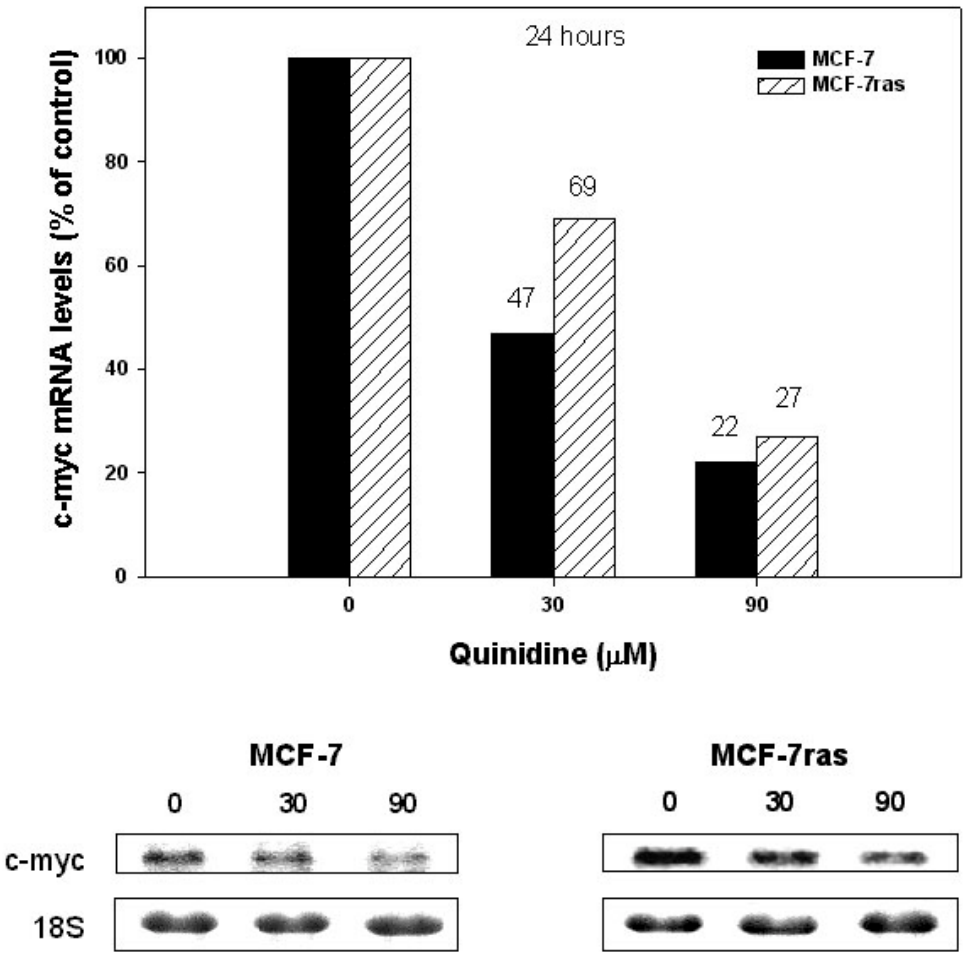


Figure 11. Suppression of *c-myc* mRNA by quinidine.

Northern blots were performed using 15 μg of total cell RNA/lane isolated from MCF-7 (solid bars) and MCF-7ras (hatched bars) cells 24 hours following sub-cultivating of confluent monolayers +/- 30 or 90 μM quinidine. The numbers above each bar represent *c-myc* mRNA levels in quinidine-treated cells as a percent of control. The *c-myc* mRNA P^{32} hybridization signals and the 18S ribosomal RNA signals from ethidium bromide stained gels are shown below the bar graph. Results are from a single experiment.

2.2. Suppression of estradiol-induced *c-myc* mRNA levels and cell cycle progression by quinidine in MCF-7 cells.

Estrogen plays an important role in etiology of breast cancer. About 30-40% of human breast tumors are dependent on estrogen for growth in initial state of the disease (van der Burg et al., 1992). Stimulation of *c-myc* gene by estradiol in estrogen-dependent MCF-7 cells is critical to initiate progression of cells through G1 phase of the cell cycle and to activate cell proliferation (Prall, O. et al, 1998). *c-myc* mRNA is rapidly (within 1 hour) induced by 2 nM estradiol in MCF-7 cells (Dubik, D. and Shiu, R., 1988).

The objective of this experiment was to test if quinidine can suppress estradiol-induced *c-myc* mRNA levels and cell cycle progression. MCF-7 cells were re-plated from the confluent state into the T-75 tissue culture flasks at the density of $2.6x10^6$/flask in 10 ml of DMEM-5% FBS (for Northern blots) or into 60 mm^2 tissue culture dishes at the density of $6x10^5$/dish in 5 ml of DMEM-5% FBS (for flow cytometry). Cells were allowed to attach for 5 hours, then cell monolayers were rinsed twice with 1xHBSS buffer to remove all traces of the medium and 10 ml (per T-75) or 5 ml (per 60 mm^2 dish) of fresh phenol red free (PRF) - DMEM containing 2% of the charcoal/dextran treated FBS (2% stripped serum) was added. The medium was changed two more times 20 and 30 hours later to remove the endogenous estrogens present in the cells. The total incubation period of the cells in the 2% stripped medium was 40 hours, similar to the conditions described in literature (Dubik,D. and Shiu, P., 1992). Finally, at the end of the 40 hours, fresh 2% stripped medium +/- drugs was added to the cells. Control cells (C) received 0.01% ETOH (vehicle), estradiol-treated cells (E2) received 2 nM estradiol, and cells treated with both estradiol and quinidine (Q+E2) received 2 nM estradiol + different concentrations of quinidine.

For Northern blot total cellular RNA was isolated 1 hour after addition of the drugs. Fifteen μg of RNA was loaded into each well and separated on 1.2% agarose-5.4% formaldehyde gels. The RNA transfer to nitrocellulose paper, hybridization procedure, and quantification of *c-myc* mRNA signals was performed as described in the experiment 2.1. As shown in the Figure 12, quinidine caused rapid concentration-dependent suppression of estradiol-induced *c-myc* mRNA levels in MCF-7 cells, with the maximum inhibition by 60% achieved with 90 μM quinidine.

For cell cycle analysis cells were harvested 30 hours after addition of the drugs. Cell nuclei were stained with propidium iodide and flow cytometry was performed as described in Materials and Methods. The results are shown in Figure 13. Most (80%) of the control cells were in G1/G0 phase of the cell cycle due to the low (2% stripped FBS) serum condition. Only 11% of the control cells progressed into the S phase. Two nM estradiol caused a significant increase in S phase cells (34 % of total) with a concomitant decrease in G1/G0 phase cell population (49% of total). Quinidine significantly (by 59%) suppressed estradiol-stimulated S phase progression. Seventy-five percent of Q+E2 cells were arrested in G1/G0, and only 14% reached S phase.

In summary, these results show that quinidine inhibits both estradiol-stimulated *c-myc* mRNA levels and G1 to S phase progression in MCF-7 cells. There was a good correlation between decreases in *c-myc* mRNA (60%) and S phase cells (59%) by quinidine, suggesting the important role of *c-myc* in quinidine-induced cell cycle arrest.

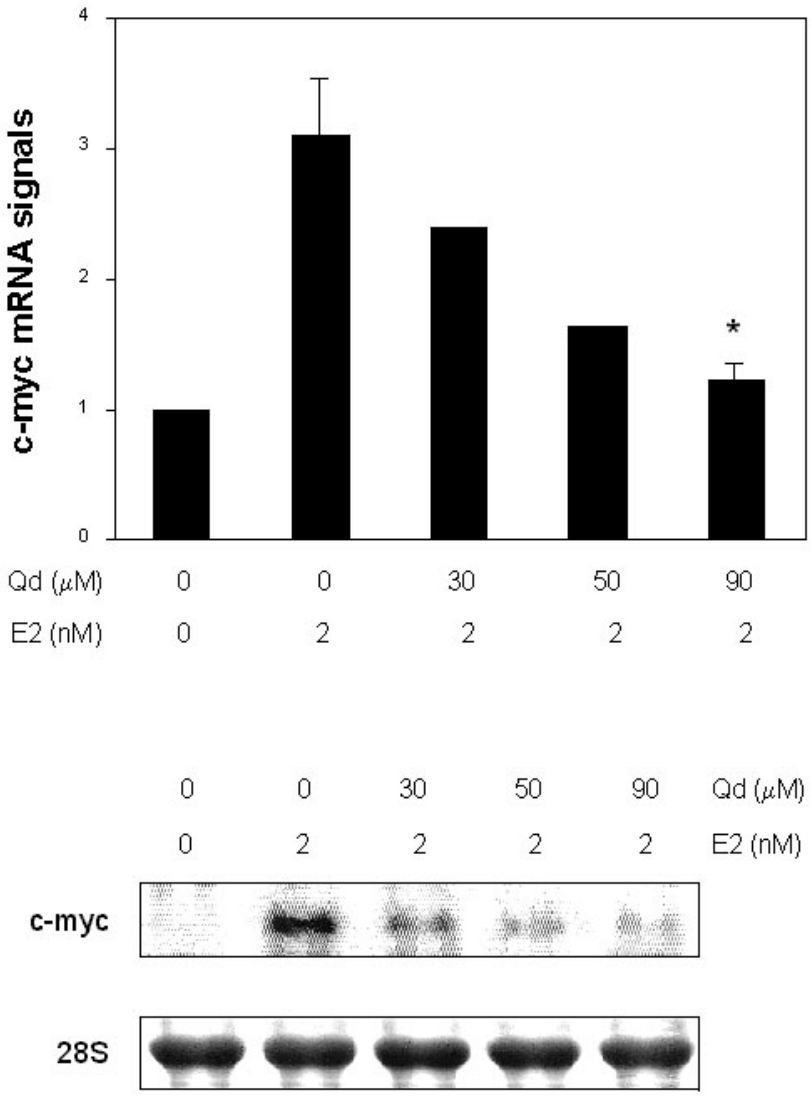


Figure 12. Suppression of estradiol-stimulated *c-myc* mRNA by quinidine.

Confluent MCF-7 cells were sub-cultivated in PRF-DMEM/2% stripped serum, and estrogen-depleted by replacing this medium twice during the total of 40 hours time period. Induction of *c-myc* mRNA by 2 nM estradiol +/- different concentrations of quinidine was measured after 1 hour by isolating total cell RNA and Northern blotting. Myc mRNA signals were normalized to the 28S ribosomal signal in the ethidium bromide stained gel. Data shown on the bar graph are the mean +/- S.D. of n = 3, except for 30 and 50 μM quinidine that are from a single experiment. Values for E2 and E2+Qnd treated groups are reported relative to control group (control=1). The differences between the percentages of estradiol- and quinidine+estradiol-treated cells were statistically significant for 90 μM quinidine ($p<0.05$).

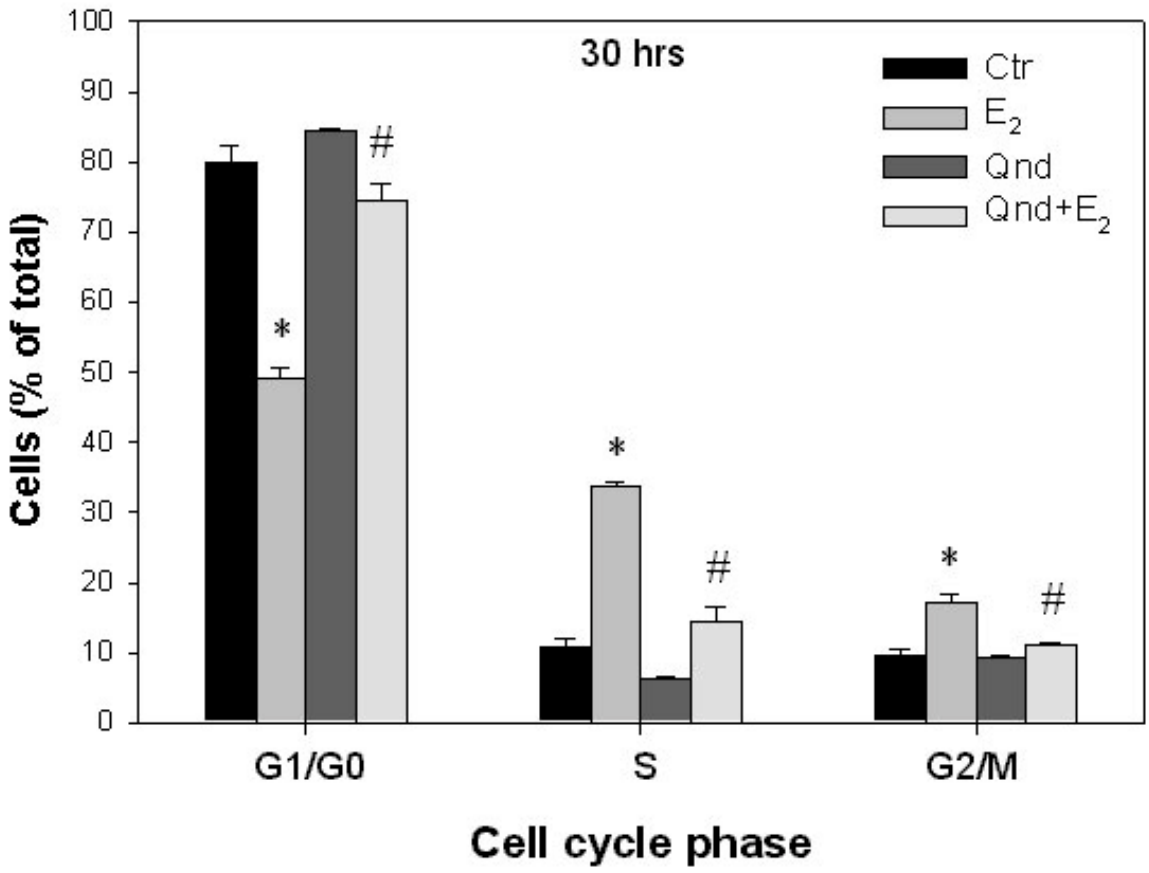


Figure 13. Suppression of estradiol-stimulated S phase progression by quinidine.

Confluent MCF-7 cells were sub-cultivated in PRF-DMEM/2% stripped serum, and estrogen-depleted by replacing this medium twice during the total of 40 hours time period. Cells were treated with Ctr-0.01% ETOH (vehicle), Qnd- 90 μM quinidine, E_2- 2nM estradiol, Qnd+E_2- 90 μM quinidine + 2nM E_2. Cell cycle phase distribution was analyzed by flow cytometry 30 hours later. Data shown on the bar graph are mean +/- S.D. of n = 4 experiments.

∗ Significantly different from control cells ($p<0.05$)

Significantly different from E2 cells ($p<0.05$)

3. Quinidine Inhibits *c-myc* Promoter Activity.

3.1. Quinidine inhibits *c-myc* promoter activity in MCF-7 cells

To determine whether the suppression of *c-myc* mRNA levels is mediated by effects of quinidine on the activity of *c-myc* promoter, transient transfection assays were performed in MCF-7 cells using a *myc*-luciferase plasmid. The construct containing 2.8 kb human *c-myc* promoter linked to the luciferase reporter gene (Del-1) was kindly provided by Dr. Bert Vogelstein, Johns Hopkins University. MCF-7 cells were plated in 5 ml DMEM/5%FBS at the density of 8 $x10^5$ cells per 60 mm^2 tissue culture dish. Seventeen hours later, the medium was replaced with PRF-DMEM/2% stripped FBS medium and, one hour later, transfections were started using DOTAP transfection reagent and 5 μg of plasmid DNA according to the procedure described in the Materials and Methods. Transfections were terminated in 6-7 hours by replacing the medium with fresh DMEM/5%FBS +/- different concentrations of quinidine. Cells were harvested 24 hours later, when the maximal levels of *myc*-driven luciferase activity occurred. Luciferase activity was measured in cell extracts containing 200 μg of proteins using an Auto Lumat (LB 953, EG and G-Bertholdt). Luciferase activity in the cells transfected with transfection reagent alone or with promoterless construct was always less then 0.01% of the activity in the control cells transfected with *myc*-luc construct. Light generation from purified luciferase was used as the standard in each experiment to insure that all determinations were performed under linear assay conditions. As demonstrated in Figure14, quinidine treatment caused a concentration-dependent decrease in the activity of the *c-myc* promoter. Ninety μM quinidine decreased *c-myc* promoter activity by 60%. This experiment provide an evidence that the activity of *c-myc* promoter is suppressed over the same range of quinidine concentrations that suppress levels of *c-myc* mRNA and protein.

The next goal was to identify the minimal region of the *c-myc* promoter, a putative quinidine responsive element (QRE), which is sufficient to confer quinidine responsiveness of the *c-myc* promoter.

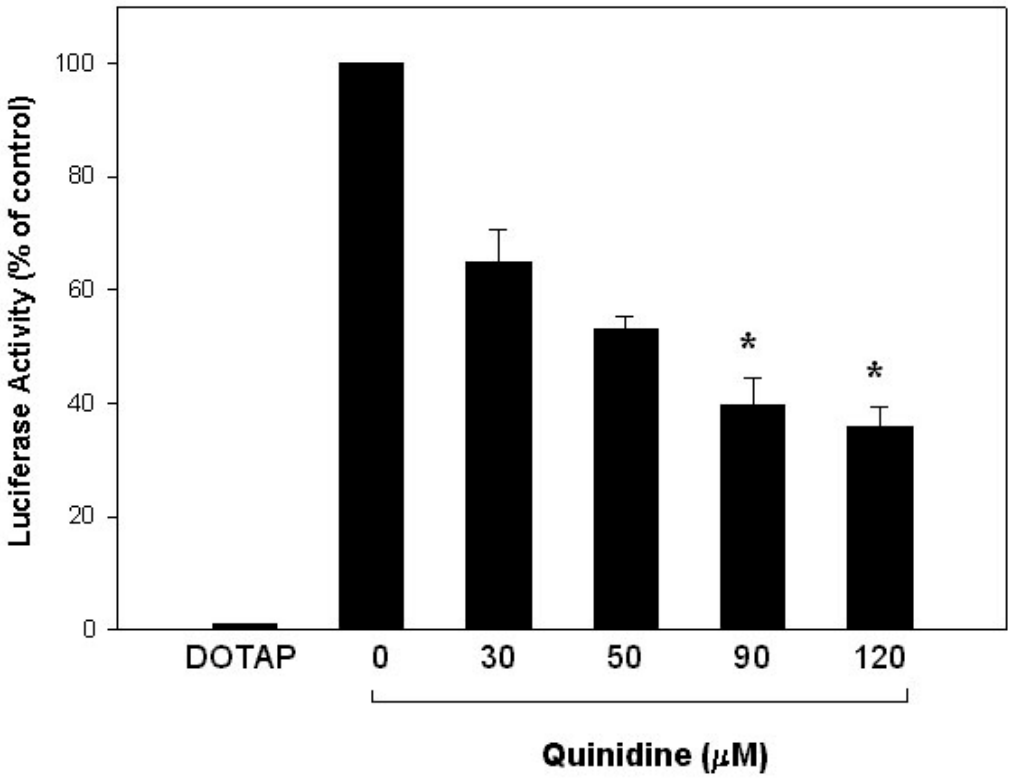


Figure 14. Quinidine suppresses *c-myc* promoter activity.

Confluent MCF-7 cells were sub-cultivated, allowed to attach, transferred into PRF-DMEM/2% stripped serum, and transfected with 5 μg/dish of 2.8 kb human *c-myc* promoter-luciferase reporter plasmid or with the transfection mix alone (DOTAP). At the end of transfection cells were incubated for 24 hours in DMEM/5%FBS + the indicated concentrations of quinidine before preparing cell extracts for luciferase assay. Data shown are the mean +/- S.D. of n =3 experiments (90 and 120 μM Qnd) and n=2 experiments (30 and 50μM Qnd). Luciferase activity was measured in extracts containing 200 μg of protein, and the data expressed as a percent of luciferase activity in control cells (100%). Luciferase activity in mock-transfected cells (DOTAP) was < 0.01% that of control cells.
∗ Significantly different from control (0 quinidine) cells ($p<0.05$)

3.2. QRE is located within 168 bp region of the *c-myc* promoter between -100 and +68 with respect to P1.

To determine the minimal region of the *c-myc* promoter that is sufficient for quinidine responsiveness, several *c-myc* promoter 5'-deletion mutants of the original Del-1 construct were used. The mutant constructs were kindly provided by Dr. Bert Vogelstein, Johns Hopkins University. Their structures are shown in Figure 15. These constructs were transiently transfected into MCF-7 cells and their relative luciferase activities in response to quinidine were assayed 24 hours later in an identical manner as described in 3.1. The basal (control) levels of *c-myc* promoter activity varied among these mutant constructs (Figure 15B), presumably due to the presence of different negative and positive regulatory elements. For example, the Del-1 has lower levels of basal luciferase activity compared to the Del-2 and Del-4, due to the presence of the negative repressor binding site in its promoter sequence that correlates with DH I (see Table 2) located 1851 bp upstream of the P1. The lower levels of basal promoter activity in the Frag-E compared to the Del-2 and Del-4 are explained by the deletion of the five tandem CT-boxes (CCCTCCCC) located 101 bp upstream P1. These CT-boxes are required to promote transcription from P1 and for maximal activity of P2 (see Table 3). In all these deletion constructs quinidine inhibited *c-myc* promoter activity by 60-70% (Figure 16A). In contrast, quinidine had a little effect on the promoterless construct (Luc, Figure 16B), suggesting that effects of quinidine are specific for *c-myc* promoter. The results from this experiment suggest that the putative QRE is located within 168 bp region of the Frag-E construct, since this region was sufficient to confer quinidine responsiveness of the *c-myc* promoter. This region contains two important cis-elements: the TGF β1 control element (TCE) and region from -60 to -37 that contains Sp1/Sp3 binding site (see Table 3).

The first element is involved in inhibition of *c-myc* P1 transcription initiation in response to TGF β1, whereas the presence of intact second element is essential for P1 transcription. The further fine mapping of QRE within the Frag-E using specific PCR primers could help in understanding the importance of these binding sites in the quinidine-mediated regulation of the *c-myc* promoter.

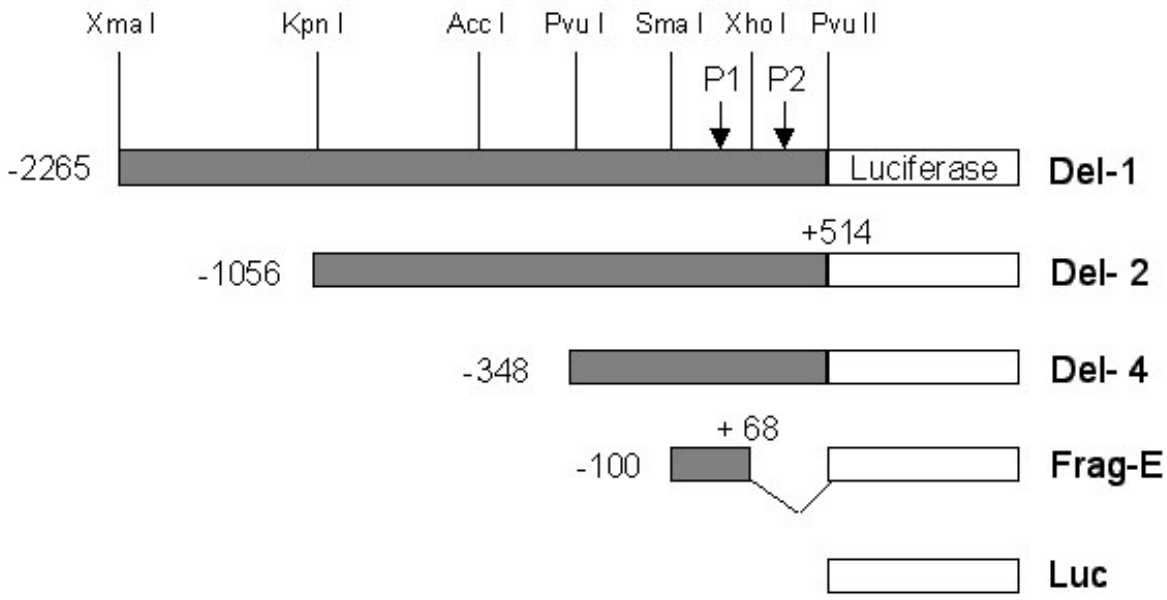


Figure 15. The structures of the 5'-deletion mutants of the original Del-1 construct.

The gray and white rectangles represent *c-myc* promoter and luciferase coding region, respectively. P1 and P2 are the sites of the transcription initiation from the respective promoters. The restriction enzymes and their cut sites are shown above the Del-1 construct. The locations of 5' and 3'-ends of the constructs are given with respect to the P1.

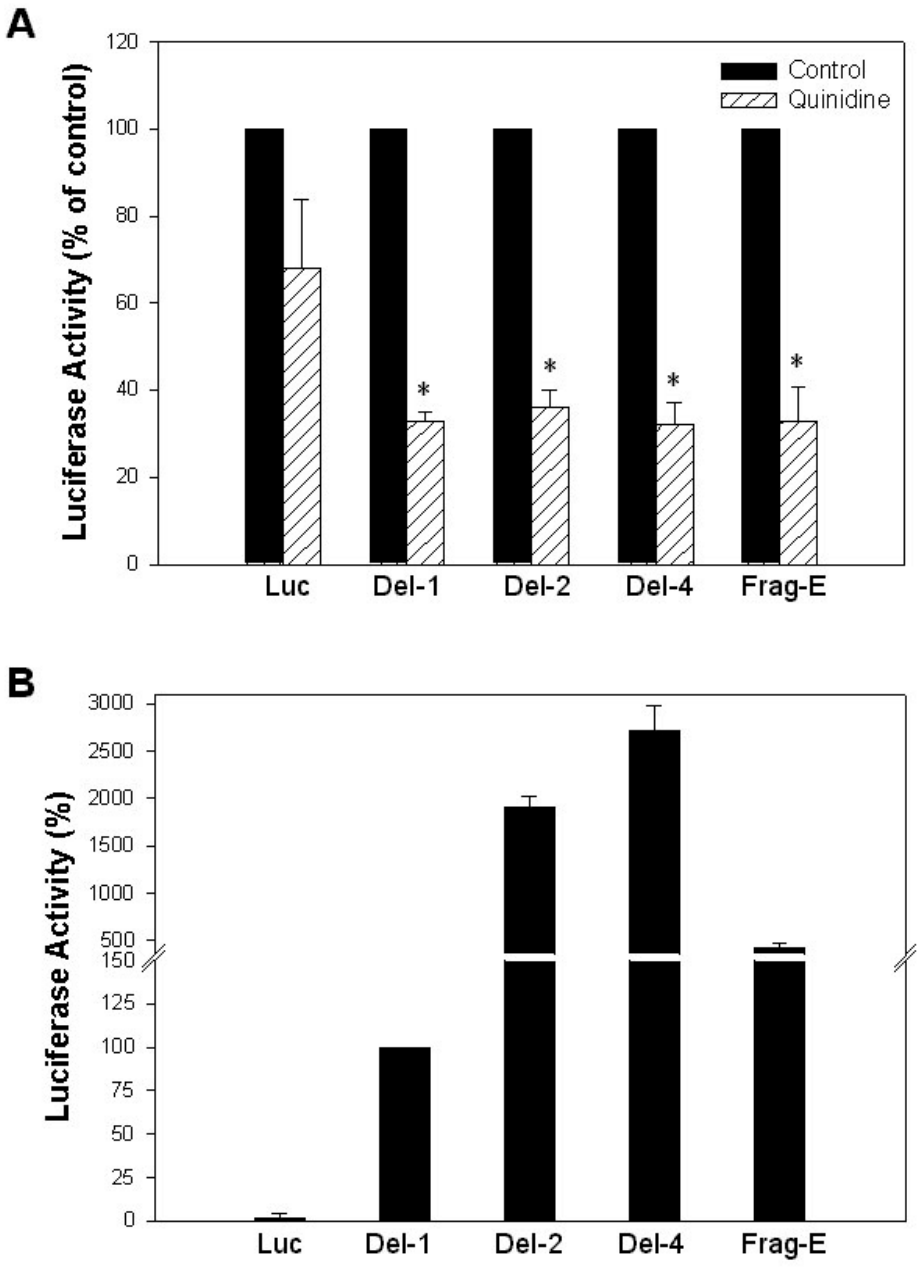


Figure 16. Inhibition of *c-myc* promoter activity in 5'-deletion mutants of the Del-1 by quinidine.

Confluent MCF-7 cells were sub-cultivated, allowed to attach, transferred into PRF-DMEM/2% stripped serum, and transfected with 5 μg/dish of the different *c-myc* mutant constructs. At the end of transfection cells were incubated for 24 hours in DMEM/5%FBS

+/- 90uM quinidine before preparing cell extracts for luciferase assay. Luciferase activity was measured in cell extracts containing 200 μg of protein.
A. Luciferase activities in quinidine-treated cells (hatched bars) are expressed as percent of respective controls (solid bars) for each construct. Data shown on the bar graph are the mean luciferase activity +/- S.D. of at least n=3 experiments.
∗ Significantly different from control values ($p<0.05$).

B. Luciferase activity in the cells transfected with Del-1 was set to 100%. Luciferase activities in cells transfected with other construct are expressed relative to the Del-1. Cells transfected with promoterless construct (Luc) always had luciferase activity less then 0.01% of the activity in the control cells transfected with *myc*-luc construct. Data shown in the bar graph represent the mean +/- S.D. of at least n=3 experiments.

3.3. Quinidine does not affect activity of the cyclin D1 promoter

To examine the specificity of the effects of quinidine on the *c-myc* promoter, the human cyclin D1 promoter linked to the luciferase reporter gene was used. MCF-7 cells were transiently transfected with cyclinD1-luc construct in a similar procedure as described for the *myc*-luc plasmids in the section 3.1. Transfection reactions were terminated by replacing the medium with the fresh DMEM/5%FBS +/- 90 μM quinidine. Twenty-four hours later, luciferase activity in cell extracts was measured. As shown in Table 4A, quinidine did not cause significant changes in the activity of the cyclin D1 promoter. This is an important control for the specificity of quinidine's effects on the *c-myc* promoter.

3.4. Quinidine does not affect enzymatic activity of purified luciferase protein

The reporter gene system allows one to measure the promoter activity of the gene of interest. In this system the promoter sequence is linked to the reporter gene coding sequence. As a result, any changes in the promoter activity would cause the corresponding changes in the levels of the reporter protein, e.g. luciferase. The changes in promoter activity of the gene of interest are measured indirectly by measuring enzymatic activity of the reporter protein. Therefore, it is important to demonstrate that changes in the activity of particular promoter in response to the drug treatment are not due to the direct effect of that drug on the enzymatic activity of the reporter protein. In order to show that quinidine has no effect on the activity of luciferase protein, the extracts of the control and quinidine-treated MCF-7 cells were used. One hundred pg of purified luciferase protein was added to both cell extracts and the luciferase activities in these samples were assayed in an identical manner as described for the *myc*-luciferase construct in section 3.1. As shown in Table 4B, quinidine had no effect on the

enzymatic activity of the purified luciferase protein. These results are important, because they demonstrate that any quinidine-induced changes in the luciferase activity in the extracts of cells transfected with the *myc*-luciferase construct (see Figure 14 and 16) are likely to be due to the corresponding changes in the activity of the *c-myc* promoter.

A. Effect of quinidine on the cyclin D1 promoter activity

Treatment	**Luciferase Activity (% of control)**
Control	100
Quinidine	120 (+/- 15.3)

B. Effect of quinidine on the enzymatic activity of purified luciferase

Treatment	**Luciferase Activity (% of control)**
Control	100
Quinidine	99 (+/- 1)

Table 4. Effect of quinidine on the cyclin D1 promoter and purified luciferase activity.

Confluent MCF-7 cells were sub-cultivated, transferred into PRF-DMEM/2% stripped serum, and transfected with 5μg/dish of the human cyclin D1-luciferase plasmid (A) or with transfection reagent alone (B). Transfected cells were incubated for 24 hours in DMEM/5%FBS medium +/- 90 μM quinidine before preparing cell extracts for the luciferase assay.

A. Luciferase activity was measured in extracts containing 200 μg of protein. Luciferase activity in the quinidine-treated cell extracts is expressed as a percent of that in the control cells (100%). Data shown are the mean +/- S.D. of n = 4 experiments.

B. 100 pg of the purified luciferase protein were added to the control and quinidine-treated cell extracts containing 200 μg of proteins before luciferase activity was measured. Luciferase activity in the quinidine-treated cell extracts is expressed as a percent of that in the control cells (100%). Data shown are the mean +/- S.D. of n = 3 experiments.

3.5. The role of TGFβ1 control element and Sp1/Sp3 binding site of a QRR in the regulation of *c-myc* promoter by quinidine

The results from the experiment 3.2 suggest that putative QRE is located within 168 bp region of the *c-myc* promoter. This region contains two important cis-elements: the TGFβ1 control element (TCE) and GC-rich region from -60 to -37 that contains Sp1/Sp3 binding site (see Table 3). In the next series of experiments we tested the role of these elements in the regulation of *c-myc* promoter by quinidine. In general, the regulation of gene promoter by particular agent via a cis-element can be achieved by several mechanisms, some of which are listed here: 1) by changing the binding of trans-acting factors to the element, without altering their protein levels, 2) by changing the protein levels of trans-acting factors, 3) by binding directly to the cis-element, preventing further binding of trans-acting factors.

Effects of quinidine on Sp1 and Sp3 protein levels in MCF-7 cells. The goal of this experiment was to test if quinidine alters the levels of Sp1 and Sp3 proteins. Confluent MCF-7 cells were re-plated in 60 mm^2 dishes in 5 ml DMEM-5 %FBS at the density of $1x10^6$ cells/dish and treated with 90 μM quinidine or H_2O (vehicle). Cells were harvested 90 min later for Western blot analysis. Equal amounts of proteins (10 μg for Sp1 and 20 μg for Sp3 gel) were loaded into each well of 7.5 % polyacrylamide gels. As shown in Figure 17, quinidine didn't change the levels of either protein. The results indicate that suppression of *c-myc* promoter by quinidine is not mediated by the changes in Sp1/Sp3 protein levels. This does not exclude the possibility of Sp1/Sp3 involvement in regulation of *c-myc* promoter by quinidine, since binding of these proteins to the GC-rich region of QRR but not their levels could be responsive to quinidine. Sp1 and Sp3 are ubiquitous transcription factors that are involved in regulation of a number of genes. Hence, changes in the levels of Sp1/Sp3

proteins will affect expression pattern of all those genes. The Sp1 transcription factor can regulate transcription by at least two mechanisms: 1) binding GC-rich sequence directly or 2) indirectly interacting with other transcription factors to alter gene transcription (Porter, W. et al., 1996). For example, Sp1 can form a complex with the ER, which then binds to DNA regions containing both Sp1 elements and ERE-half sits (Porter, W. et al., 1996; Wu-Peng, X. et al., 1992). Therefore, transactivating activity of Sp1 factor may be influenced by the presence of other responsive elements next to the GC-rich region. Sequence analysis of *cyclin D1* promoter revealed a presence of two consecutive Sp1 binding sites between base pairs –113 and –102. This Sp1-binding region has also been shown to bind Egr-1 (early growth response protein-1) transcription factor. A dual interplay between Egr-1 and Sp1 response elements has been described for a number of promoters (Khachigian, L. et al., 1995; Skerka, C. et al., 1995; Ebert, S. and Wong, D., 1995) including cyclin D1 promoter (Guillemot, L. et al, 2001). In this model Egr-1 displaces Sp1 at the overlapping motifs to activate transcription of its target genes (Khachigian, L. et al., 1995; Cui, M. et al., 1996) In my experiments quinidine had a minimal effect on *cyclin D1* promoter activity, indicating that quinidine does not affect binding of Sp1 protein to the *cyclin D1* promoter. The preferential regulation of Sp1/DNA interaction in *c-myc* but not *cyclin D1* promoter by quinidine could be explained by the overlapping nature of Sp1/Egr sites of *cyclin D1* promoter. This issue can be resolved by examining the changes in Sp1/DNA interaction of *c-myc* and *cyclin D1* promoters in response to quinidine using electrophoretic mobility shift assay.

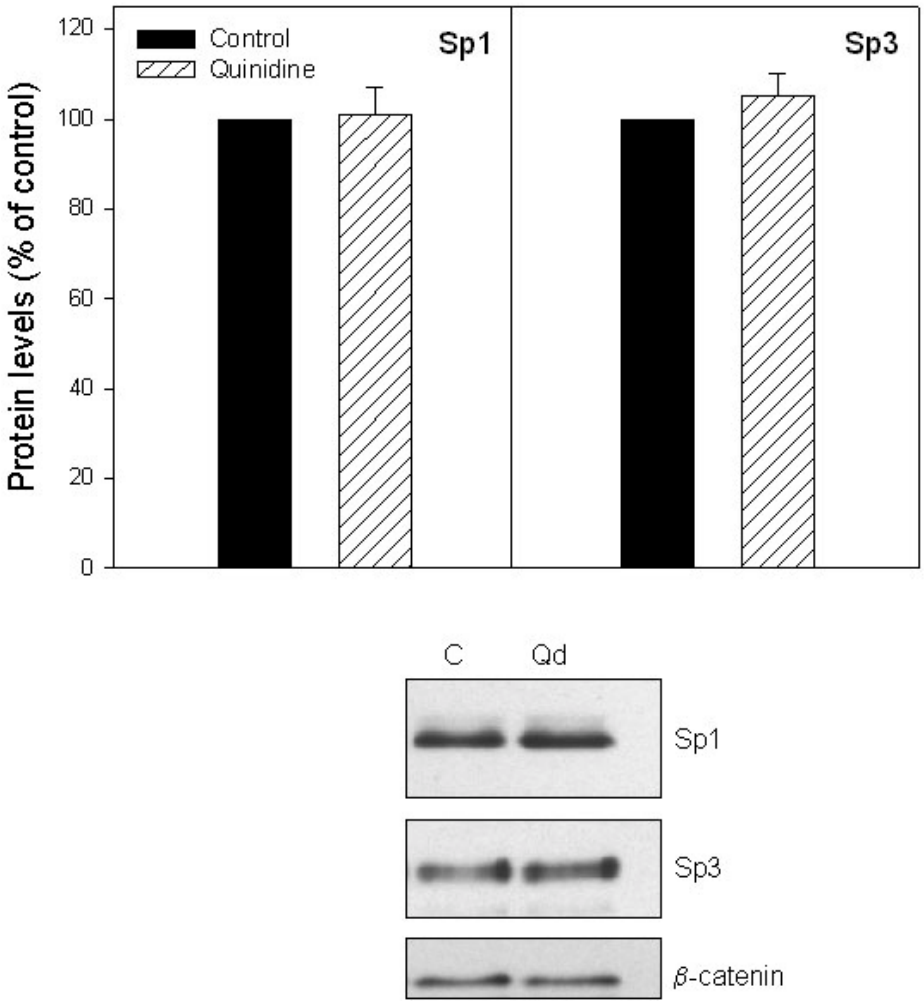


Figure 17. Quinidine does not change the levels of Sp1 and Sp3 proteins.

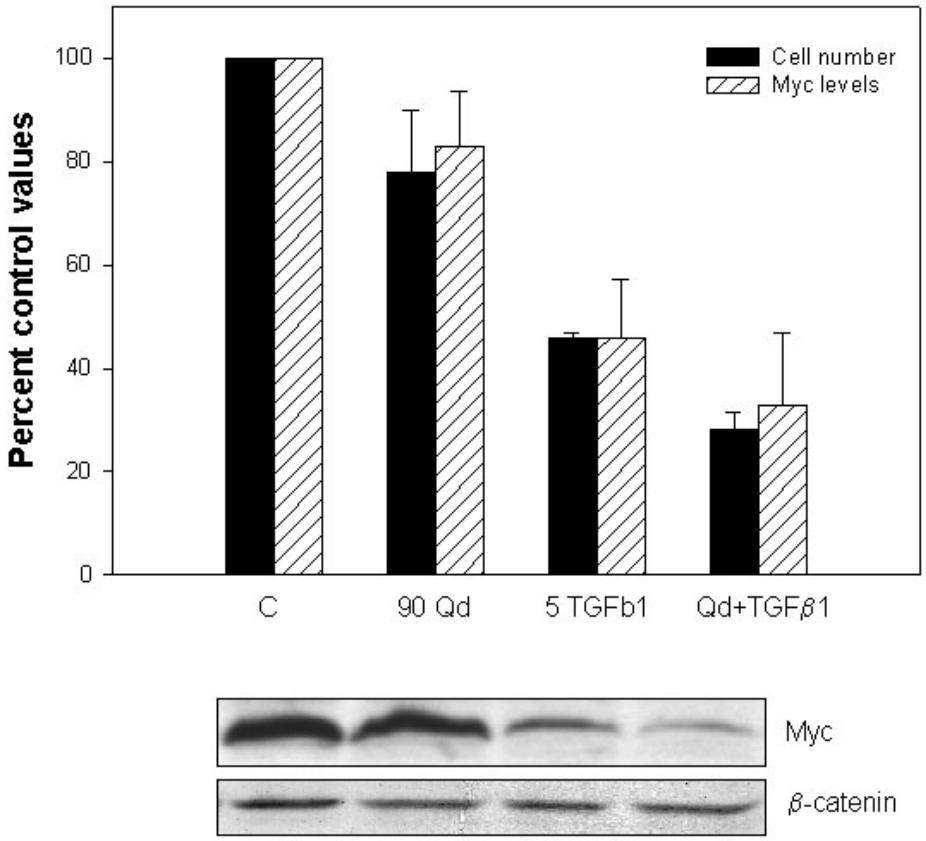
Confluent MCF-7 cells were sub-cultivated in DMEM/5% FBS +/- 90 uM quinidine. Cells were harvested 90 min later and Sp1/Sp3 proteins levels after Western blot assay were determined by densitometry. The β-catenin signals were used as a loading control. The bar graph shows Sp1 (left panel) and Sp3 (right panel) proteins levels in control (solid bars) and quinidine-treated (hatched bars) cells as the mean +/- S.D. of n=2 experiments.

The role of TCE in regulation of *c-myc* promoter by quinidine. Normal human mammary epithelial cells, MCF10A, respond to TGFβ1 treatment by rapid (within 2 hours) decrease in Myc protein levels and subsequent inhibition of cell growth. These effects are abolished in breast cancer cell lines and in MCF10A cells transformed with Ras/ErbB2 oncogenes (Chen, C. et al, 2001). Down-regulation of *c-myc* promoter activity through TCE has been reported to play a key role in TGFβ1 mediated growth inhibition in different cell lines (Pietenpol, J. et al, 1991; Chen, C. et al, 2001). In order to test the role of TCE in the regulation of *c-myc* promoter by quinidine, a series of experiments were performed to compare effects of quinidine and TGFβ1 on proliferation and Myc protein levels in different cell lines.

MCF10A cells were re-plated from confluent T-75 flasks at the density of 6 x 10^5 cells/60 mm^2 (for Western blot) or 1 x 10^5 cells/35 mm^2 (for cell count) tissue culture dishes in 5 or 3 ml MEGM medium, respectively. Cells were treated with: vehicle (4mM HCl, 1mg/ml BSA - 1μl/ml of medium), 90 μM quinidine, 5ng/ml TGFβ1, 90 μm quinidine + 5ng/ml TGFβ1. For Western blot assay, cells were harvested 24 hours later and equal amounts of proteins (60 or 70 μg) for each sample were separated on 10% polyacrylamide gels. For cell count assay, cells were harvested 72 hours later. As shown in Figure 18, quinidine had little effect on cell number and Myc protein levels, whereas TGFβ1 caused a significant reduction of both. Quinidine+TGFβ1 had additive effects on cell number and Myc levels, which indicates that these agents work through the different pathways. Also, quinidine, but not TGFβ1, inhibited growth of MDA-468, MDA-231, and MCF-7 breast cancer cells (Figure 19). MDA-468 cell line has a defective TGFβ1 pathway due to the lack of an important mediator of TGFβ1 signaling, Smad4 transcription factor (Chen, C. et al,

2001). Jointly, these results suggest that regulation of *c-myc* and subsequently cell growth by quinidine and TGFβ1 is mediated by different mechanisms in the described cell lines.

In summary, the results presented in this section imply that suppression of *c-myc* promoter by quinidine is not mediated through TGFβ1 responsive element (TCE), due to the different responses of cells to TGFβ1 and quinidine (Figures 18 and 19). By eliminating TCE as a mediator of quinidine's responses on *c-myc* promoter, the only other known regulatory element remaining within the proposed 168 bp QRE is the GC-rich region. This region has been shown to be essential for the transcription from the P1 promoter and can bind Sp1/Sp3 transcription factors. Our results showed that quinidine does not affect the levels of these proteins (Figure 17). It is possible, that binding of these factors to the GC-rich region, but not their levels are altered by quinidine.



	Contorl	90 uM quinidne	5ng/ml TGFb1	90 Qd + 5TGFb1
Cell count data (% of control)	100	78 (12)	46 (0.9)	28 (3.4)
Myc protein levels (% of control)	100	83 (10.6)	46 (11.2)	33 (14.5)

Figure 18. Quinidine and TGFβ1 have distinct effects on proliferation and Myc protein levels in MCF10A cells.

Confluent MCF10A cells were sub-cultivated in MEGM +/- vehicle (4mM HCl, 1mg/ml BSA - 0.5 µl/ml of medium), 90 µM quinidine, 5ng/ml TGFβ1, 90 µm quinidine + 5ng/ml TGFβ1. Cells were harvested 24 (Western blot assay) and 72 (cell count assay) hours later. Myc protein levels were determined by densitometry and normalized to the β-catenin signals. The bar graph shows cell number (solid bars) and Myc protein levels (hatched bars) in control and treated cells. The data in the table represent mean +/- STDEV of n=2 experiments.

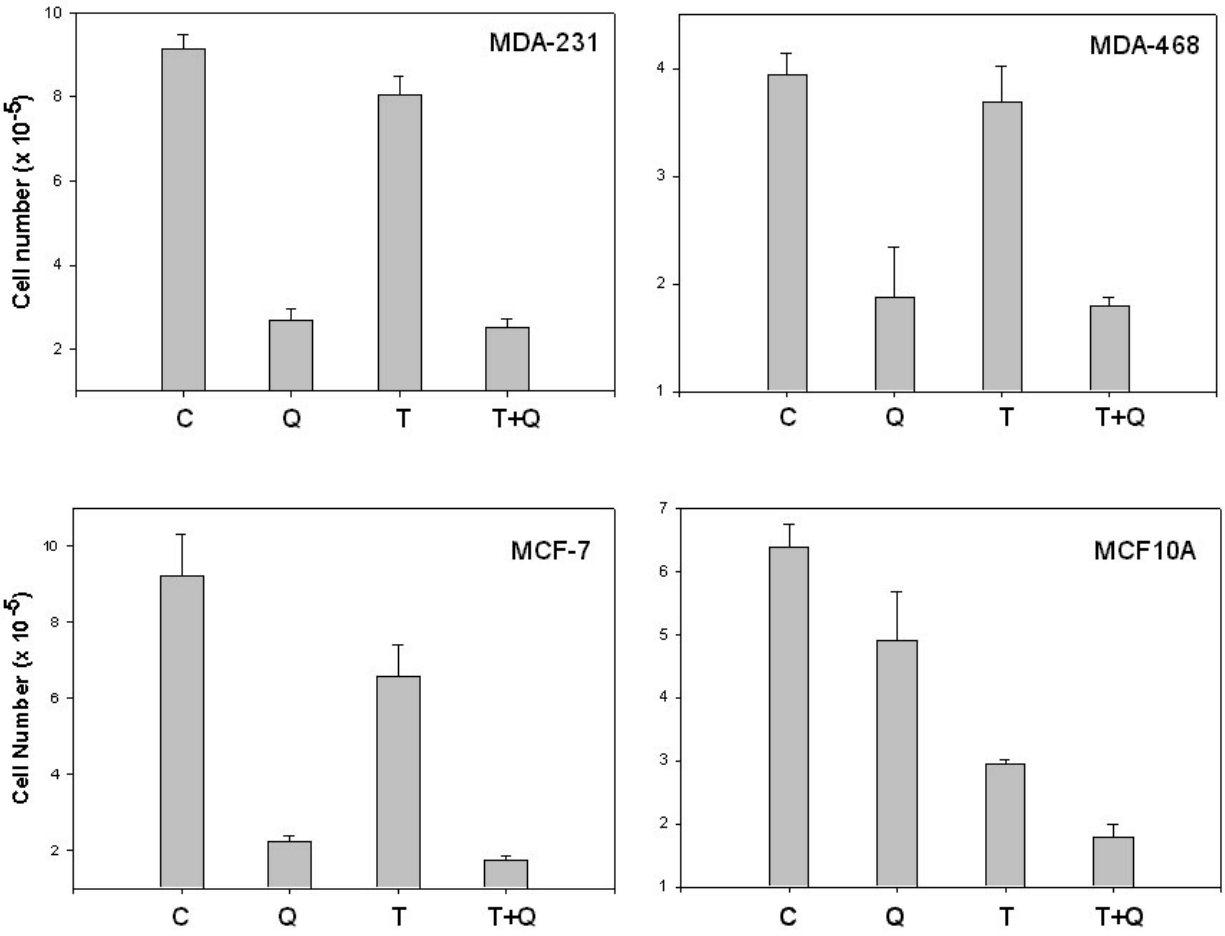


Figure 19. Quinidine and TGFβ1 have distinct effects on proliferation in normal (MCF10A) and tumor (MDA-231, MDA-468, MCF-7) breast epithelial cell lines.

Confluent cultures of MDA-231, MDA-468, MCF-7, and MCF10A cells were sub-cultivated at the density of $1x10^5$ (MDA-231, MDA-468, MCF10A) or $2x10^5$ (MCF-7) per 35 mm^2 tissue culture dish in the presence of: C-vehicle (4mM HCl, 1mg/ml BSA - 0.5 μl/ml of medium), Q- 90 μM quinidine, T-5ng/ml TGFβ1, T+Q-90 μm quinidine + 5ng/ml TGFβ1. Cells were harvested 72 or 96 (for MCF-7) hours later and counted by hemocytometer. The bar graphs show cell numbers as mean+/- STDEV of n=2 experiments.

4. Quinidine Inhibits Growth and Induces More Differentiated phenotype in Human Breast Cancer Cells

Another objective of this study was to determine the long-term effects of quinidine in breast cancer cells. *c-myc* plays an important role in cellular differentiation. It has been shown in many cells that down-regulation of *c-myc* accompanies terminal differentiation and permanent withdrawal from the cell cycle (Canelles, M. et al, 1997; Griep, A and Westphal, H., 1988; Holt, J. e al, 1988; Prochownik, E. et al, 1988). My results demonstrate that quinidine inhibits Myc protein and mRNA levels in human breast cancer cells, probably by regulating the activity of *c-myc* promoter. Therefore, we tested whether quinidine also induces differentiation in these cells. The effects of quinidine on Ki-67 antigen expression and accumulation of the cytoplasmic lipid droplets have been tested.

4.1. Quinidine decreases the percentage of the MCF-7 cells expressing the Ki-67 antigen

Ki-67 is a nuclear protein found in cells that are actively engaged in the cell cycle (van Dierendonck, et al, 1989). The turnover rate of Ki-67 protein is less than 1 hour (Bruno and Darzynkeiwicz, 1992); therefore, its absence from cells is an indication that cells have exited the cell cycle and entered G0 or non-proliferative state that is associated with the cellular differentiation.

To test the effects of quinidine on the expression of Ki-67 protein, MCF-7 cells were replica plated at the density of 2 x 10^5/35 mm^2 tissue culture dish in 3 ml of DMEM/5%FBS medium +/- 90 μM quinidine. Twelve, 18, 24, 30, 48, and 72 hours later cells were fixed and stained for Ki-67 immunoreactivity as detailed in the Materials and Methods. Figure 20

shows a representative picture of the control and quinidine-treated MCF-7 cells after immunohistochemical staining of Ki-67 antigen (brown color) and counter-staining with Mayer's hematoxylin (blue color). Brown cells (Ki-67-positive) represent cell population in G1, S, G2, and M phase of the cell cycle. Blue cells (Ki-67-negative) represent the population of cells in G0 or non-proliferative state. The Ki-67-negative and positive cells were scored using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). A minimum of 500 cells were counted per experimental condition. The results are shown in Figure 21. The quinidine-treated cells showed a significant increase in Ki-67 negative cells between 48 and 72 hours, compared to the control cells. These data suggest that quinidine-treated MCF-7 cells begin to accumulate in the G0 state after 24 hours of treatment.

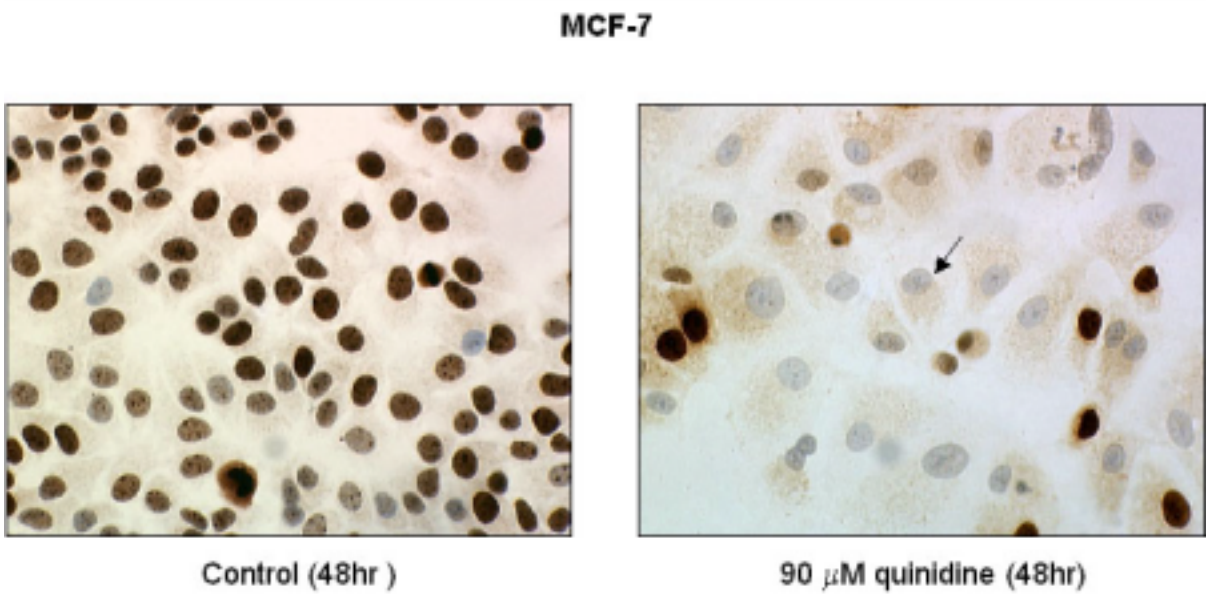


Figure 20. Ki-67 immunohistochemical staining of MCF-7 cells.

Control or 90 μM quinidine-treated MCF-7 cells were grown for 48 hours on the glass cover slips and assayed for Ki-67 immunoreactivity. Brown staining indicates Ki-67 positive or cycling cells. Blue staining indicates Ki-67 negative or G0 cells.

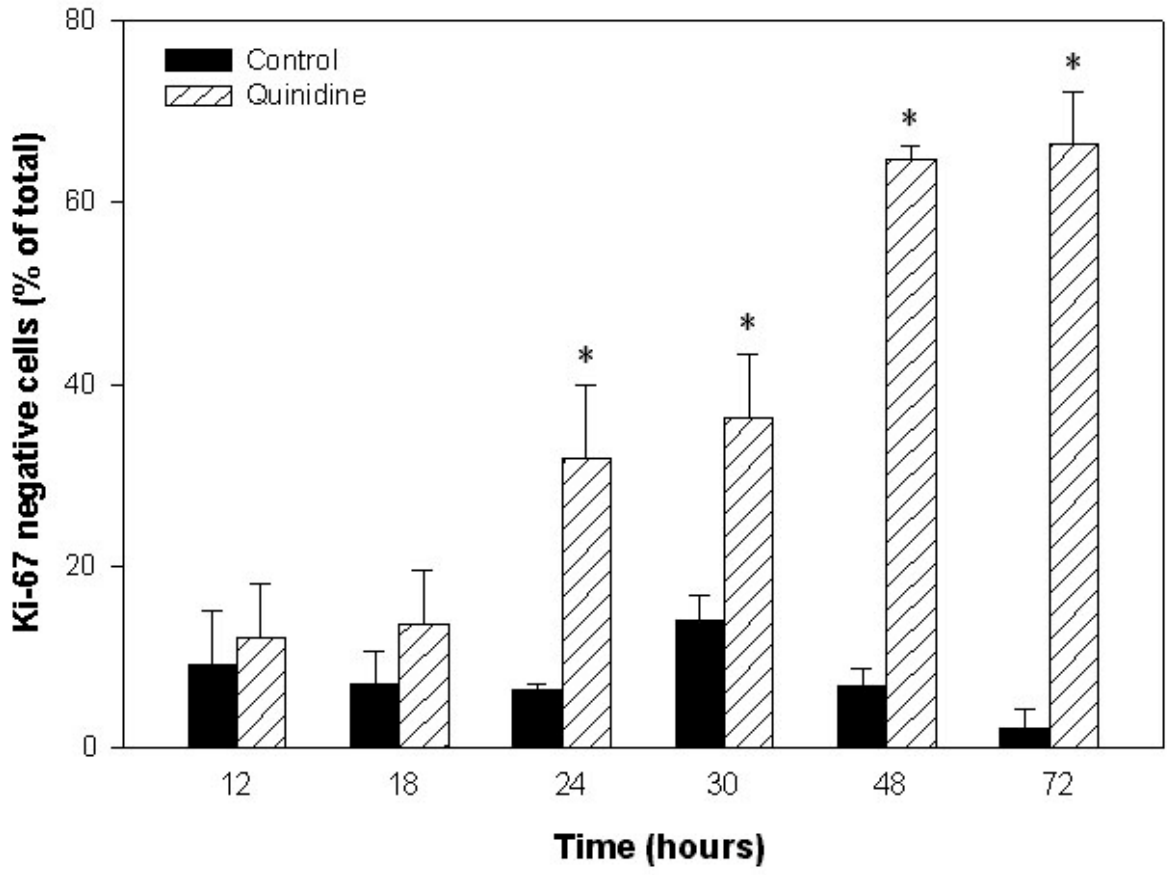


Figure 21. Increase in G0 cell population in MCF-7 cells treated with quinidine.

Control or 90μM quinidine-treated MCF-7 cells were grown on the glass cover slips. At the indicated times, cells were stained for Ki-67 immunoreactivity, and Ki-67 positive and negative cells were counted. Between 200 and 700 cells were counted per experimental condition. The bars represent the percentage of Ki-67 negative cells in control (solid bars) and quinidine-treated cells (hatched bars). The data are mean +/- SEM of two to five experiments.

∗ Significantly different from control values ($p<0.05$).

Wang, S., et al, 1998.

4.2. Quinidine causes accumulation of cytoplasmic lipid droplets in MCF-7 cells.

Lipid droplets are found in the cytoplasm of normal mammary epithelium (Jing, Y. et al, 1996), and cytoplasmic lipid droplet accumulation occurs in a variety of differentiating cell systems. Induction of differentiation in human breast cancer cell lines by retinoic acid (Bacus. S. et al, 1990), the vitamin D analog, 1-α- hydroxyvitamin D5 (Mehta, R. et al, 2000), and oncostatin M (Douglas, A. et al, 1998) is accompanied by the accumulation of cytoplasmic lipid droplets.

To determine whether quinidine treatment induces the accumulation of cytoplasmic lipid droplets, the MCF-7 cells were replica plated at the density of 2 x 10^5/35 mm^2 tissue culture dish in 3 ml of DMEM/5%FBS medium +/- 90 μM quinidine. Seventy-two, 96, and 120 hours later cells were fixed and the Oil-Red O staining of the cells was performed as described in the Material and Methods. Figure 22 shows a representative picture of the control and quinidine-treated MCF-7 cells after the Oil-Red O staining and counter-staining with Mayer's hematoxylin to visualize the cytoplasmic lipid droplets (red color) and the cell nuclei (blue color), respectively. The Oil-Red O staining of the normal human mammary epithelial cells (HMEC), performed in an identical manner as for MCF-7 cells is also shown in the Figure 21. This picture demonstrates the morphological similarities between the quinidine-treated MCF-7 cells and untreated HMEC, implying that quinidine-treated tumor cells acquire a more differentiated phenotype. MCF-7 cells were scored as positive or negative for the presence of cytoplasmic lipid droplets using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). A minimum of 500 cells was counted per experimental condition. The results are shown in Figure 23. Quinidine caused a significant increase in lipid

droplets-positive MCF-7 cells at 70, 96, and 120 hours of treatment, compared to the control cells.

In summary, the presented data show that quinidine treatment after 24 hours caused accumulation of MCF-7 cells in G0 state with the induction of the cytoplasmic lipid droplets after 48 hours. Since in general these changes are observed during cellular differentiation, we conclude, that quinidine induces a more differentiated phenotype in MCF-7 human breast cancer cells.

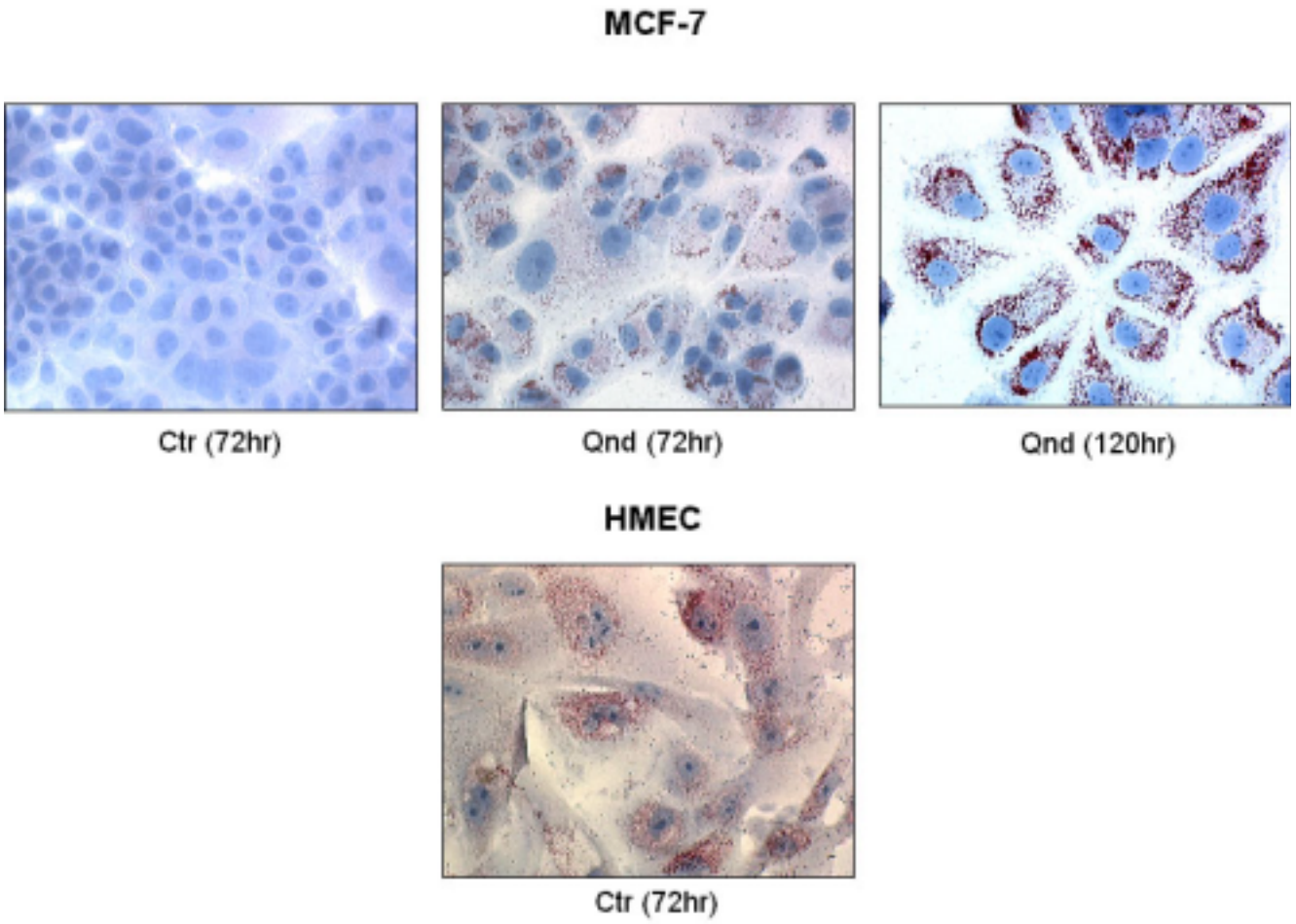


Figure 22. Oil Red O staining of the MCF-7 and HMEC.

Control or 90 μM quinidine-treated cells were grown on glass cover slips. At the indicated times cells were stained with the Oil Red O stain (red dots in the cytoplasm) and counterstained with the Mayer's hematoxylin (blue nuclei).

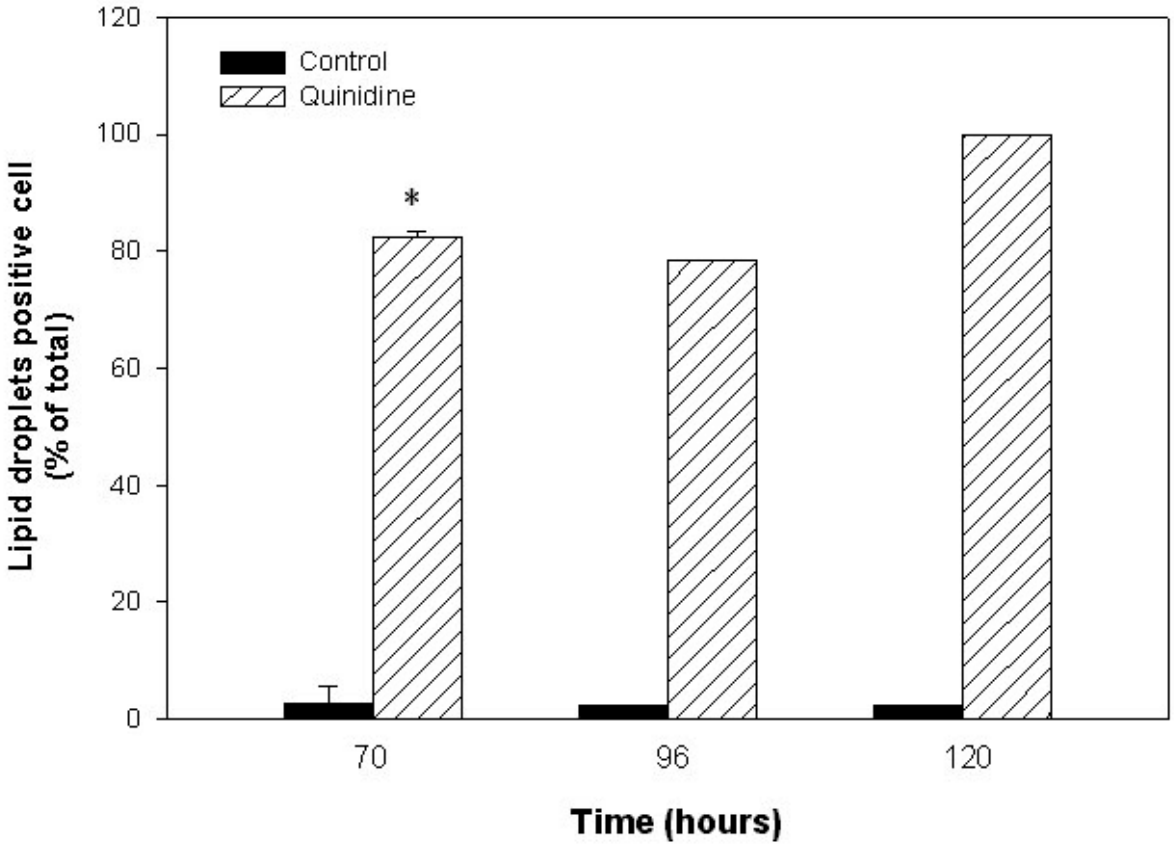


Figure 23. Quinidine causes accumulation of cytoplasmic lipid droplets in the MCF-7 cells.

Control or 90 μM quinidine-treated MCF-7 cells were grown on the glass cover slips. At the indicated times cells were stained with Oil Red O to monitor accumulation of cytoplasmic lipid droplets. Lipid droplet positive and negative cells were counted. The bars represent the percentage of lipid droplet positive cells in control (solid bars) and quinidine-treated (hatched bars) cells. At least 500 cells were counted per experimental condition. Data shown in the histogram are the mean +/- S.D. of n = 3 experiments for 70 hours and results from single experiments for 96 and 120 hours.

∗ Significantly different from control values ($p<0.05$).

4.3. Quinidine induces more differentiated phenotype in other breast cancer cell lines.

The objective of this experiment was to test if quinidine induces more differentiated phenotype in other breast tumor cell lines. MCF-7, T47D, MDA-231, and MDA-435 cells were replica-plated at the densities of 0.8-3 x 10^5/35 mm^2 tissue culture dish in 3 ml of DMEM/5%FBS +/- 90 μM quinidine or 10 μM retinoic acid for 96 hours. The retinoic acid has been shown to induce differentiation in human breast cancer cells (Bacus. S. et al, 1990). Therefore, it was used as a positive control in this experiment. The Nile Red fluorescent stain, instead of the Oil Red O, was used to monitor lipid droplet formation in response to quinidine. The cells were counterstained with fluorescein-conjugated antibodies to phalloidin that bind actin filaments to assay for the changes in the actin cytoskeleton. The results are shown in Figure 24. Lipid droplet accumulation was weak or absent in the control cell lines and increased by retinoic acid and quinidine. Lipid droplets 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.

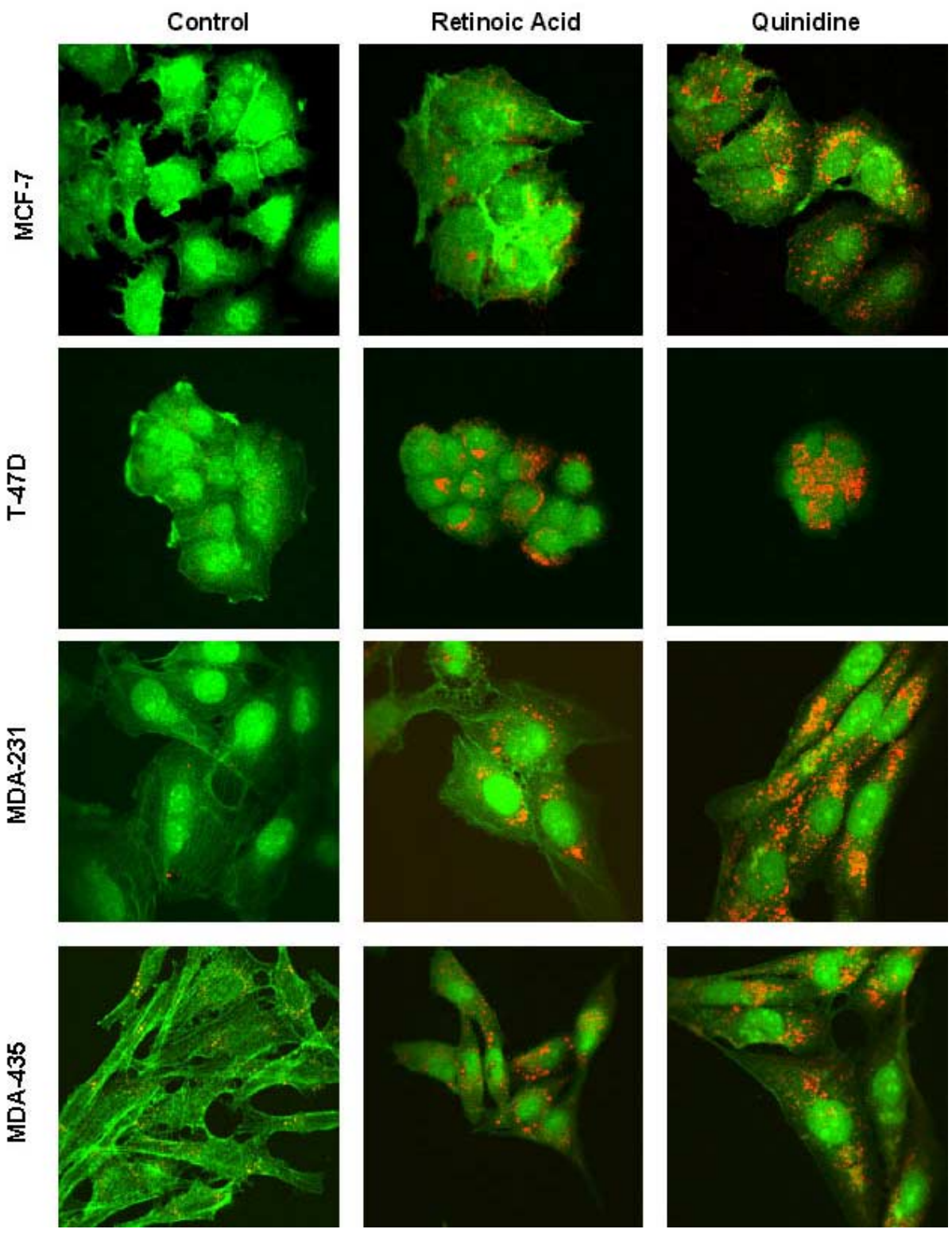


Figure 24. Accumulation of cytoplasmic lipid droplets in mammary tumor cell lines in response to quinidine.

MCF-7, T47D, MDA-231, and MDA-435 cells were grown on glass cover slips in medium containing 0.01% ethanol (control), 10 μM retinoic acid, or 90 μM quinidine. After 96 hours, cells were fixed and stained with the Nile Red and fluorescein-phalloidin to identify lipid droplets (orange color) and actin filaments (green color), respectively. Images were obtained by confocal microscopy.

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4.4. Quinidine inhibits proliferation in human breast tumor cell lines

The data presented above suggest that quinidine induces differentiation in breast tumor cell lines. In general, the process of cellular differentiation is associated with an exit from the cell cycle and cessation of proliferation. Therefore, we next tested if quinidine also inhibits the growth in human mammary tumor cells. The effects of quinidine on cell growth was assayed by two different methods. In the first method breast cancer (MCF-7, MDA-231, MDA-435) and normal (HMEC, MCF10A) cells were replica-plated at the density of 1-1.5 $x10^5$/35 mm^2 dish in the DMEM/5% FBS (tumor cells) of MEGM (normal cells) medium +/- 90 μM quinidine. Cell numbers were counted using a hemocytometer as described in the Materials and Methods. In the second method, cells were plated in a 96-well dishes at the following densities, depending upon the relative growth rates of the cell lines: HMEC (2000), MCF10A (1000), MCF-7 (1000), MDA-231 (500), T47D (1500). Cell growth was monitored using the MTS enzymatic assay as detailed in the Materials and Methods. The results are shown in Figure 25. Quinidine suppressed growth in all breast tumor cell lines, while having no effect in normal breast epithelial cells.

The data presented above suggest that quinidine induces more differentiated phenotype and selectively inhibits cell growth in four different tumor, but not in normal breast epithelial cell lines.

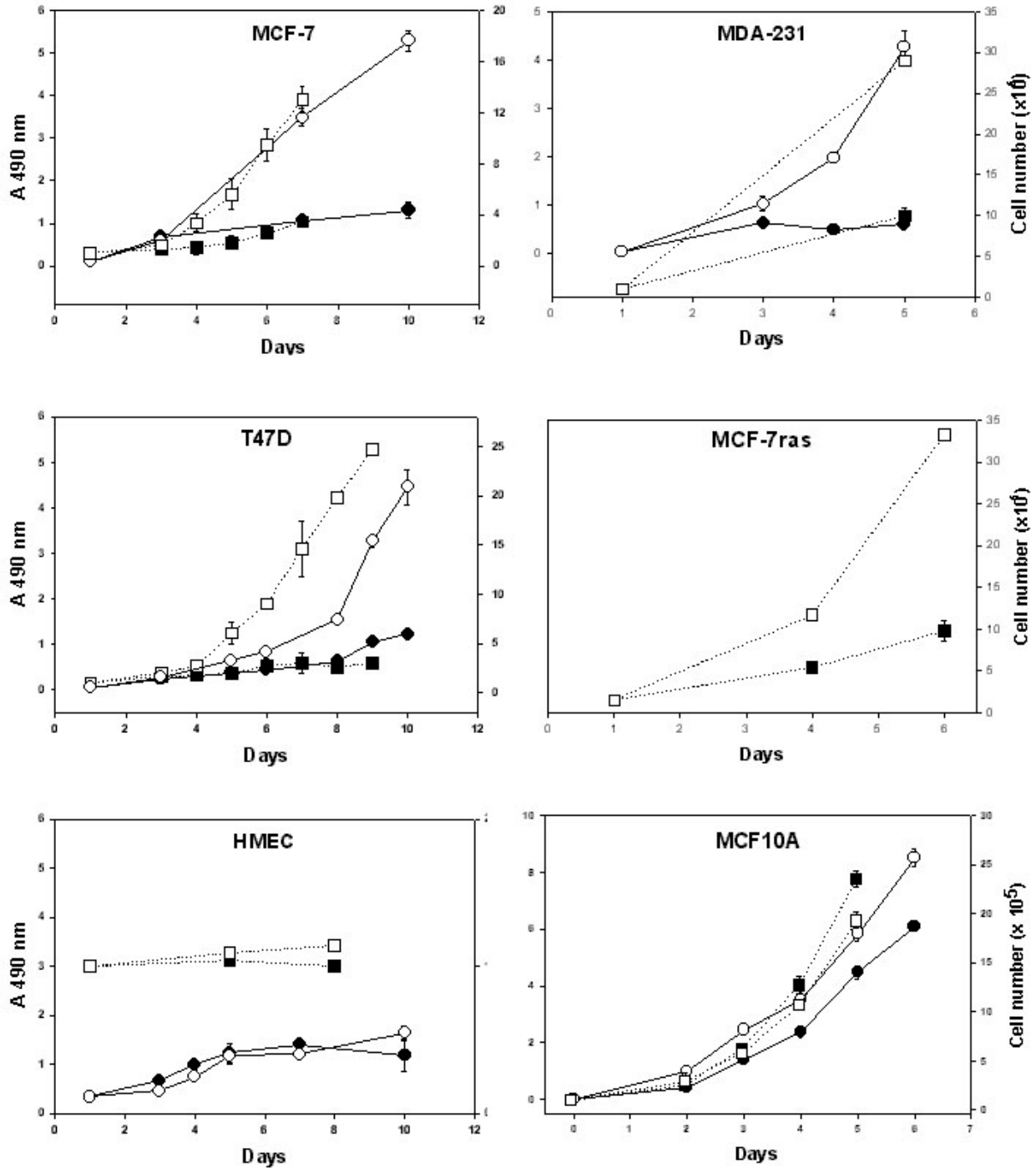

Figure 25. Quinidine selectively inhibits growth in human breast tumor, but not in normal cell lines.

MCF-7, MDA-231, T47D, MCF-7ras tumor cells, and normal HMEC and MCF10A cells were replica-plated in 96-well plates for the MTS assay, or in 35 mm^2 dishes for the cell count assay in control (open symbols) or 90 μM quinidine-containing medium (solid symbols). For the MTS assay (circles), results are the average of quadruplicates in one experiment. For the cells count assay (squares), the results represent the mean and SEM of three independent experiments for the MCF-7, T47D, and MDA-231 cell lines in duplicate dishes (MCF-7 and T47D), or single dishes (MDA-231) for each experiment. MCF-7ras and MCF10A data are the mean +/- range of two experiments performed in duplicate dishes. HMEC cell count data are from one experiment performed in single dishes.

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4.5. *c-myc* antisense oligonucleotides mimic the effects of quinidine on lipid droplets formation in the MCF-7 cells

The objective of this experiment was to test if suppression of Myc is sufficient to induce a more differentiated phenotype in breast tumor cells similar to quinidine. In fact, blocking of Myc activity using antisense oligonucleotides was shown to induce differentiation in several cell lines (Griep, A and Westphal, H., 1988; Holt, J. e al, 1988). MCF-7 cells were plated at the density of 2.2 x 10^5/35 mm^2 tissue culture dish on glass cover slips in DMEM/5%FBS. Cells were allowed to attach to the cover slips and then the medium was exchanged to DMEM/2%FBS +/- 9 μM *c-myc* antisense oligonucleotides or +/- *c-myc* sense oligonucleotides (negative control). Ninety-six hours later cells were fixed and Oil Red O staining was performed as described in the Materials and Methods. The results are shown in Figure 26. MCF-7 cells treated with *c-myc* antisense oligonucleotides showed accumulation of cytoplasmic lipid droplets, whereas control and *c-myc* sense oligonucleotides-treated cells were predominantly lipid droplet-negative. These results suggest that specific down-regulation of the Myc activity is sufficient to induce lipid droplet formation in MCF-7 cells. Thus, it is conceivable, that inhibition of *c-myc* by quinidine is responsible for the morphological changes associated with more differentiated phenotype observed in quinidine-treated breast tumor cells. The presented results, as well as the data from other laboratories, suggest a critical role for Myc in regulation of cellular growth and differentiation in breast tumor cells. Therefore, it is important to understand the precise molecular mechanisms regulating *c-myc* gene expression by quinidine, which may reveal new pharmacological targets for breast cancer therapy.

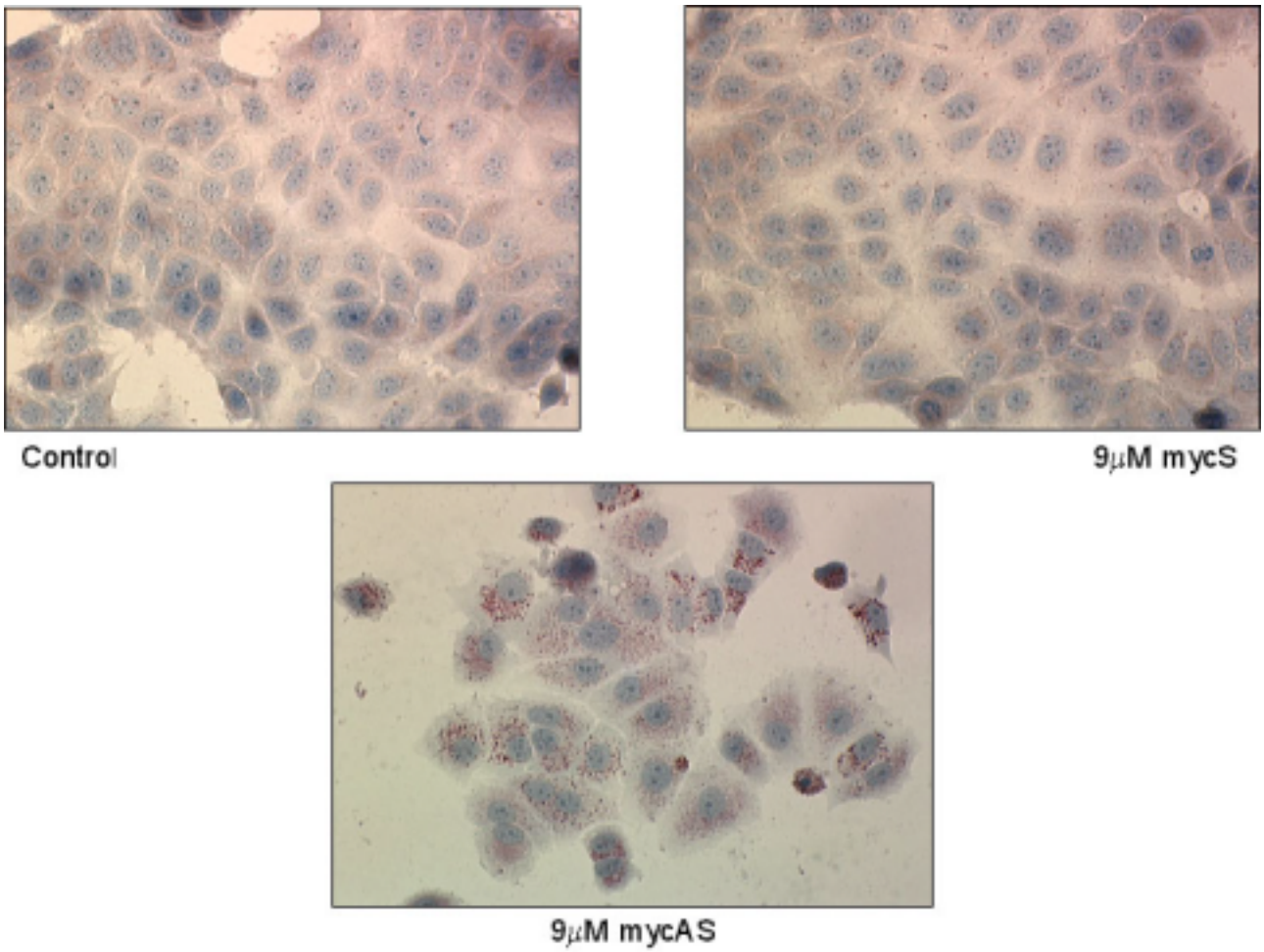

Figure 26. *c-myc* antisense oligonucleotides cause accumulation of cytoplasmic lipids in MCF-7 cells.

MCF-7 cells were plated on glass cover slips and treated with nothing (control), 9 μM *c-myc* antisense oligonucleotides (mycAS), or 9 μM *c-myc* sense oligonucleotides (mycS). 96 hours later cells were stained with Oil Red O (red color) and counterstained with Mayer's hematoxylin (blue color) to visualize cytoplasmic lipid droplets and cell nuclei, respectively. The results are representative of n= 2 experiments.

IV. DISCUSSION

Previous studies indicate that quinidine causes G1/G0 cell cycle arrest and inhibition of proliferation in MCF-7 human breast cancer cell line (Woodfork, K. et al, 1995). The goal of studies presented here was to further understand the mechanisms underlying cell cycle arrest and inhibition of growth by quinidine. The regulation of *c-myc* gene by quinidine in human tumor and normal breast epithelial cell lines were studied.

C-myc is one of the most common oncogene aberrations in breast cancers (Deming, S. et al, 1999; Garcia, I. et al, 1989; Escot, C. et al, 1986). The abnormal expression of *c-myc* was reported in 32% of breast cancers (Liu, E. et al, 1989), suggesting its important role in genesis and/or progression of breast cancer. Hence, understanding the mechanisms of *c-myc* gene regulation is important and may lead to the discovery of new targets for breast cancer therapy. *In vivo* studies with *c-myc* antisense oligonucleotides showed a significant increase in cancer-free survival of Eμ-transgenic mice compared to the animals treated with scrambled (negative control) oligonucleotieds (Akhtar, S. and Agrawal, S., 1997). In addition, mice with human melanoma xenografts showed a significant decrease in tumor size after systemic administration of *c-myc* antisense oligonucleotides (Leonetti, C. et al, 2001). My results demonstrate significant suppression of Myc by quinidine in four different human breast cancer cell lines growing in tissue culture (Figure 8). A large body of evidence established an important role of *c-myc* in cellular differentiation. Studies in different cell lines had shown that down-regulation of *c-myc* by antisense oligonucleotides or expression of dominant negative *c-myc* gene accompanies terminal differentiation and permanent withdrawal from the cell cycle (Ebinuma, H. et al, 1999; Canelles, M. et al, 1997; Griep, A. and Westphal, H., 1988; Holt, J. et al, 1988; Prochownik, E. et al, 1988). Conversely,

independent studies showed that ecotpic c-Myc expression from a transfected gene is sufficient to block the induction of differentiation in MEL, 3T3-L1 preadipocyte, F9, PC12 neuronal, and U-937 monoblastic cell lines (Coppola, J.A., and Cole, M.D., 1986; Dmitrovsky, E. et al, 1986; Freytag, S., 1988; Larsson, L., et al, 1988; Maruyama, K. et al, 1987, Onclercq, R. et al, 1989; Prochownik, E. and Kukowska, J., 1986). In consistence with these data, my results demonstrate that suppression of Myc by quinidine was associated with inhibition of growth and induction of a more differentiated (less malignant) phenotype in four different breast tumor cell lines (Figures 24 and 25).

The induction of differentiation (differentiation therapy) with a vitamin A derivative, all-*trans* retinoic acid (ATRA) has been a major advance and become the first choice drug in the treatment of acute promyelocytic leukemia (APL) (Tobita, T. et al, 1997; McBurney, M. et al, 1993). The major principle behind the differentiation therapy is a pharmacologically induced conversion of poorly differentiated cancer cells back to the original differentiated or non-proliferative state. Since normal cells are already differentiated, ideally, they should not be affected by differentiation-inducing agents, leading to the less side effects compared to traditional cytotoxic chemotherapy. The successful treatment of APL with all-*trans* retinoic acid had led to attempts to apply the same principle for the treatment of other cancers. Unfortunately, differentiation therapy for solid cancers, such as breast cancer, generally, has been proven to be ineffective. Therefore, induction of a more differentiated phenotype in breast cancer cells by quinidine is a promising finding, suggesting a potential role for quinidine-like compounds in the therapy of breast cancer. In addition, differentiation-inducing agents, such as retinoids, have been shown to be very effective in chemopreventive studies in breast cancer, cervical cancer, head and neck cancer, and lung cancer (Whelan, P.,

1999). Chemoprevention is a relatively new branch of oncology, which aims to use chemical agents to reduce the incidence of malignant diseases in humans by suppressing carcinogenesis in premalignant lesions. In analogy to retinoids, quinidine-like compounds may also be effective as breast cancer chemopreventive agents. A better understanding of factors and mechanisms regulating differentiation (such as down-regulation of the *c-myc*), as well as their disruption in carcinogenesis would reveal new possibilities to design novel therapeutic approaches for chemopreventive and differentiation therapy of solid tumors. The results from my experiments indicate that quinidine suppresses the activity of *c-myc* promoter over the same range of concentrations that suppress levels of *c-myc* mRNA and protein (Figure 14). Myc protein, mRNA levels, and promoter activity were suppressed by 68% (Figure 9), 78% (Figure 11), and 60-70% (Figure 16A) respectively by 90μM quinidine. The changes in *c-myc* mRNA and protein levels in response to quinidine may therefore be attributed to its effects on *c-myc* promoter. Furthermore, a 168 bp region (from –100 to +68 in respect to P1) of the *c-myc* promoter was identified as a putative quinidine response region (QRR) (Figures 15 and 16). This region contains two important cis-elements: the TGF β1 control element (TCE) and region from -60 to -37 that contains Sp1/Sp3 binding site (Nishikura, K., 1986, see Table 3). The first element is involved in inhibition of *c-myc* P1 transcription initiation in response to TGF β1 (Pietenpol, J. et al, 1991), whereas the presence of intact second element is essential for P1 transcription (Nishikura, K., 1986). Quinidine had no effects on the levels of Sp1 or Sp3 proteins in MCF-7 cells (Figure 17). These results indicate that suppression of *c-myc* promoter by quinidine is not mediated by the changes in Sp1/Sp3 protein levels. However, it does not exclude the possibility of Sp1/Sp3 involvement in regulation of *c-myc* promoter by quinidine, since binding of these proteins to the GC-rich

region of QRR but not their levels could be responsive to quinidine. This issue can be resolved by electrophoretic mobility shift assay, that directly examines protein/DNA interactions in the promoter region of interest. In addition, my results suggest that regulation of *c-myc* and subsequently cell growth by quinidine and TGFβ1 is mediated by different mechanisms in breast cancer cell lines. It is also possible that suppression of the *c-myc* promoter in response to quinidine is mediated by the region within QRR that has not been described yet. To identify this potential new element, the fine mapping of QRR should be done by making small promoter deletions using polymerase chain reaction (PCR) and targeted single-stranded DNA primers. Determination of QRE may lead to the identification of transcription factors that mediate suppression of *c-myc* promoter activity by quinidine. This in turn may reveal the signaling pathways that are involved in the regulation of *c-myc* gene and are targeted by quinidine.

Alternative mechanism, by which quinidine can alter the expression of *c-myc* gene is via changing the acetylation of histones associated with *c-myc* promoter or transcription factors that bind to it. The chromatin structure and consequently the transcriptional activity of genes can be modulated by two families of proteins, via changes in the acetylation status of histones (Grunstein, M., 1997; Davie, J. and Chadee, D., 1998). Deacetylation of histones by histone deacetylases (HDACs) is, in general, associated with more condensed or transcriptionally inactive chromatin. In contrast, the hyperacetylation of histones by histone acetylases (HATs) has been linked to the transciptionally active chromatin (Kornberg, R. and Lorch, Y., 1999; Kouzarides T., 1999; Grunstein, M., 1997). A large body of evidence demonstrated, that agents that inhibit HDAC enzymatic activity, such as trichostatin A (TSA), superoylanilide hydroxamic acid (SAHA), trapoxin, and phenyl butyrate induce

differentiation in a number of cell lines (Marks, P. et al, 2000; Medina, V. et al, 1997; Richon, V. et al, 1998; Sambucetti, L., 1999). The results from our laboratory showed that quinidine causes rapid degradation of HDAC-1, followed by histone hyperacetylation in MCF-7 cells (Zhou, Q. et al, 2000). Several studies, which used Raji cells (B cell line) stably transfected with episomal *c-myc* gene within self-replicating Epstein-Barr virus-derived vector, showed that mRNA transcription from both *c-myc* promoters, P1 and P2, is induced by two HDAC inhibitors, sodium butirate (Albert, T. et al, 2001) and tricostatin A (Madisen, L. et al, 1998). Therefore, suppression of *c-myc* promoter by quinidine is unlikely to be a result of quinidine-induced histone hyperacetylation. On the other hand, degradation of HDAC-1 by quinidine can alter the acetylation and subsequently DNA binding or transactivating activities of transcription factors that regulate *c-myc* promoter activity. In fact, the activities of several transcription factors have been reported to depend on their acetylation status (Gu, W. and Roeder, R., 1997; Munshi, N. et al, 1998; Waltzer, L. and Bienz M. et al, 1998).

One of the major problems of current chemotherapeutic drugs for breast cancer treatment is their lack of selectivity for tumor tissue and associated with it toxicity in normal tissues. Importantly, quinidine had minimal effects on Myc levels or proliferation in normal breast epithelial cell lines (Figures 10 and 25). The difference in responses of tumor and normal cells to quinidine could be due to the lack of quinidine's target in normal cells or its overexpression in tumor cells. For example, if changes in membrane potential in response to quinidine are involved and ultimately lead to the suppression of Myc, then the levels of expression of quinidine-sensitive potassium channels could be different in normal and cancer cells. In fact, the unique linear hyperpolarized current, which is inhibited by quinidine in

MCF-7 cells, is overexpressed in their Ha-ras transformed counterpart (Klimatcheva, E. and Wonderlin, W., 1999). The differential regulation of Myc in normal versus tumor cells could also be attributed to the differences in *c-myc* promoter sequence in these cells.

In the studies presented here, as well as in the previous studies the maximal responses to quinidine were achieved at 90 μM concentration. The IC_{50} values of quinidine for growth inhibition, G0/G1 arrest, and suppression of Myc protein levels were 37.15 (Woodfork, K. dissertation, 1993), 24 +/-14 (Wang, S., 1998), and 27.3 +/- 13 μM (Figure 9), respectively. These values are above the therapeutic concentrations of quinidine as antiarrhythmic drug, which range from 2 to 6 μg/ml (5.3 to 15.8 μM). Additionally, 6 μg/ml (15.8 μM), 9 μg/ml (24 μM), and 14 μg/ml (37 μM) concentrations of quinidine caused side effects in 10, 30, and 50 % of patients, respectively (Goodman and Gilman's, VII edition, 1985). The IC_{50} values of quinidine for G0/G1 (24 μM) arrest and suppression of Myc (27μM) are close to the dose (24 μM) that causes toxic effects in only 30 % of patients. To minimize the toxic effects, the lower doses of quinidine can be used in combination with other differentiation-inducing agents, such as retinoids. In fact, most of the chemotherapeutic regiments employ combination or polychemotherapy, consisting of the use of two or more compounds to achieve maximum kill of tumor cells with minimal toxicity.

In summary, the studies presented here demonstrate a significant suppression of Myc levels by quinidine, which followed by inhibition of proliferation and induction of a more differentiated phenotype in four different human breast cancer cell lines growing in tissue culture. In contrast, quinidine had minimal effects on Myc protein levels and proliferation in normal breast epithelial cells. Differentiation-inducing agents, such as retinoids, have been successfully used for the treatment of acute promyelocytic leukemia, but not solid cancers. In

addition, retinoids, have been shown to be very effective in chemopreventive studies in breast cancer, cervical cancer, head and neck cancer, and lung cancer. Furthermore, *in vitro* and *in vivo* studies with *c-myc* antisense oligonucleotieds have shown promising results in treatment of several cancers. Therefore, suppression of Myc and induction of a more differentiated phenotype in breast cancer cells by quinidine is a promising finding, suggesting a potential role for quinidine-like compounds in chemopreventive and differentiation therapies of breast cancer.

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VI. CURRICULUM VITAE

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Awards

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Work experience	1996 - present	West Virginia University	Morgantown, WV
	Research Assistant		
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Skills

Tissue culture techniques, experience with primary and established cell lines, cell viability/cytotoxicity and proliferation assays with 96-well plates, apoptosis detection assays, FACS analysis, stable and transient cell transfection, RNA, DNA and protein isolation, Northern blot, Western blot, plasmid amplification and isolation, cloning techniques, immunohistochemistry, histochemistry, reporter gene assays, clonogenic survival assay. Experienced in using Phosphorimager, Luminometer, Densitometer, Fluorimager, Zeiss fluorescence and scanning laser confocal microscopes. Molecular Modeling.

Experienced in using MS Word, Excel, Power Point, Sigma Plot, Adobe

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Publications

Papers:

Melkoumian, Z.K., Strobl, J.S. Regulation of *c-myc* gene expression in human breast tumor and normal breast epithelial cell lines by quinidine. (in preparation).

Zhou, Q., **Melkoumian, Z**., Lucktong, A., Moniwa, M., Davie, J., and Strobl, J. Rapid Induction of histone acetylation and cellular differentiation in human breast tumor cell lines following degradation of histone deacetylase-1. *J. Biol. Chem.*, 275: 35256-35263, 2000.

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