

AD \_\_\_\_\_

Award Number: DAMD17-02-1-0622

TITLE: Augmentation of the Differentiation Response to Antitumor Antimalarials

PRINCIPAL INVESTIGATOR: Rayhana Rahim, B.A.  
Jeannine S. Strobl, Ph.D.

CONTRACTING ORGANIZATION: West Virginia University Research Corporation  
Morgantown, WV 26506-6845

REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040112 120

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

|  |   |  |   |                                  |
|--|---|--|---|----------------------------------|
| <b>1. AGENCY USE ONLY</b><br>(Leave blank)   |   | <b>2. REPORT DATE</b><br>July 2003                             | <b>3. REPORT TYPE AND DATES COVERED</b><br>Annual Summary (1 Jul 2002 - 30 Jun 2003)) |                                  |
| <b>4. TITLE AND SUBTITLE</b><br>Augmentation of the Differentiation Response to Antitumor Antimalarials  |   |  | <b>5. FUNDING NUMBERS</b><br>DAMD17-02-1-0622   |                                  |
| <b>6. AUTHOR(S)</b><br>Rayhana Rahim, B.A.<br>Jeannine S. Strobl, Ph.D.  |   |  |   |                                  |
| <b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b><br>West Virginia University Research Corporation<br>Morgantown, WV 26506-6845<br><br>E-Mail: rrahim@wvu.edu  |   |  | <b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>                                       |                                  |
| <b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b><br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012   |   |  | <b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>                               |                                  |
| <b>11. SUPPLEMENTARY NOTES</b><br>Original contains color plates: ALL DTIC reproductions will be in black and white  |   |  |   |                                  |
| <b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b><br>Approved for Public Release; Distribution Unlimited   |   |  |   | <b>12b. DISTRIBUTION CODE</b>    |
| <b>13. ABSTRACT (Maximum 200 Words)</b><br>An impending challenge to breast cancer drug therapies is the availability of more effective and less toxic chemotherapeutic agents that do not relay harm to neighboring normal breast cells and tissues. We have shown that the quinoline antimalarials chloroquine (CQ) and hydroxychloroquine (HCQ) inhibit proliferation and induce differentiation in breast cancer cell lines without toxicity to normal MCF-10A cells. The purpose of this project is to derive more efficacious antitumor agents and enhance the differentiation response by using CQ and HCQ in combination with the demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza-dC), or with the differentiating agent, all-trans-Retinoic acid (ATRA). Cell survival, cellular differentiation, and histone H4 acetylation status were measured. Future experiments will analyze histone deacetylase (HDAC) protein degradation and the acetylation levels at specific lysine sites in order to generate the overall histone acetylation profiles for the drug combinations. Results show that the combination of 5-Aza-dC or ATRA with the antimalarials sensitized both the MDA-MB-231 and MCF-7 breast cancer cell lines to growth inhibition, enhanced cellular differentiation, and elevated histone H4 acetylation. These results support the use of CQ or HCQ with other tumor differentiating agents in combination to provide a more effective and less toxic therapeutic regimen for breast cancer intervention. |   |  |   |                                  |
| <b>14. SUBJECT TERMS</b><br>Histone deacetylase, quinoline antimalarials, chloroquine, hydroxychloroquine, differentiation, hyperacetylation   |   |  |   | <b>15. NUMBER OF PAGES</b><br>16 |
|  |   |  |   | <b>16. PRICE CODE</b>            |
| <b>17. SECURITY CLASSIFICATION OF REPORT</b><br>Unclassified   | <b>18. SECURITY CLASSIFICATION OF THIS PAGE</b><br>Unclassified | <b>19. SECURITY CLASSIFICATION OF ABSTRACT</b><br>Unclassified | <b>20. LIMITATION OF ABSTRACT</b><br>Unlimited  |                                  |

## Table of Contents

|  |             |
|--|-------------|
| <b>Cover.....</b>                        | <b>1</b>    |
| <b>SF 298.....</b>                       | <b>2</b>    |
| <b>Table of Contents.....</b>            | <b>3</b>    |
| <b>Introduction.....</b>                 | <b>4</b>    |
| <b>Body.....</b>                         | <b>5</b>    |
| <b>Key Research Accomplishments.....</b> | <b>15</b>   |
| <b>Reportable Outcomes.....</b>          | <b>15</b>   |
| <b>Conclusions.....</b>                  | <b>15</b>   |
| <b>References.....</b>                   | <b>16</b>   |
| <b>Appendices.....</b>                   | <b>none</b> |

**Introduction:**

The purpose of this project is to use combination drug therapy to augment the differentiation response of breast cancer cells to the antitumor antimalarials: chloroquine (CQ) and hydroxychloroquine (HCQ). We hypothesized that the use of a drug combination modulating epigenetic events would lower the concentration of CQ or HCQ needed to produce the differentiation response. Hence, the proposed work seeks to use these two promising agents in combination with the demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza-dC), or with the differentiating agent, all-trans-Retinoic acid (ATRA) in order to lower the threshold for chemotherapy-induced cell death in breast cancer cells. Human breast cancer cell lines (MCF-7 and MDA-MB-231) will be treated with either CQ or HCQ at their IC<sub>25</sub> and IC<sub>50</sub> MTS values  $\pm$  5-Aza-dC or ATRA. Cellular differentiation will be measured by exit from the cell cycle to a Ki67 negative state and by accumulation of cytoplasmic lipid droplets. Cell survival, histone H4 acetylation status, and HDAC (histone deacetylase) 1 and 2 protein degradation will be measured. In addition, the development of a new and highly sensitive assay for histone H4 acetylation by mass spectrometry will be pivotal in further developing HDAC inhibitors for the prevention and treatment of breast cancer. This new approach will illustrate the level and the specific lysine sites that get modified (acetylated/deacetylated) by the chemotherapeutic agents.

**Body:**Research Accomplishments

Task #1 of this project is to determine optimal conditions for cellular differentiation using CQ or HCQ  $\pm$  5-Aza-dC or ATRA (or a vitamin D-derivative as an alternative drug of choice if the other agents do not cause differentiation).

Cell survival was first measured using the MTS assay in which cells were pretreated with 5-Aza-dC or ATRA 48hrs before treatment with CQ. The combination of ATRA with CQ sensitized both the MDA-MB-231 (estrogen receptor, ER-) and MCF-7 (ER+) cell lines to growth inhibition. The combination of 5-Aza-dC (at its lowest concentration, 1 $\mu$ M) with CQ sensitized the MDA-MB-231 (ER-) cells, but failed to elicit growth inhibition in the uncloned early passage MCF-7 (ER+) cell line. However, the combination of 5-Aza-dC with CQ did sensitize clonal ER- and ER+ MCF-7 cell lines to growth inhibition indicating that the response is not dictated by ER expression. To show the effect of the antimalarials in combination with the lowest concentrations of either 5-Aza-dC or ATRA on breast cancer cell growth, cell viability was measured to generate growth curves (Figure 1,2). More than the MTS assay, clonogenic survival is a highly sensitive assay for determining dose response. Clonogenic survival assays measure the ability of cells to survive and form discrete colonies at a low plating density; therefore, it is the closest method to correlating *in vitro* to *in vivo* studies. Thus, this method was adopted to show the effect of the antimalarials with 5-Aza-dC or ATRA on tumor survival (Figure 3). Overall, these results show that the combination of 5-Aza-dC or ATRA with CQ decreases breast cancer cell survival more than CQ alone.

A primary marker for cellular differentiation entails the accumulation of cytoplasmic lipid droplets, which can be detected using Oil Red-O histochemistry. As evidence of cellular differentiation, both CQ and HCQ  $\pm$  5-Aza-dC or ATRA led to the accumulation of cytoplasmic lipid droplets detected by Oil Red-O staining in both the MCF-7 and MDA-MB-231 breast cancer cell lines after 48 hours of treatment (Figure 4,5). The results showed that the combination of 5-Aza-dC (10 $\mu$ M) or ATRA (10 $\mu$ M) with CQ or HCQ caused enhanced lipid droplet accumulation at both their IC<sub>25</sub> and IC<sub>50</sub> values than either antimalarial alone.

Another primary marker for cellular differentiation is cell cycle arrest in G<sub>1</sub> as measured by Ki67 histochemistry. Ki67 is a large nuclear protein expressed in all proliferating cells, in normal as well as in tumor cells. It is also expressed during all active phases of the cell cycle (G<sub>1</sub>, S, G<sub>2</sub>, and M-phases), but is absent in resting, or differentiating cells (G<sub>0</sub> phase) (Scholzen and Gerdes, 2000). Therefore, it makes it an excellent marker in determining the growth fraction of a given cell population and is used widely as a diagnostic tool in distinct neoplasms. For this reason, Ki67 protein was measured in both MCF-7 and MDA-MB-231 breast cancer cells after 48 hours of treatment with CQ or HCQ at IC<sub>25</sub> and IC<sub>50</sub> MTS values  $\pm$  5-Aza-dC or ATRA (Figure 6). The results showed that the combination of 5-Aza-dC (10 $\mu$ M) or ATRA (10 $\mu$ M) with CQ or HCQ displayed an enhanced Ki67 index which is indicative of a greater percentage of cells that have exited from the cell cycle into a differentiated Ki67 negative state.

In conclusion, both CQ and HCQ serve as selective breast tumor differentiating agents and the combination of low concentrations of 5-Aza-dC or ATRA with these antitumor antimalarials

decreases breast cancer cell survival and augments the differentiation response more than either CQ or HCQ alone. Task #1 is complete.

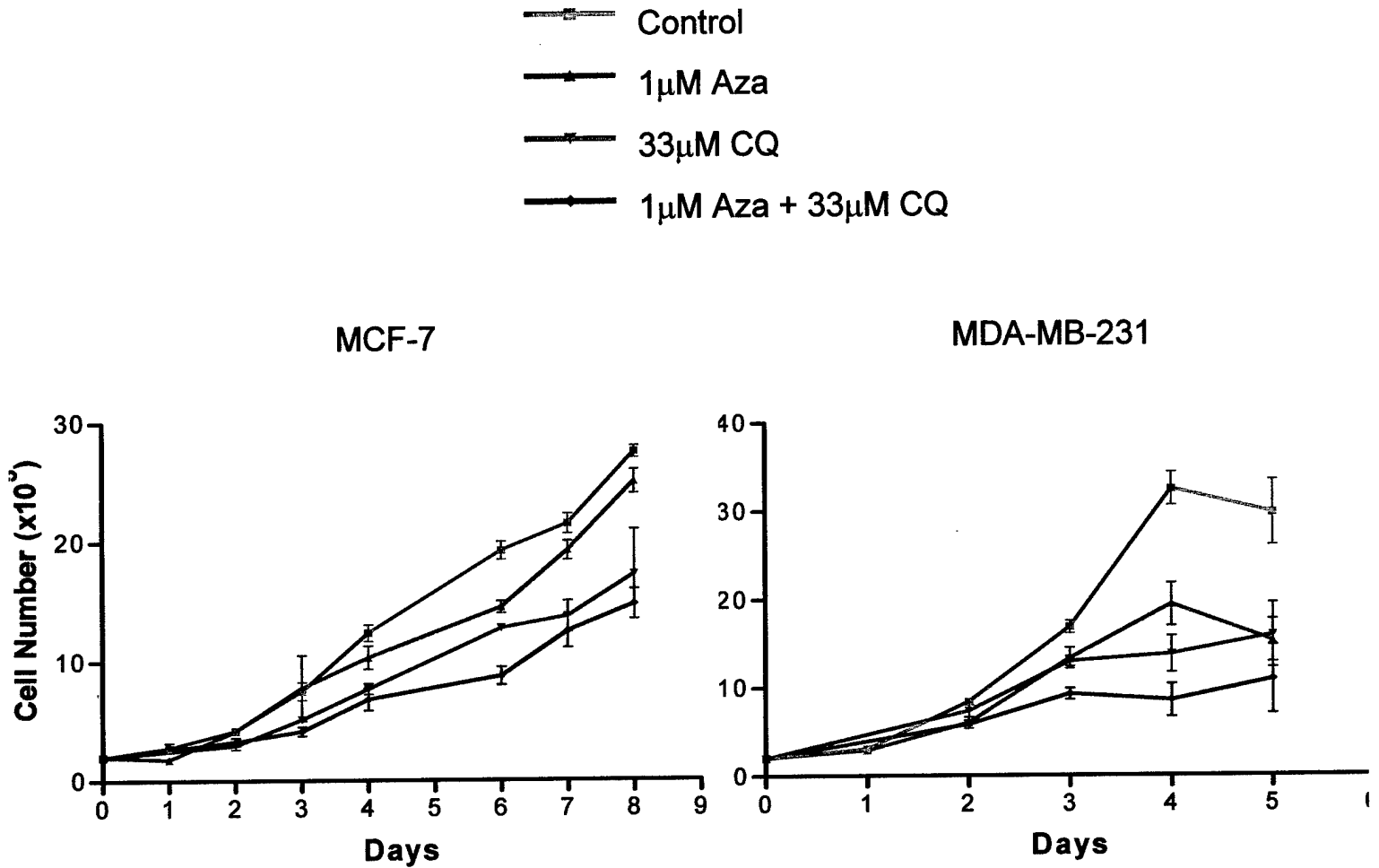
Task #2 of this project is to determine the optimum conditions for histone H4 hyperacetylation. Using Western blotting, histone H4 acetylation status and HDAC 1 and 2 protein levels will be measured in breast cancer cells treated with CQ or HCQ  $\pm$  5-Aza-dC or ATRA. The acid-extraction protocol from Upstate (Upstate Biotechnology, NY) was adopted to isolate total histone proteins. Western blotting was performed on MCF-7 breast cancer cells treated with CQ at its IC50 MTS value  $\pm$  ATRA (Figure 7). After quantitation, the results showed greater than 4 fold increase in the levels of acetylated histone H4 upon combination treatment with CQ and ATRA (100 $\mu$ M). The levels were similar to that of the positive control, TSA (trichostatin A, 300nM), showing that combination treatment caused a greater hyperacetylation response. Objective #1 of this project is complete. Objective #2 and #3 will be completed once the histone acetylation status of the antimalarials combined with 5-Aza-dC is measured and the HDAC 1 and 2 protein levels assessed.

In addition, future work will also include using another new and promising antitumor agent, NSC69603 (received from the National Cancer Institute (NCI), MD) which has structural similarity to that of CQ. NSC69603 has shown to be a very potent differentiating agent with extremely low toxicities as determined by *in vivo* studies done by NCI. Using Oil-Red-O and Ki67 histochemistry assays, NSC69603 caused lipid droplet accumulation and exit from the cell cycle to a Ki67 negative state at very low concentrations. In order to confirm G1 cell cycle arrest, alterations in the regulation of a key regulatory cell cycle protein, E2F, was measured by Western blotting (Figure 8). E2F, which is tightly bound to the retinoblastoma (Rb) protein, interacts with HDAC to enhance cell cycle progression and is differentially regulated in breast cancer cell lines versus normal mammary cell lines (Melkoumian, Z. et al., 2002). The results show significant downregulation of E2F-1 protein at 48 hours, which is indicative of an enhanced differentiation response. Future work will explore the effects of NSC69603  $\pm$  5-Aza-dC or ATRA on cellular differentiation and histone H4 hyperacetylation.

#### Training Accomplishments

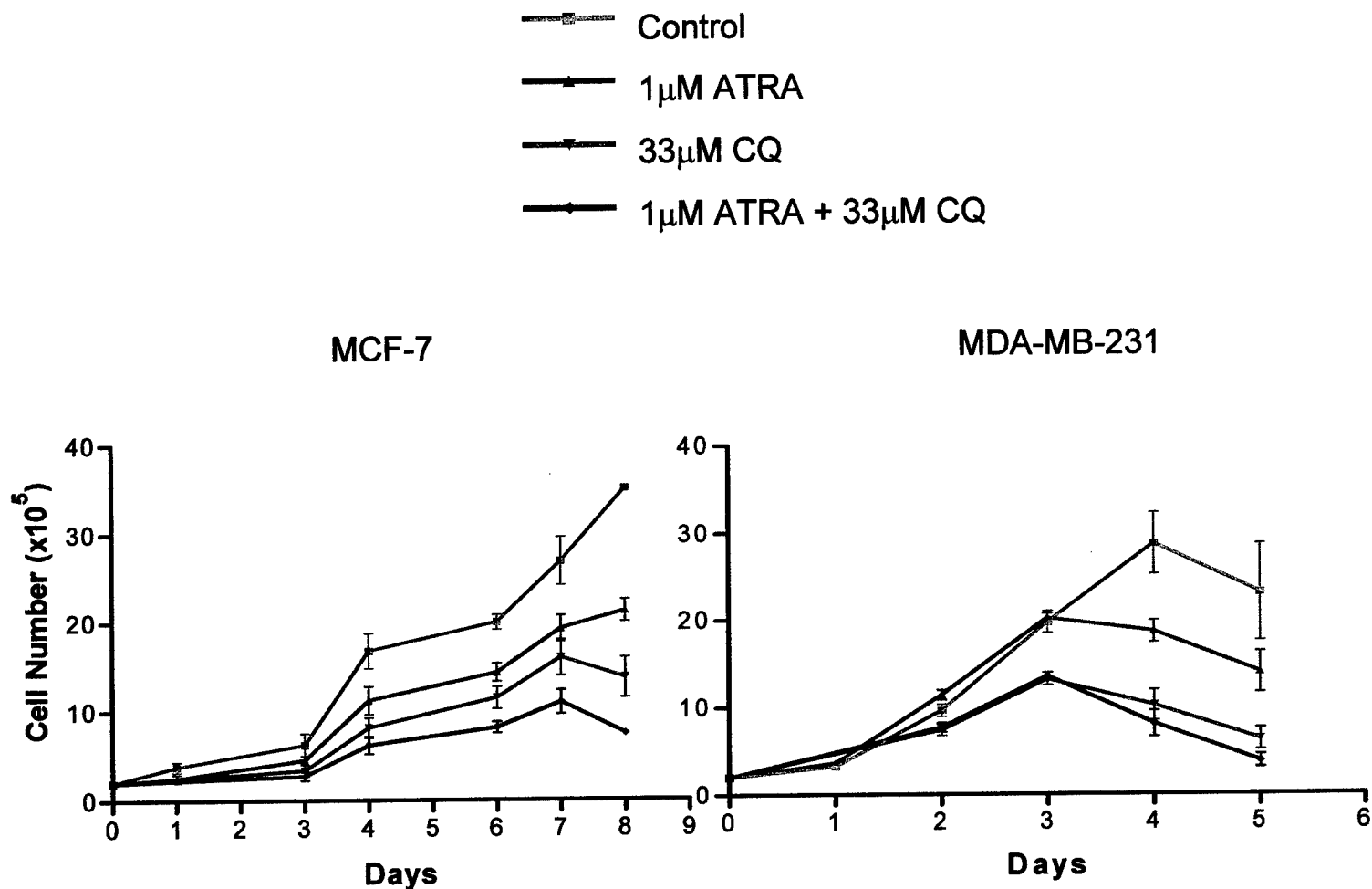
In addition to the above stated research accomplishments, the P.I. has made several training achievements during the July 1, 2002 through June 30, 2003 funded year. Per the requirements of the P.I.'s Pharmacology and Toxicology Ph.D. Program, a written and oral proposal defense was successfully completed in August of 2002. Results attained during the funded year were also presented in the departmental seminar in May of 2003. The P.I. also completed two semesters of required courses and presented these findings in the departmental research forums each semester. The P.I. attended the American Association for Cancer Research meeting held in Washington, D.C. in July of 2003 and participated in the abstract submission and poster presentation.

**Figure 1: Breast Cancer Cell Survival Following Antimalarial and 5-Aza-2'-deoxycytidine Treatments**

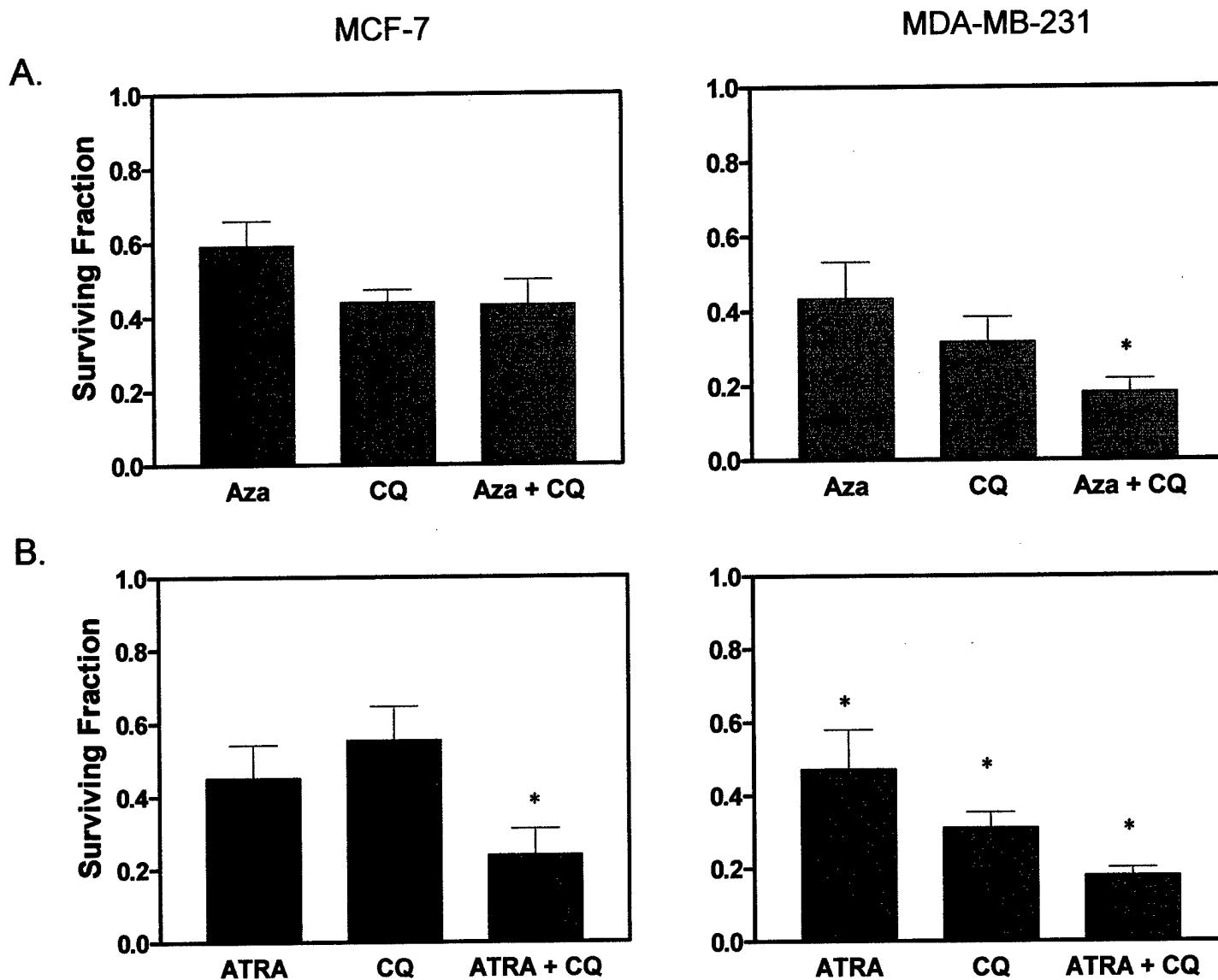


**Figure 1: Effect of 5-Aza-dC combined with the Chloroquine antimalarial on tumor cell growth.**  $2 \times 10^5$  MDA-MB-231 and MCF-7 cells were plated in 3ml of 5% FBS DMEM in 35mm dish plates. Following drug treatments, cell counting and viability were assessed on the indicated days to generate the growth curves. Data represents  $n=3$  (duplicates per each individual experiment)  $\pm$  SEM.

**Figure 2: Breast Cancer Cell Survival Following Antimalarial and Retinoid Treatments**

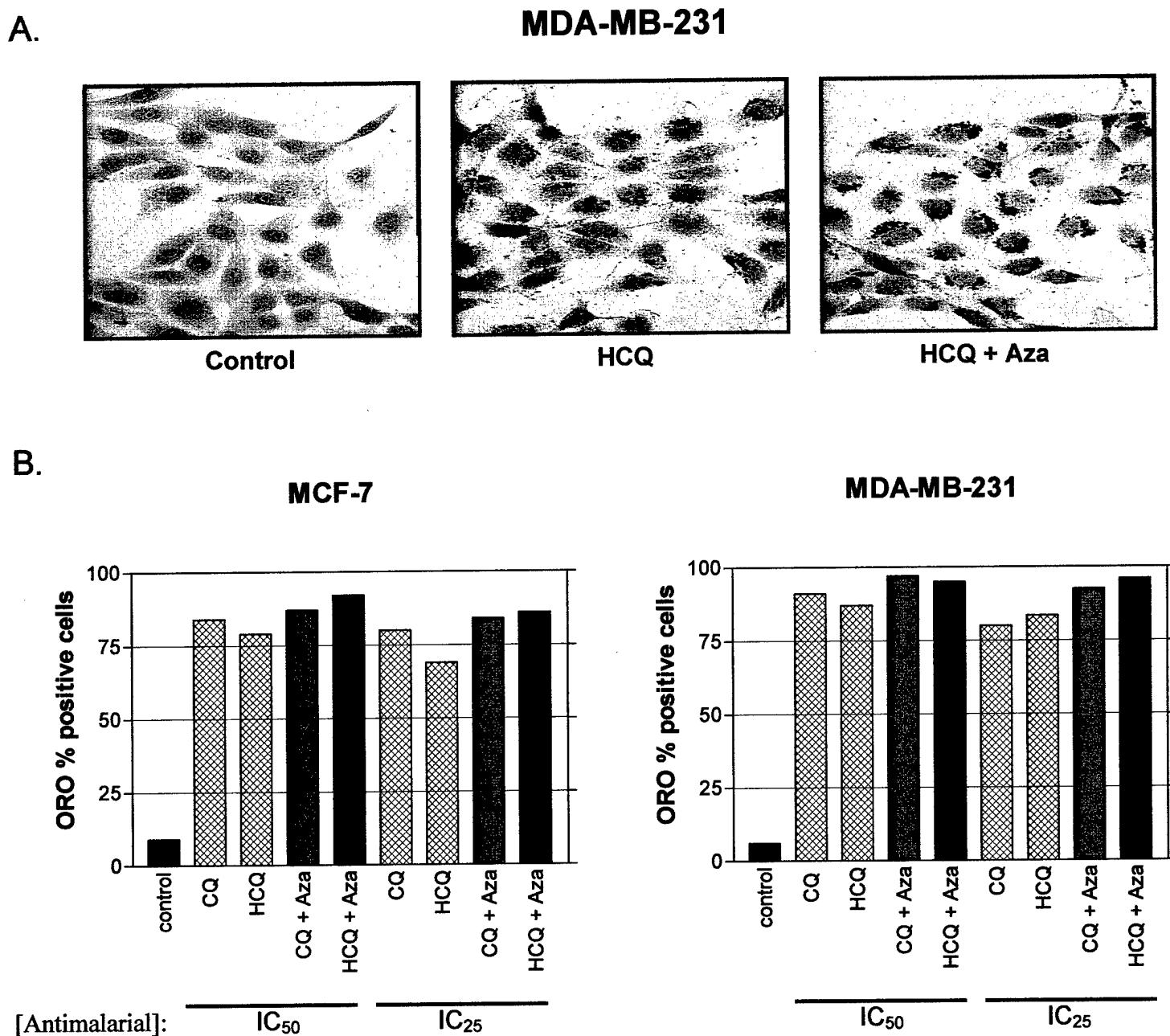


**Figure 2: Effect of ATRA combined with the Chloroquine antimalarial on tumor cell growth.**  $2 \times 10^5$  MDA-MB-231 and MCF-7 cells were plated in 3ml of 5% FBS DMEM in 35mm dish plates. Following drug treatments, cell counting and viability were assessed on the indicated days to generate the growth curves. Data represents  $n=3$  (duplicates per each individual experiment)  $\pm$  SEM.

**Figure 3: Clonogenic Survival of Breast Cancer Cells**

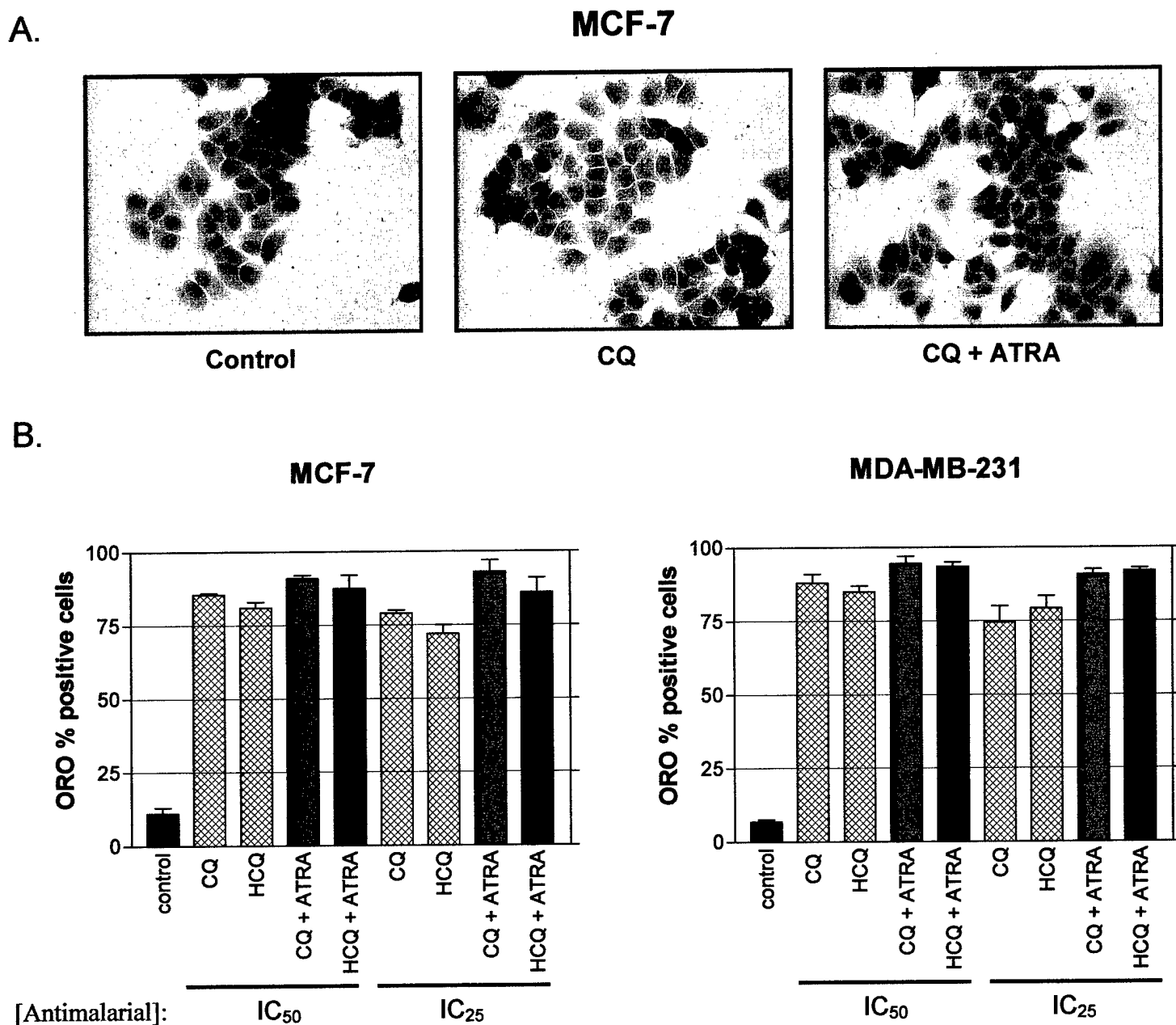
**Figure 3A,B:** Effect of Chloroquine antimalarial  $\pm$  5-Aza-dC or ATRA on tumor clonogenic survival.  $6 \times 10^3$  MCF-7 or MDA-MB-231 cells pretreated with CQ ( $33 \mu\text{M}$ ), 5-Aza-dC ( $1 \mu\text{M}$ ), or ATRA ( $1 \mu\text{M}$ ) were plated in 5ml of 10% FBS DMEM in 60mm dish plates. MCF-7 and MDA-MB-231 cells were then subjected to a 7 or 5 day (undisturbed) incubation period, respectively. To visualize colonies, dishes were stained with 3 ml of 0.5% crystal violet, 5% formalin, 50% ethanol, 0.85% NaCl for 3 minutes, then rinsed with tap water. Colonies were scored using a Nikon Eclipse TS100 microscope at 20X magnification with  $\geq 20$  MCF-7 cells and  $\geq 50$  MDA-MB-231 cells = 1 colony. Data represents  $n=3$  (duplicates per each individual experiment)  $\pm$  SEM (\* $p < 0.001$ ).

**Figure 4: Augmentation of Cellular Differentiation in Breast Cancer Cells Following Combination Treatment of 5-Aza-2'-deoxycytidine with Antimalarials**



**Figure 4A,B: Oil-Red-O (ORO) Histochemistry: Effect of Chloroquine or Hydroxychloroquine antimalarials ± 5-Aza-dC on cellular differentiation.** A.) HCQ (IC<sub>25</sub>) ± 10μM 5-Aza-dC B.) CQ or HCQ (IC<sub>50</sub> and IC<sub>25</sub>) ± 10μM 5-Aza-dC. MCF-7 and MDA-MB-231 cells were plated on 5x5 mm<sup>2</sup> 95% ethanol-washed glass coverslips in 35 mm<sup>2</sup> dishes at 2x10<sup>5</sup> and 1x10<sup>5</sup> cells per dish, respectively. 48 hours after drug treatment, the ORO assay was performed and lipid droplet accumulation in the cytoplasm was visualized by light microscopy. At least 300 cells were counted visually. Data represents 1 experiment.

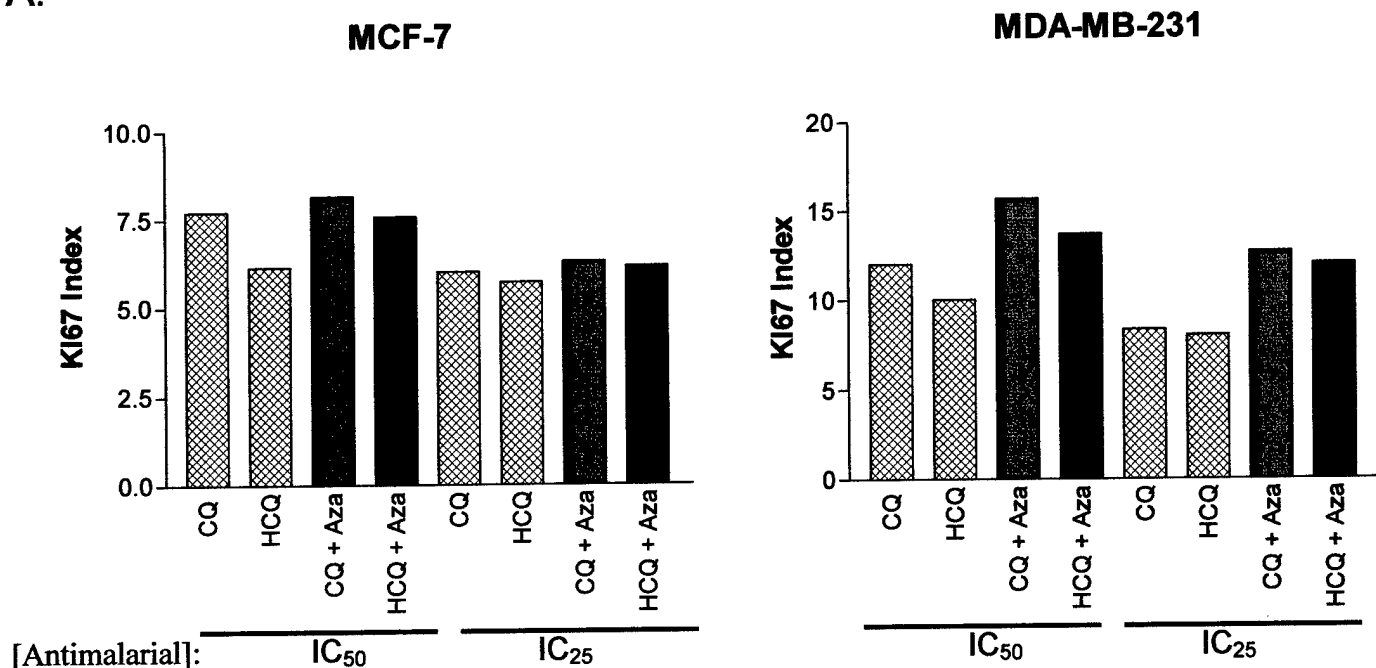
**Figure 5: Augmentation of Cellular Differentiation in Breast Cancer Cells Following Combination Treatment of all-trans-Retinoic Acid with Antimalarials**



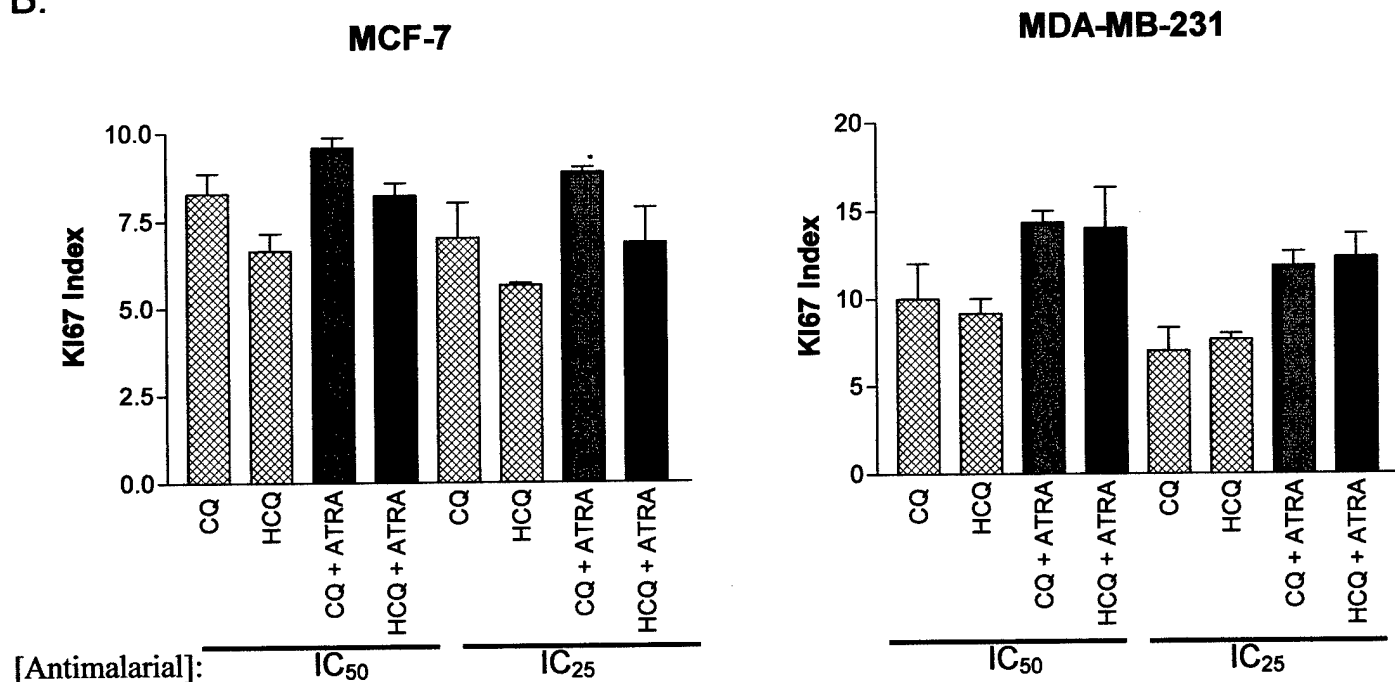
**Figure 5A,B: Oil-Red-O (ORO) Histochemistry: Effect of Chloroquine or Hydroxychloroquine antimalarials ± ATRA on cellular differentiation. A.) CQ (IC<sub>25</sub>) ± 10µM ATRA B.) CQ or HCQ (IC<sub>50</sub> and IC<sub>25</sub>) ± 10µM ATRA. MCF-7 and MDA-MB-231 cells were plated on 5x5 mm<sup>2</sup> 95% ethanol-washed glass coverslips in 35 mm<sup>2</sup> dishes at 2x10<sup>5</sup> and 1x10<sup>5</sup> cells per dish, respectively. 48 hours after drug treatment, the ORO assay was performed and lipid droplet accumulation in the cytoplasm was visualized by light microscopy. At least 300 cells were counted visually. Data represents the mean of 2 independent experiments (+/- SEM).**

**Figure 6: Induction of Cell Cycle Arrest in Breast Cancer Cells Following Combination Treatment of 5-Aza-2'-deoxycytidine or all-trans-Retinoic Acid with Antimalarials**

A.



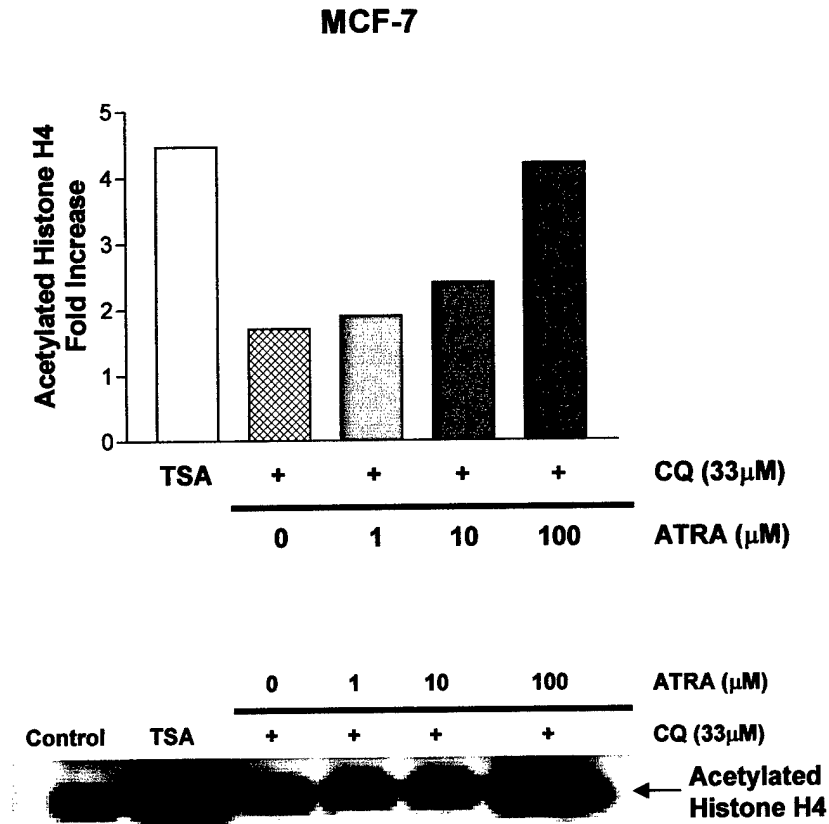
B.



**Figure 6A,B: KI67 Histochemistry: Effect of Chloroquine or Hydroxychloroquine antimalarials ± 5-Aza-dC or ATRA on cellular differentiation. A.) CQ or HCQ (IC<sub>50</sub> and IC<sub>25</sub>) ± 10 $\mu$ M 5-Aza-dC; n=1 B.) CQ or HCQ (IC<sub>50</sub> and IC<sub>25</sub>) ± 10 $\mu$ M ATRA; n=2 (+/- SEM). MCF-7 and MDA-MB-231 cells were plated on 5x5 mm<sup>2</sup> 95% ethanol-washed glass coverslips in 35 mm<sup>2</sup> dishes at 2x10<sup>5</sup> and 1x10<sup>5</sup> cells per dish, respectively. 48 hours after drug treatment, KI67 immunohistochemistry was performed. KI67 negative percentage of the cell population was determined by visual counting. At least 500 cells were counted. (KI67 Index=%KI67 negative drug treated/ %KI67 negative control).**

**Figure 7: Histone H4 Hyperacetylation in MCF-7 Cells Following Combination Treatment of all-trans-Retinoic Acid with Chloroquine**

A.



**Figure 7: Effect of Chloroquine antimalarial ± ATRA on histone H4 acetylation status.**  $1 \times 10^7$  MCF-7 cells were treated with trichostatin A (TSA, 300nM) or chloroquine (CQ,  $IC_{50}=33\mu M$ ) ± ATRA for 24 hours. Total histone proteins were isolated using acid-extraction protocol from Upstate. 20μg of purified histone proteins were resolved on a 12% acrylamide gel and analyzed by Western blot. Signals were quantitated by FluoroChem (Alpha Innotech, San Leandro, CA) spot densitometry using automatic background subtraction.



**Key Research Accomplishments:**

- Conditions for cellular differentiation optimized. (Task #1)
- Optimization of cell survival assay and histone isolation. (Task #1, Objectives #1-3)
- Western blot: Histone H4 hyperacetylation response measured. (Objective #1 and #3)

**Reportable Outcomes:**

- Abstract and poster presentation- American Association for Cancer Research Meeting; July 11-14, 2003; Washington, D.C. "Combination drug therapy with antitumor antimalarials augment the differentiation response to breast cancer cells." Bata, R.R., Zhou, Q., and Strobl, J.S. Abstract #3725. Proceedings of 94<sup>th</sup> Annual American Association for Cancer Research, Vol. 44, 2<sup>nd</sup> edition.
- Liu, C., Strobl, J.S., Schilling, J.K., McCracken, M., Chatterjee, S.K., Rahim-Bata, R., and Kingston, D.G.I. "Design, synthesis and bioactivities of steroid linked taxol analogs as potential targeted drugs for prostate and breast cancer." (Manuscript submitted June 2002), Journal of Natural Products.

**Conclusions:**

The proposed work focused on using two promising antitumor antimalarials in combination with the demethylating agent, 5-Aza-dC, or with the differentiating agent, ATRA in order to lower the threshold for chemotherapy-induced cell death in breast cancer cells by augmenting their differentiation response. Cell survival, cellular differentiation, and histone H4 acetylation status were measured to show increased sensitivity of breast cancer cell lines to growth inhibition, differentiation, and histone H4 hyperacetylation upon combination treatment of the antimalarials with 5-Aza-dC or ATRA. Experiments to assess the level of HDAC1,2 and E2F proteins will be performed and also include the addition of NSC69603. Finally, normal mammary cell lines will be treated with these drug combinations to determine the selectivity of the agents. All of these experiments will aid in the development of a new and highly sensitive assay for histone H4 acetylation by mass spectrometry.

**References:**

Melkoumian, Z.K., Martirosyan, A.R., and Strobl, J.S. (2002) *Myc protein is differentially sensitive to quinidine in tumor versus immortalized breast epithelial cell lines*. International Journal of Cancer, 102(1): 60-69.

Scholzen, T., and Gerdes, J. (2000) *The Ki67 protein: from the known and the unknown*. Journal of Cellular Physiology, 182: 311-322.