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TITLE: Sildenafil and Phosphodiesterase-5 Inhibitors to Reduce Cardiotoxicity and Enhance the Response of Breast Tumor Cells to Doxorubicin

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14. ABSTRACT

In the current work, we report that sildenafil does not protect breast tumor cells from adriamycin based on multiple assays (viable cell number, clonogenic survival, cell cycle distribution and DNA damage). Furthermore, clonogenic survival assays support the conclusion that sildenafil sensitizes breast tumor cells lacking functional p53 to Adriamycin. Since breast cancer frequently is shown to express mutant p53, these findings support the potential utility of sildenafil as a cardioprotectant that is unlikely to interfere with the antitumor actions of Adriamycin (or other chemotherapeutic agents; see previous report). Sildenafil does not increase Adriamycin toxicity to bone marrow cells or macrophages. Again, these findings indicate that sildenafil is unlikely to increase host toxicity of Adriamycin. Adriamycin has the capacity to produce multiple modes of cell death in the breast tumor cells. We now have evidence that Adriamycin also functions through the generation of free radicals to promote senescence. These findings raise questions relating to the selectivity of Adriamycin against the tumor cell versus the heart that require further exploration.

15. SUBJECT TERMS
Adriamycin; sildenafil; macrophages; bone marrow; DNA damage; cell cycle distribution; senescence

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INTRODUCTION

Our proposed work was based on the observations that phosphodiesterase-5 inhibitors, specifically erectile dysfunction drugs such as sildenafil, could protect cardiomyocytes and the heart from the toxicity of the anthracycline antibiotic, adriamycin (1). Furthermore, we had generated preliminary data that sildenafil did not appear to protect breast tumor cells from adriamycin and, in fact, may have promoted sensitivity to this drug. Our research goals were to extend these observations and to delineate the mechanistic basis for these differential effects in the tumor and in the heart.

We have requested and received a 1 year no-cost extension, in part in order to be able to complete the proposed studies in animal model systems. It was not feasible to perform these experiments previously due to problems with the approval of the study protocols by our university IACUC system. These problems have now been resolved and we anticipate being able to carry out the in-vivo experiments. However, it is likely that we will use another phosphodiesterase-5 inhibitor with a longer half life than sildenafil (possibly vardenafil) for these in-vivo studies.

In view of our no-cost extension, this report will cover the recent 1 year's work (annual) rather than the work performed during the entire period of funding (final report to be submitted in 2010).

BODY

In our current work, we have continued to explore the interaction(s) between sildenafil and adriamycin in breast tumor cells and have extended our studies to macrophages and bone marrow cells. In addition to potential cardiotoxicity, adriamycin also is responsible for toxicity to bone marrow precursors, which causes it to be immunosuppressive. Consequently, in anticipation of the possibility of using sildenafil in combination with adriamycin in the clinic for the treatment of breast cancer, it is important to evaluate whether sildenafil might exacerbate these additional elements of toxicity.

<u>Studies in Breast Tumor Cells</u>

In Figures 1 through 2, we substantiate the combining sildenafil with adriamycin (doxorubicin) in p53 wild-type MCF-7 breast tumor cells as well as p53 mutant MDA-MB231 breast tumor cells does not compromise the activity of Adriamycin. In Figures 3-4, we have extended this work to clonogenic survival assays, which are the gold standard for drug and radiation action. As in our earlier studies, sildenafil failed to protect against suppression of self-renewal capacity by adriamycin in MCF-7 cells and in MCF-7 cells engineered to express the executioner caspase, caspase 3, associated with promotion of apoptosis (2,3). However, in the case of p53 mutant MDA-MB231 cells and p53 null MCF-7/E6 cells, we observe clear evidence of potentiation of drug action. Since breast cancer patients present with both wild type and mutant p53, these studies indicate that the use of sildenafil (or other phosphodiesterase-5 inhibitors) as cardioprotective agents should not compromise the antitumor activity of Adriamycin. In studies presented in the previous annual report, we established that sildenafil also did not compromise the effectiveness of other antitumor drugs or ionizing radiation.

In further support of these findings, we evaluated whether sildenafil might influence either the extent of DNA damage or the cell cycle arrest induced by Adriamycin. In a previous report, we measured the effects of adriamycin combined with sildenafil on gamma H2AX formation, a well established indicator of DNA damage (4). In the current work, we monitored 53BP1 foci formation as an additional indicator of DNA damage (5). As shown in Figure 5, Adriamycin markedly increased foci formation, an effect that was not attenuated by exposure to sildenafil.

Figure 6 indicates that Adriamycin produced a reduction in the S phase population as well as an increase in the G2/M cell population, consistent with previous reports from our laboratory (6). Sildenafil failed to modify the impact of adriamycin on cell cycle distribution, as would be expected given its lack of interference with breast tumor cell sensitivity to adriamycin.

Studies in Bone Marrow Cells

In our proposal, we indicated our intent to evaluate the impact of sildenafil on toxicity of Adriamycin to bone marrow cells. The bone marrow cells were isolated from the femurs and tibias of BALB/c nude mice. In the current work (Figure 7), we present data indicating that Adriamycin does reduce viability of bone marrow cells, as expected for an agent that suppresses bone marrow function in patients (7) . However, there was no increased toxicity to the bone marrow cells when sildenafil was included in the incubation medium, again supporting the potential utility of sildenafil as a cardioprotective agent.

In addition, we evaluated the impact of Adriamycin with sildenafil on cell cycle distribution of the bone marrow cells. Figure 8 indicates that Adriamycin treatment suppressed the S phase fraction and promoted accumulation of cells in the G2M phase. Sildenafil treatment did not significantly alter this distribution.

Studies in Macrophages

In addition to cardiotoxicity and in association with bone marrow suppression, Adriamycin, like other antitumor drugs, can suppress the immune system (8). As an indicator of immune function, we evaluated the impact of exposure to Adriamycin with and without sildenafil on the viability of macrophages obtained from B6C3 mice. Figure 9 shows that, as with the studies in bone marrow cells, there was no enhancement of Adriamycin toxicity by sildenafil.

We were also interested in examining whether Adriamycin toxicity to macrophages was through the generation of free radical species. Figure 10 shows that the free radical scavengers, N-acetyl cysteine and glutathione failed to interfere with the toxicity of Adriamycin. This is an unexpected observation that suggests that the effects on macrophages are not through the generation of reactive oxygen species. As a positive control, we demonstrate that N-acetyl cysteine was partially protective against the toxicity of hydrogen peroxide.

Additional Studies Relating to Adriamycin Action in Breast Tumor Cells

In our previous studies with Adriamycin in breast tumor cells, we reported on the promotion of senescence as a key element in the response to this drug treatment (9). However, we originally found that senescence was observed only in cells expressing wild type (functional) p53 (9). In subsequent work, we determined that breast tumor cells where p53 was either null (MCF-7/E6)

or mutated (MDA-MB231), residual cells that survived exposure to Adriamycin were in a state of senescence (Figure 11) (10). Furthermore, as with our findings in p53 wild type cells (11), we observed recovery of proliferative capacity from the senescent cell population (Figure 12). This is an important observation as we hypothesize that senescence and proliferative recovery may have relevance to tumor dormancy and disease recurrence, respectively, in the patient.

Finally, a critical point that is relevant to the cardiotoxicity of Adriamycin is that it is thought to be cardiotoxic through the generation of reactive oxygen species (12,13) while its antitumor action is thought to occur primarily through inhibition of topoisomerase II (14). However, we have now generated data that indicates that the senescence effect of Adriamycin also appears to be mediated through free radical generation. Figure 13 shows that Adriamycin-induced senescence can be suppressed using the free radical scavengers, N-acety cysteine and glutathione, and that suppression of free radical generation (at least partially) protects the cells from the toxicity of Adriamycin (Figure 14). Studies are now planned to identify the nature of the free radical species associated with senescence induction by Adriamycin in breast tumor cells.

KEY RESEARCH ACCOMPLISHMENTS

1. Sildenafil fails to protect various breast tumor cell lines against the toxicity of adriamycin (using multiple assays including clonogenic survival).
2. Sildenafil enhances the response to adriamycin in breast tumor cells lacking functional p53.
3. Sildenafil does not protect breast tumor cells against DNA damage induced by adriamycin.
4. Sildenafil does not alter cell cycle perturbations produced in breast tumor cells by adriamycin.
5. Sildanafil does not increase Adriamycin toxicity to bone marrow cells.
6. Sildanafil does not increase Adriamycin toxicity to macrophages.
7. Proliferative recovery is evident after senescence induced by adriamycin in breast tumor cells lacking functional p53.
8. Senescence induced by Adriamycin in breast tumor cells is associated with the generation of reactive oxygen species, similar to the mechanism of its cardiotoxicity.

REPORTABLE OUTCOMES

Di X, Newsham I, Shiu R and Gewirtz DA. Apoptosis, autophagy and accelerated senescence in the response of human breast tumor cells to Adriamycin. Biochem. Pharmacol. 77(7):1139-50. 2009.

Di X, Bright AT, Bellott R, Gaskins E, Robert J, Holt S, Gewirtz D, Elmore L. A chemotherapy-associated senescence bystander effect in breast cancer cells. Cancer Biol Ther. 2008. 7(6):864-872.

Gewirtz D, Holt SE and Elmore LW. Accelerated senescence: An emerging role in tumor cell response to chemotherapy and radiation. Biochemical Pharmacology 2008. 76(8): 947-957.

Xu Di will receive his PhD degree in Pharmacology and Toxicology (June 2009).

Submission of RO1 grant to NIH on Adriamycin induced senescence in breast cancer cells (March 2009)

CONCLUSIONS

1. Sildenafil fails to protect breast tumor cells from adriamycin based on multiple assays (viable cell number, clonogenic survival, cell cycle distribution and DNA damage). The clonogenic survival assays support the conclusion that sildenafil appears to sensitize breast tumor cells lacking functional p53 to Adriamycin. Since breast cancer frequently is shown to express mutant p53, these findings support the potential utility of sildenafil as a cardioprotectant that is unlikely to interfere with the antitumor actions of Adriamycin (or other chemotherapeutic agents; see previous report).

2. Sildenafil does not increase Adriamycin toxicity to bone marrow cells or macrophages. Again, these findings indicate that sildenafil is unlikely to increase host toxicity of Adriamycin.

3. Adriamycin has the capacity to produce multiple modes of cell death in the breast tumor cells. We now have evidence that Adriamycin also functions through the generation of free radicals to promote senescence. These findings raise questions relating to the selectivity of Adriamycin against the tumor cell versus the heart.

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SUPPORTING DATA

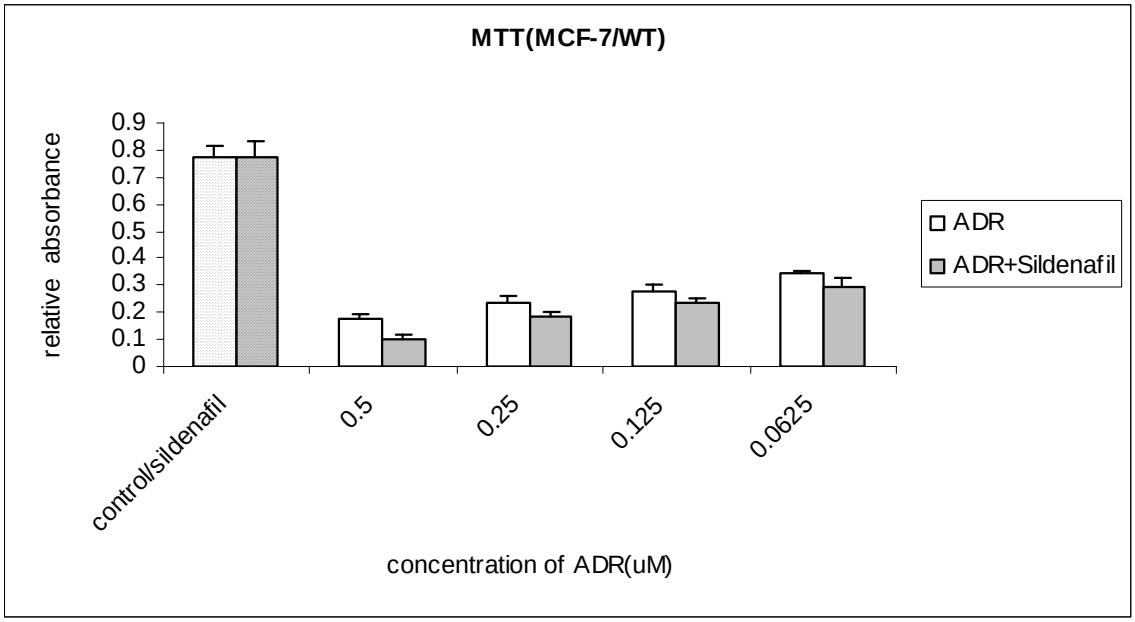


Figure 1: Lack of protection of MCF-7 breast tumor cells from adriamycin by sildenafil. Cells were exposed to sildenafil (10μM) for 1 hour prior to incubation with adriamycin (various concentrations) for 72 hr. Relative absorbance was measured by the MTT assay and indicates the number of viable cells.

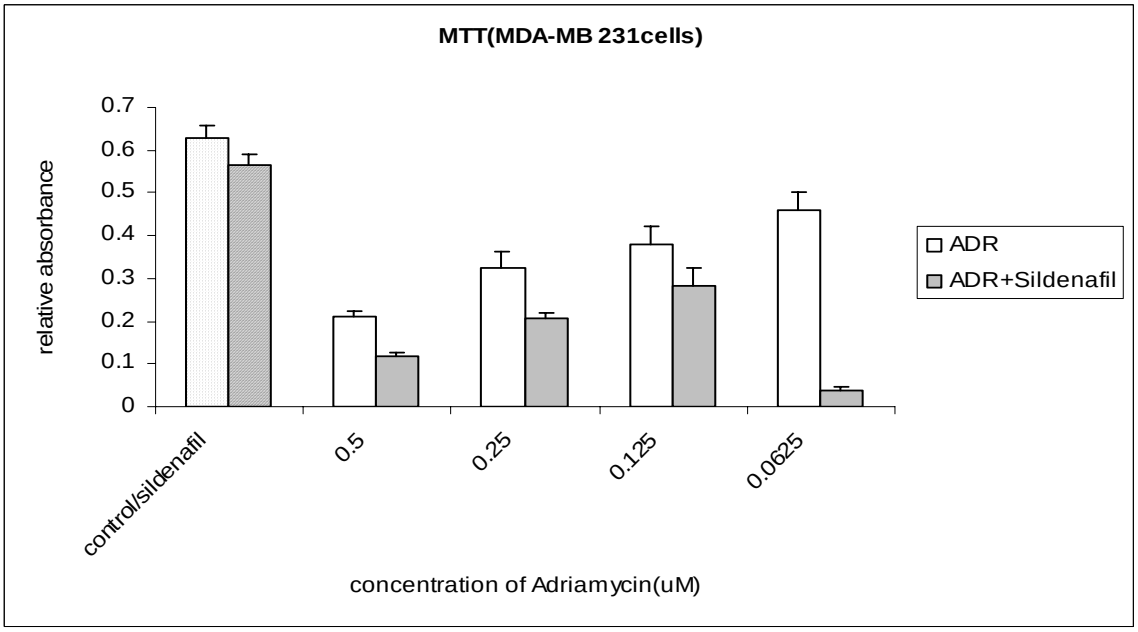
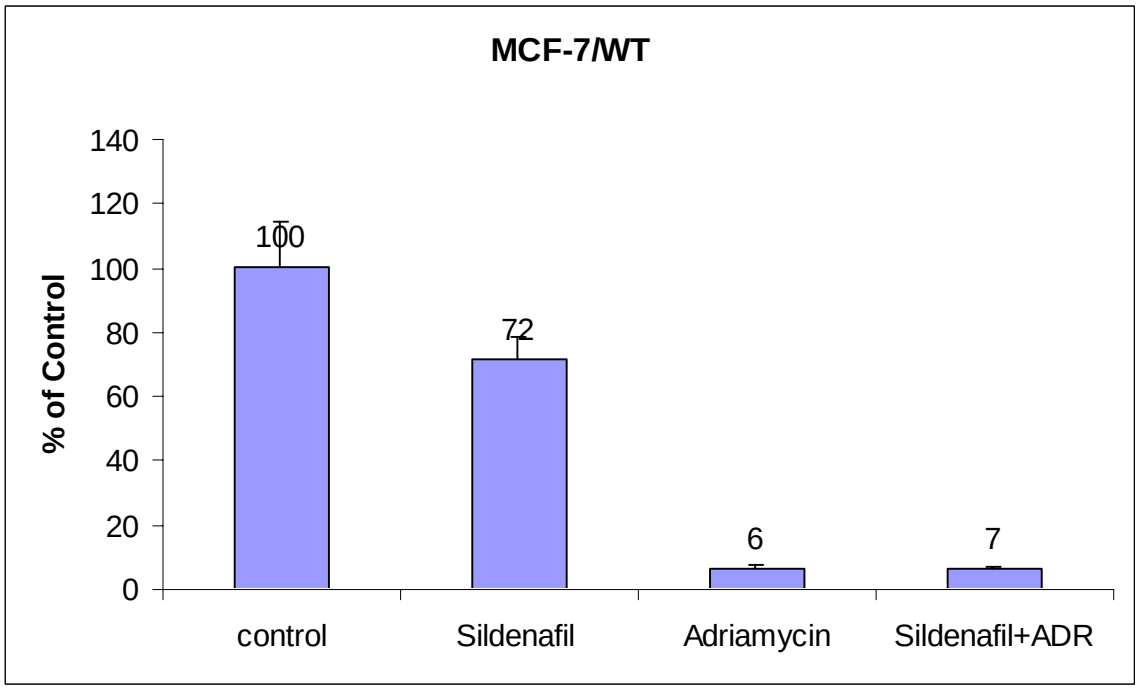


Figure 2: Sensitization of MDA-MB231 breast tumor cells to adriamycin by sildenafil. Cells were exposed to sildenafil (10μM) for 1 hour prior to incubation with adriamycin (various concentrations) for 72 hr. Relative absorbance was measured by the MTT assay and indicates the number of viable cells.



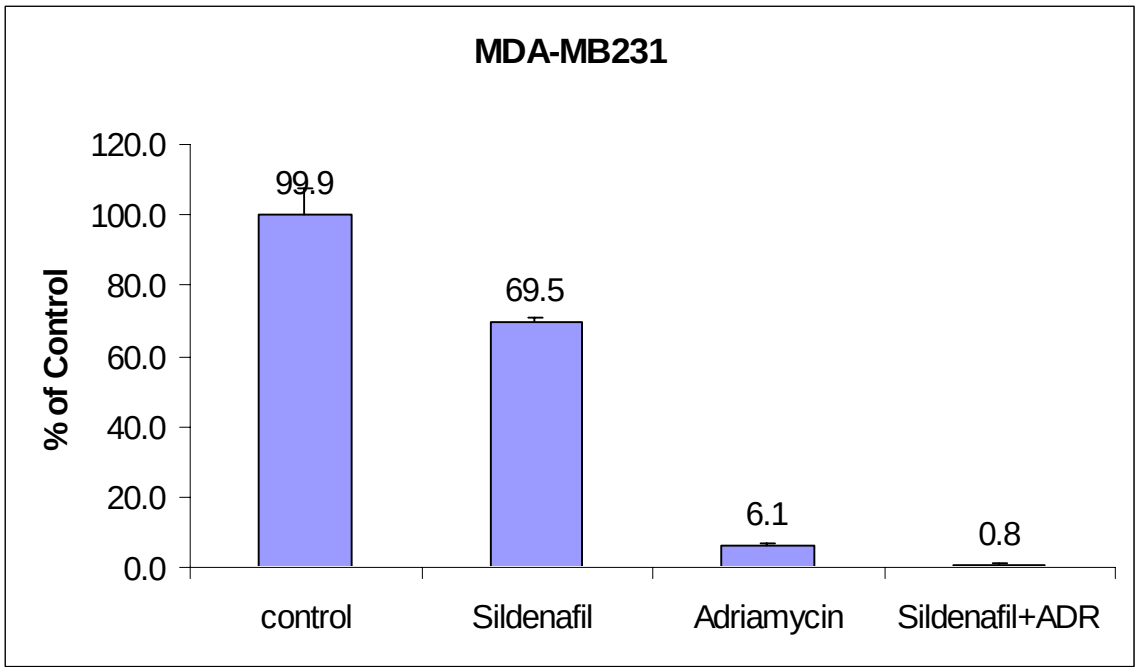


Figure 3: Lack of protection of MCF-7 cells and sensitization of MDA-MB231 breast tumor cells to adriamycin by sildenafil. Cells were exposed to sildenafil (10μM) for 1 hour prior to incubation with adriamycin (1μM) for 72 hr. Clonogenic survival was evaluated after 14 days.

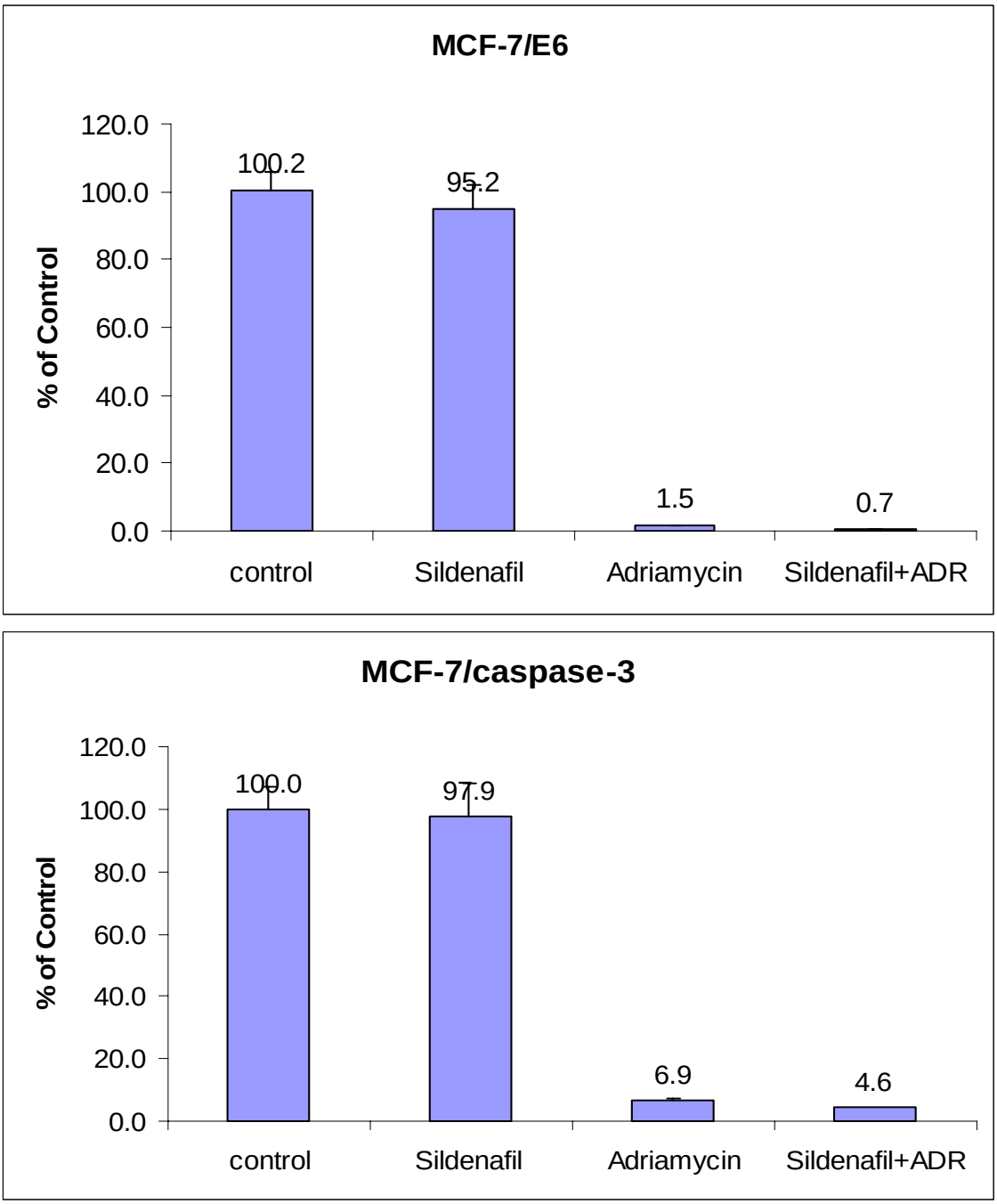


Figure 4. Lack of protection of MCF-7/caspase 3 cells and sensitization of MCF-7/E6 breast tumor cells to adriamycin by sildenafil. Cells were exposed to sildenafil (10μM) for 1 hour prior to incubation with adriamycin (1μM) for 72 hr. Clonogenic survival was evaluated after 14 days.

53bp-1 immunofluorescence staining (MCF-7)

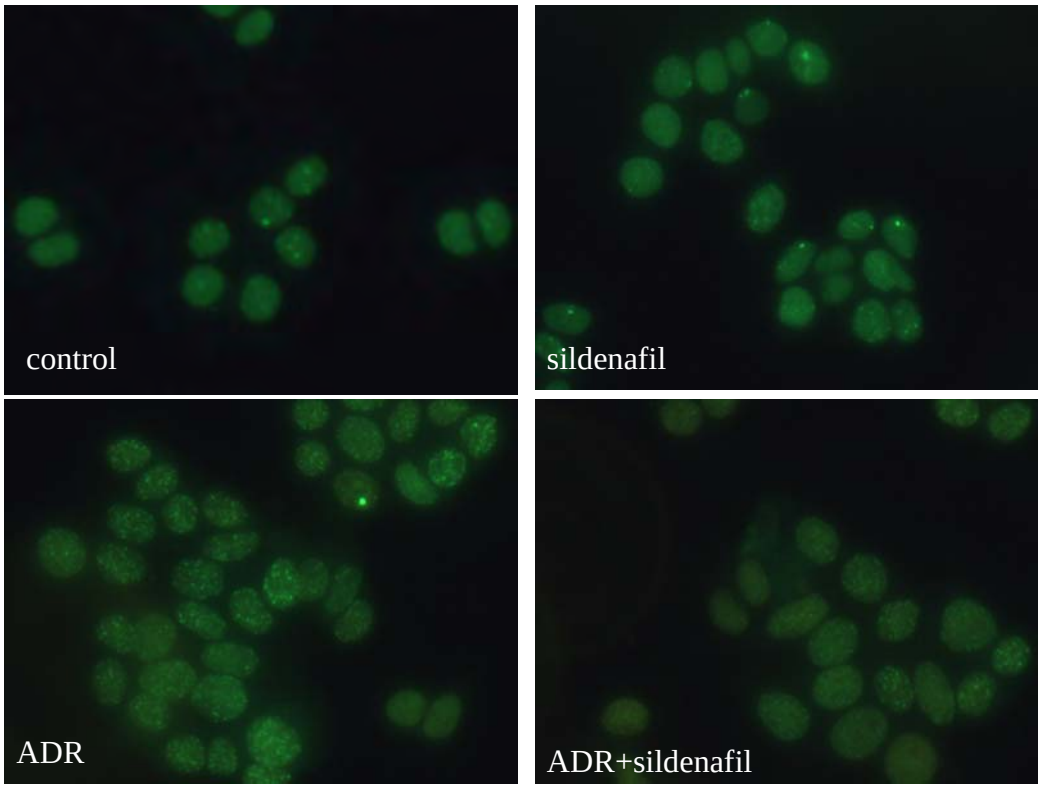


Figure 5. Sildenafil does not alter DNA damage induced by Adriamycin. 53BP1 staining in MCF-7 breast tumor cells. Cells were exposed to adriamycin alone (1μM), sildenafil alone (10μM) or sildenafil + adriamycin and foci formation was assessed after 24 hr.

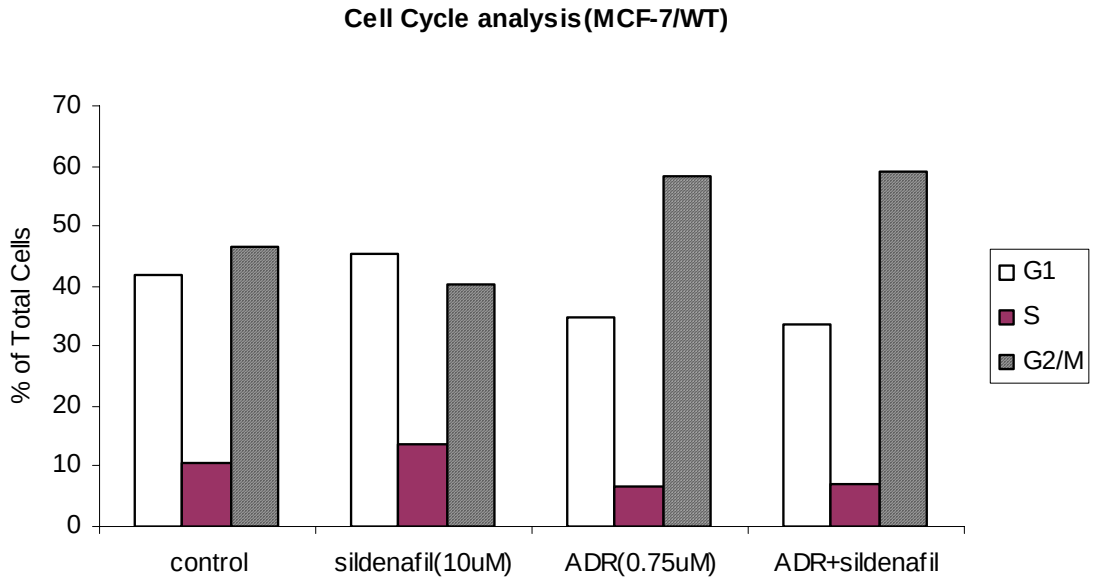


Figure 6. Sildenafil does not alter cell cycle arrest induced by Adriamycin. MCF-7 cells were exposed to adriamycin alone (1μM), sildenafil alone (1μM) or sildenafil + adriamycin and cell cycle distribution was assessed after 24 hr.

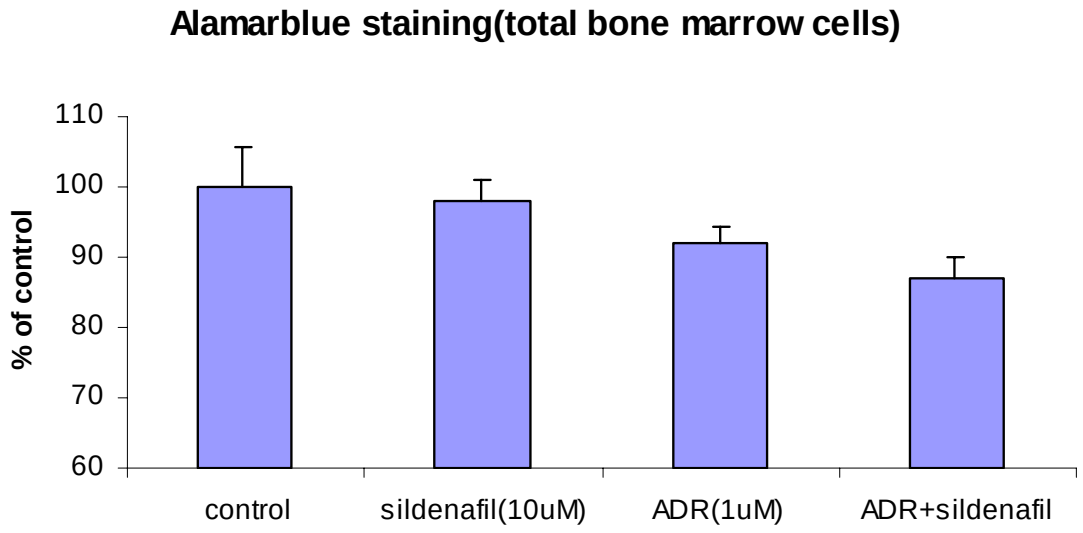


Figure 7. Sildenafil does not increase Adriamycin toxicity to bone marrow cells. Bone marrow cells were isolated from the femurs and tibias of BALB/c nude mice. Cells were cultured in IMDM medium and exposed to adriamycin alone (1μM), sildenafil alone (10μM) or sildenafil + adriamycin. Alamar blue staining, indicative of viable cells number, was assessed after 48 hr.

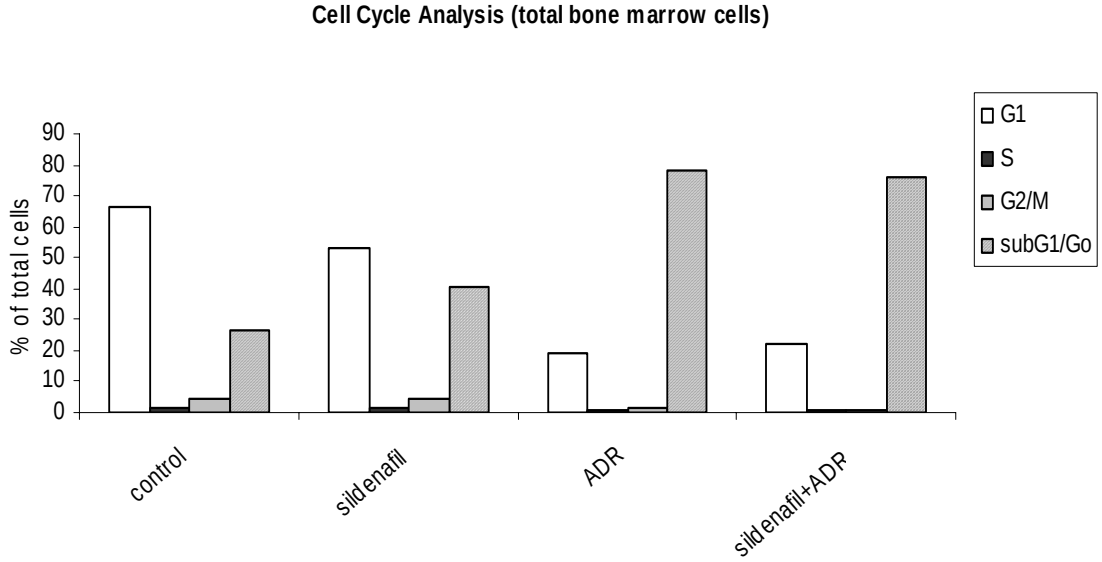


Figure 8. Sildenafil does not alter cell cycle arrest induced by Adriamycin in bone marrow cells. Bone marrow cells were isolated as described above and exposed to adriamycin alone (1μM), sildenafil alone (10uM) or sildenafil + adriamycin. Cell cycle distribution was evaluated after 48 hr.

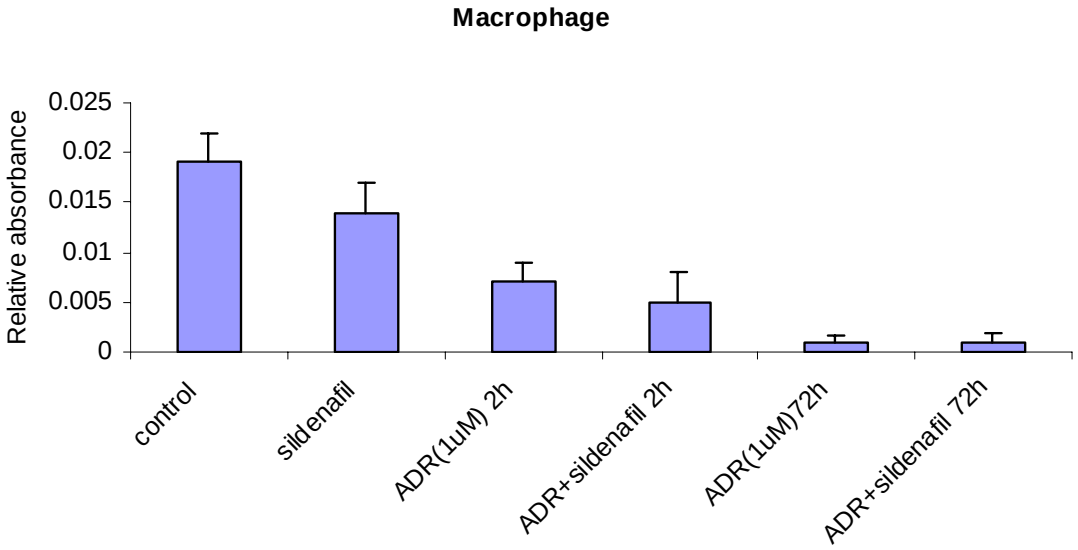


Figure 9. Sildenafil does not increase Adriamycin toxicity towards macrophages. Macrophages were obtained from B6C3 mice and cultured in DMEM medium. Cells were exposed to adriamycin alone (1uM), sildenafil alone (10uM) or sildenafil + adriamycin. Viable cells number was assessed by MTT assay after 2 hr and after 72 hr.

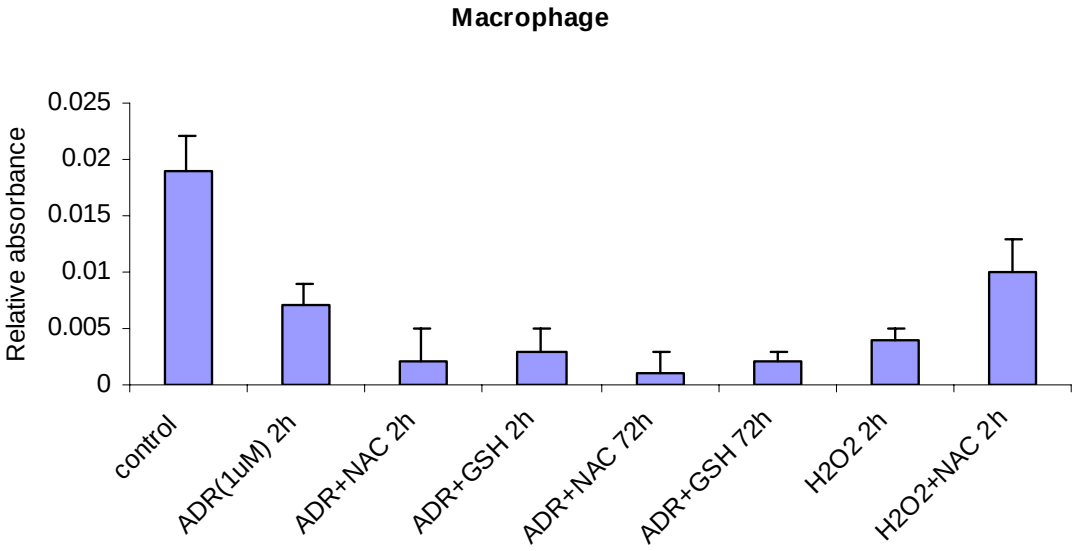


Figure 10. Adriamycin toxicity to macrophages is not mediated via free radical generation. Macrophages were obtained from B6C3 mice and cultured in DMEM medium. Cells were exposed to adriamycin alone (1μM) or adriamycin followed preceded by N-acetyl cysteine (10mM) or glutathione (10mM). Treatment with H_2O_2 alone or with prior exposure to N-acetyl cysteine was used as a positive control. Viable cells number was assessed by MTT assay after 2 hr and after 72 hr.

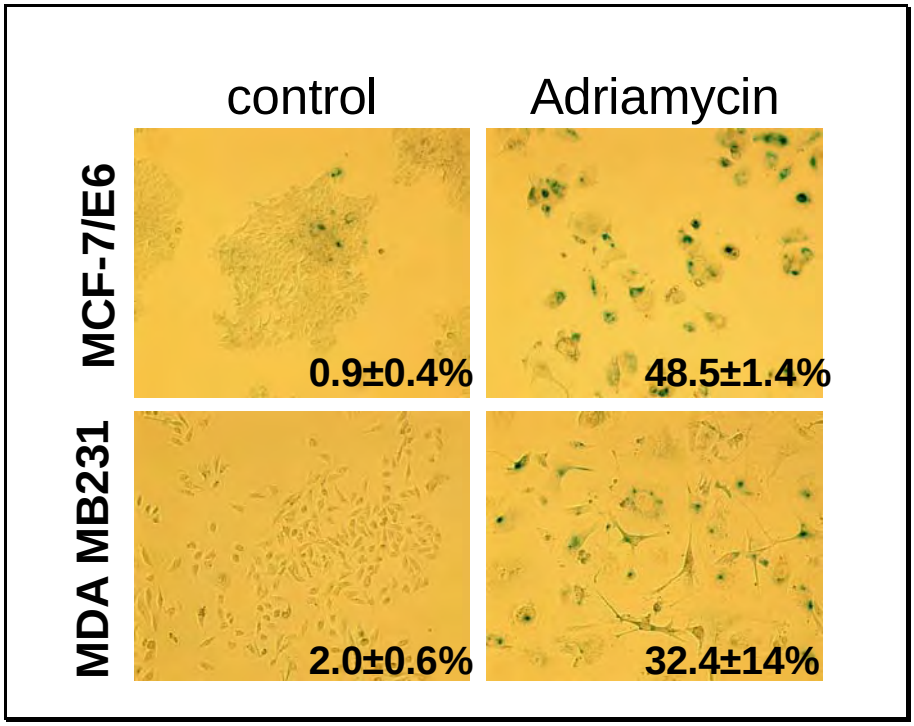


Figure 11. **Senescence in residual surviving MCF-7/E6 (p53 null) and MDA-MB231 (p53 mutant) breast tumor cells.** The percent senescent population at 6 days post exposure to Adriamycin is indicated.

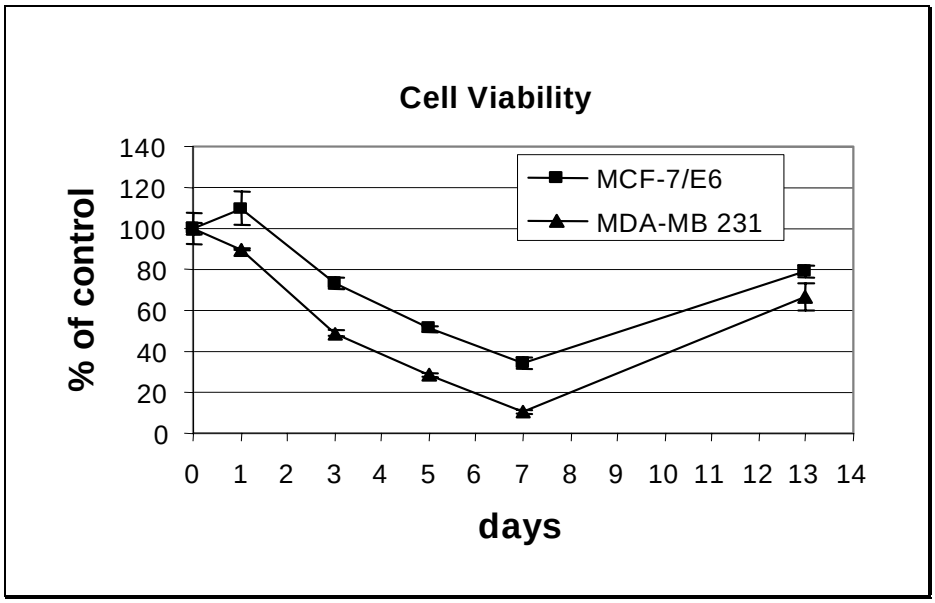


Fig. 12. Proliferative recovery after Adriamycin in MCF-7/E6 and MDA-MB231 breast tumor cells. Cells were exposed to 1 μM Adriamycin for 2 hours, washed free of drug and viable cell number monitored for the indicated days.

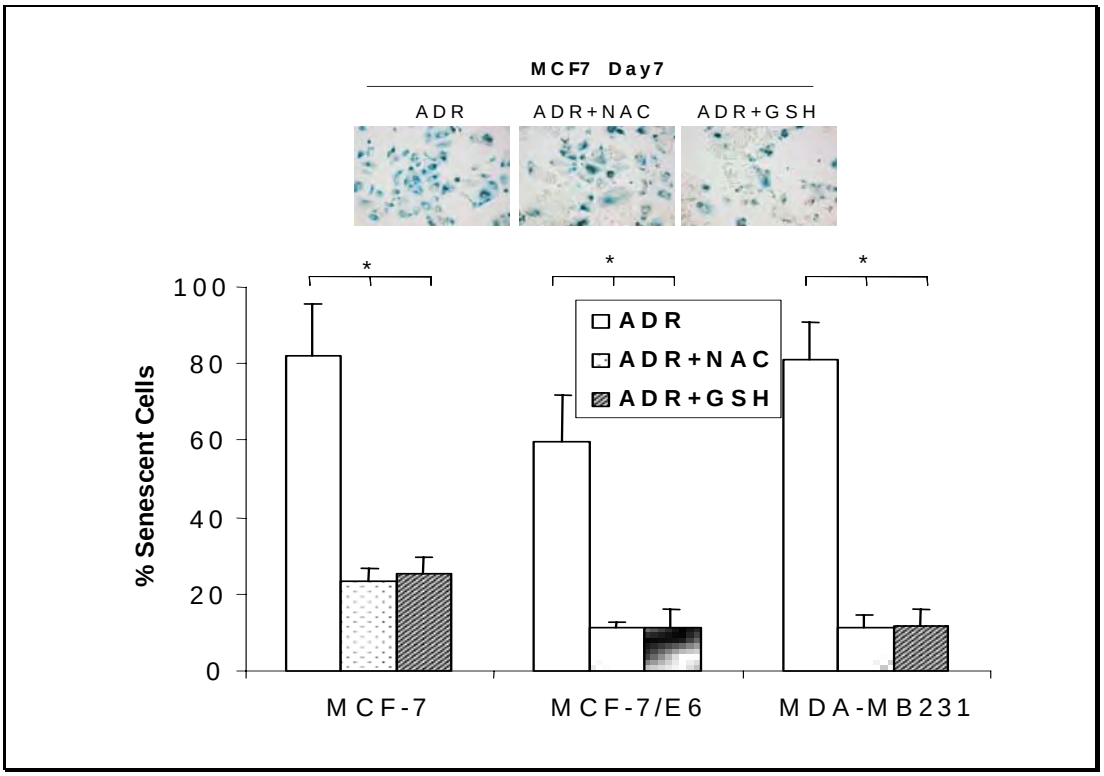


Figure 13. Influence of free radical scavengers on senescence induced by Adriamycin in MCF-7, MDA-MB231 and MCF-7/E6 breast tumor cells. MCF-7, MDA-MB231 and MCF-7/E6 cells were treated with either 10mM glutathione or 10mM N-acetyl cysteine for 1 hour prior to exposure to 1μM Adriamycin for 2 hours. Senescence was monitored based on cell morphology and expression of beta galactosidase. Upper portion of figure: β-galactosidase staining in MCF-7 cells. Lower portion of figure: Quantitation of senescence in the absence and presence of GSH and NAC. * P< 0.05.

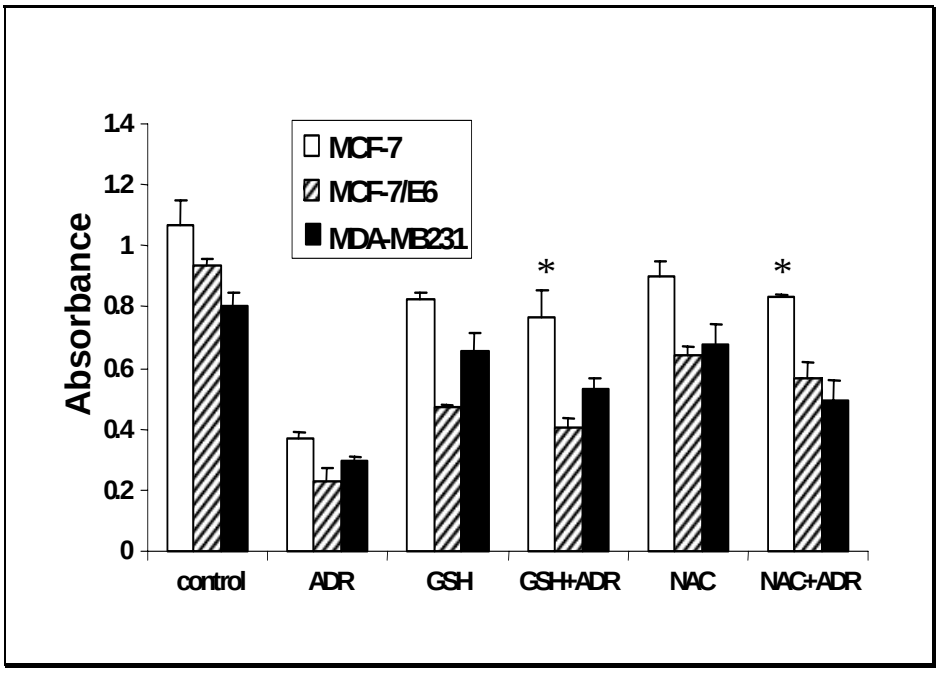


Figure 14. Protection of MCF-7 breast tumor cells from Adriamycin by NAC and GSH. Cells were treated with 1μM Adriamycin in the presence or absence of 20mM GSH or 20mM NAC for 2 hours. The MTT assay was performed 3 days post Adriamycin treatment. Absorbance (y-axis) is indicative of cell number. * p<0.05, ADR vs. GSH+ADR or ADR vs. NAC+ADR in MCF-7 cells.