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13. ABSTRACT ***(Maximum 200 Words)***

This project aims to develop improved strategies for using doxorubicin in the treatment of multidrug resistant advanced breast cancer while simultaneously minimizing the risk of cardiotoxicity. The wild type and multidrug resistant MCF7 human breast cancer cells were characterized and the HPLC methods standardized for use in pharmacokinetic studies. Electron paramagnetic resonance was utilized to detect free radical production by MCF-7 and MCF-7/ADR cells during doxorubicin metabolism. The interaction of tamoxifen with doxorubicin was investigated *in vitro*. Tamoxifen potentiated the cytotoxicity of doxorubicin towards multidrug resistant MCF-7/ADR cells but not the wild type MCF-7 cells. The wild type MCF-7 and its multidrug resistant variant cells yielded different amounts of free radicals (as judged by spin trapping experiments) when incubated with doxorubicin. The antiestrogen tamoxifen did not affect free radical production from doxorubicin. Interpretation of these results was complicated by the fact that the cell viability differed considerably in the two cell types upon treatment with doxorubicin. Tamoxifen acted as an antioxidant and inhibited free radical initiated lipid peroxidation in cardiac and hepatic microsomal preparations. Tamoxifen may reverse multidrug resistance and exert its antioxidant properties to protect against cardiac tissue damage.

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Rajagopalan Sridhar 6-30-2000

PI - Signature Date

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V. INTRODUCTION

The anti-cancer drug doxorubicin (adriamycin) causes severe adverse effects at high doses and loses its efficacy against multidrug resistant tumors including breast cancer. Potentially fatal cardiotoxicity can develop in patients when the cumulative dose of the drug exceeds 450 mg/m^2. For optimum use of doxorubicin it is necessary to overcome multidrug resistance of tumor with simultaneous protection against cardiotoxicity. Cardiotoxicity may result from the tendency of doxorubicin and its metabolites to accumulate in the heart and trigger free radical mediated injury (1-3). At present, R-verapamil, tamoxifen, raloxifene and toremifene and dipyridamole are being considered as chemosensitizers for reversing tumor resistance to doxorubicin. It is important to identify and avoid conditions under which they preferentially sensitize the tumor without subjecting the heart to the deleterious effects. Free radicals are known to induce apoptosis. If free radicals are the cause of cardiotoxicity, then R-verapamil, tamoxifen, toremifene, raloxifene and dipyridamole should protect the heart by virtue of their anti-peroxidant action. If apoptosis is the cause of cardiac injury then the alleged anti-apoptotic action of these chemicals should be beneficial to the heart. If the cardiotoxicity is the result of altered kinetics and metabolism of doxorubicin to the more toxic doxorubicinol, then certain metabolic inhibitors would afford protection. Apoptosis in the tumor is desirable, whereas apoptosis in the heart is detrimental. Methods for decreasing the free radical burden in the heart and preventing the accumulation of doxorubicin and its metabolites in the heart may improve the clinical usefulness of doxorubicin. This may be achievable because the antitumor action of doxorubicin may occur through inhibition of DNA topoisomerase with minimal involvement of free radicals. The mechanisms involved in the actions of these pharmacologically diverse compounds may hold valuable clues for improved cancer therapy and protection against cardiotoxicity. The specific aims of the project are:

#1: To determine if R-verapamil, tamoxifen, toremifene, raloxifene and dipyridamole alter the metabolic and pharmacokinetic profiles of doxorubicin in athymic nude mice with multidrug resistant MCF-7 human tumor xenografts. High pressure liquid chromatography (HPLC) will be utilized to measure the concentration of doxorubicin, doxorubicinol and the chemomodulator in blood, tumor, heart, kidneys and liver of mice. The extent of lipid peroxidation and the concentration of reduced glutathione will be determined in heart and liver tissue by standard biochemical assays.

#2: To determine if R-verapamil, tamoxifen, toremifene, raloxifene and dipyridamole have differential effects on programmed cell death (apoptosis) in cardiac tissue compared to the tumor. The extent of apoptosis in heart tissue and tumor will be assayed *in situ* by histological methods and by DNA laddering assays in order to correlate cardiac damage with extent of apoptosis.

#3: To apply the electron spin resonance (ESR) technique of spin trapping to compare the influence of R- and S-verapamil on doxorubicin-induced free radical formation *in vitro* and *in vivo.* Alpha-(2,4,6-trimethoxyphenyl) N-tert-butylnitrone (TMeOPBN) will be administered to mice for *in vivo* studies and TMeOPBN and 5,5-dimethylpyrroline N-oxide (DMPO) will be used for *in vitro* experiments with cardiac microsomes and mitochondria isolated from animals in the different treatment groups.The long term goal is to find safer methods of treating multidrug resistant advanced breast cancer with doxorubicin while preventing cardiotoxic side effects of treatment.

VI BODY

The HPLC techniques have been developed and the necessary quality control measures have been established. These will be directly applicable to pharmacokinetic studies. A standard curve was generated for quantitative analysis of doxorubicin using daunomycin as an internal standard (Figure 1). The reproducibility of the method was also tested (Table 1 and Table 2)

Flow cytometry was used to characterize the cell lines with respect to the presence of the protein associated with multidrug resistance. As expected, the protein associated with multidrug resistance was found only in MCF-7/ADR cells but not in wild type MCF-7 human breast cancer cells.

Since multidrug resistance is associated with an ATP binding permeability glycoprotein (pgp 170), the influence of glucose on the growth of the two cell lines was examined. The two cells differed in their response to high levels of glucose (Figure 2). Increasing the glucose concentration in the medium from 5 mM to 25 mM was without effect on MCF-7/ADR cells, but stimulated further growth of MCF-7 cells. This may have some bearing on the growth and progression of tumors in diabetic individuals.

The cell lines were further characterized in terms of their sensitivity to doxorubicin in the presence and absence of tamoxifen (Figures 3-6 and Table 3). Isobologram analysis revealed synergistic interaction of doxorubicin wth tamoxifen in the case of MCF-7/ADR but not MCF-7 cells (Figures 5 and 6).

Electron spin resonance (ESR) techniques were utilized to study free radical production in cells treated with doxorubicin. The formation of semiquinone free radical from doxorubicin was detected in anaerobic incubations of cells (Figure 7). The spin trap DMPO was utilized to detect the formation of hydroxyl free radicals by cells incubated with doxorubicin and DMPO under aerobic conditions (figure 8). The dose of doxorubicin needed for demonstrating free radical production was quite cytotoxic and comparison of the results from the drug sensitive MCF-7 and the drug resistant MCF-7/ADR cells was further complicated by the differences in tolerance to doxorubicin.

The ability of tamoxifen to inhibit microsomal lipid peroxidation was studied using liver and cardiac microsomes (Figures 9-11).

The animal studies have been delayed. However, we have grown MCF-7/ADR cells as solid tumor xenografts in nude mice as part of another ongoing project dealing with *in vivo* phosphorus nuclear magnetic resonance spectroscopy. We will initiate tumor transplantation for pharmacokinetic studies and apoptosis assays in the latter half of the second year of the project and complete the experiments by the end of the third year.

VII. KEY RESEARCH ACCOMPLISHMENTS

HPLC method for measuring doxorubicin content in biological samples has been standardized.

MCF-7 and MCF-7/ADR cells have differ in their growth patterns in RPMI medium with normal (5 mM) and high (25 mM) glucose levels.

MCF-7 and MCF-7/ADR cells metabolize doxorubicin to the corresponding semiquinone free radical. However, the concentration of doxorubicin used to demonstrate this results in reproductive cell death of the cells. The semiquinone free radical was observed under anaerobic conditions. Including 5,5-dimethylpyrroline-N-oxide (DMPO) as a spin trap enabled the detection of hydroxyl radical in aerobic incubation mixtures.

Tamoxifen inhibited doxorubicin stimulated lipid peroxidation in both liver and cardiac microsomes.

Tamoxifen by itself causes reproductive cell death. Tamoxifen interacts synergistically with doxorubicin to cause reproductive cell death in MCF-7/ADR cells, but the interaction is only additive in the case of wild type MCF-7 cells.

VIII. REPORTABLE OUTCOMES

Sridhar, R., Yamamoto, M., Patel, N.A. and Cooper, D.R. MCF-7 and MCF-7/ADR cells respond differently to changes in glucose concentrations. FASEB J. 13: A920, 1999. Abstract # 682.14 presented at "EXPERIMENTAL BIOLOGY 99", Washington, DC, April 17-21, 1999.

Funded proposal: "Import of Poly ADP-Ribosylation and Free Radical Stress in Ataxia-Telangiectasia (A-T) Heterozygous Human Mammary Epithelial Cells (HMEC)"
Funding Agency: Marshall Space Flight Center, NASA, Huntsville, AL.
Principal Investigator: Rajagopalan Sridhar, 15% time and effort.
Project period: 09/01/99 to 08/31/01.

IX. CONCLUSIONS

A robust analytical method has been established for carrying out pharmacokinetic studies on tumor bearing mice. The formation of free radicals during cellular metabolism of doxorubicin has been demonstrated. However, the conditions necessary for demonstrating free radical formation also caused extensive cell death. Tamoxifen is an efficient antioxidant which interacts synergistically with doxorubicin to kill multidrug resistant MCF-7/ADR cells. This has to be confirmed *in vivo* using solid tumor xenografts of MCF-7/ADR cells in nude mice. This will be accomplished in the next phase of the project. Raloxifene and toremifene will also be tested *in vivo* along with tamoxifen. Our experiments underscore the importance of glucose levels in modulating the proliferation rate and cytotoxic response of cells.

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#15	Abstract	FASEB J. 13: A920, 1999. Abstract # 682.14 presented at "Experimental Biology 99", Washington, DC, April 17-21, 1999.

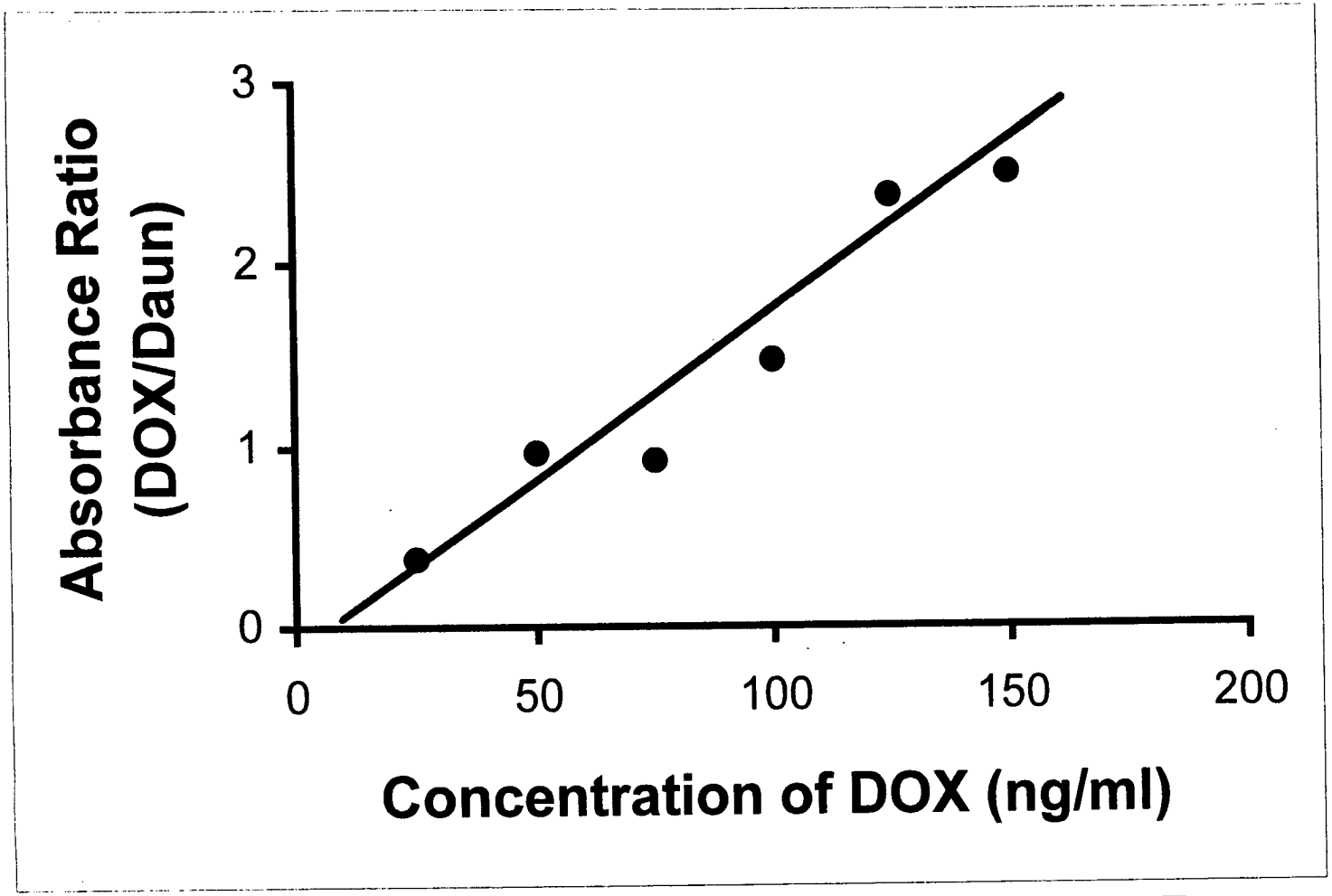

Figure 1: Calibration Curve for HPLC Analysis of Doxorubicin

HPLC analysis was carried out on samples containing known amounts of doxorubicin (DOX) and daunomycin (DAUN) as the internal standard. Spherisorb ODS-2 column (5μ 4.6 mm x 25 cm column was used in a Shimadzu HPLC system equipped with a fluorescence detector. A 70: 30 mixture of phosphate buffer (pH 0.06 M; pH 4.0) and acetonitrile was used for isocratic elution at a flow rate of 1 ml per minute, at room temperature. Fluorescence detector was at excitation and emission wave lengths of 480 nm and 560 nm respectively. Doxorubicin and daunomycin eluted after retention times of 7.5 and 17.5 minutes respectively.

APPENDIX # 2

Concentration of	Percent coefficient of variation	
Doxorubicin (ng/ml)	**Intra-day**	**Inter-day**
25	**4.2**	**6.9**
125	**5.8**	**6.0**

Table 1: Intra-day and inter-day reproducibility of HPLC analysis of doxorubicin

Plasma samples containing known amounts of doxorubicin were subjected to the acid extraction procedure and analyzed using HPLC and the inter-day and intra-day variation was checked.

APPENDIX # 3

Concentration of Doxorubicin added (ng/ml)	Concentration of Doxorubicin recovered	Percent recovery (mean ± standard deviation)
25	23.3, 26.4, 23.9	98.1 ± 5.36
50	45.9, 48.1, 53.7	98.4 ± 6.56
100	94.5, 93.8, 96.7	95.0 ± 1.23

Table 2: Efficiency of acid extraction method for recovery of Doxorubicin from mouse plasma

Plasma samples were thawed and mixed using a vortex generator. One hundred milliliters of plasma were spiked with an equal volume of daunomycin (5 μM) as an internal standard and then mixed with 300 μl of 0.6 N HCl in 90% ethanol and stored at 4°C for one hour to form a gel. The sample was then centrifuged at 20,000 *g* for 25 minutes and the clear supernatant was analyzed for doxorubicin. Since doxorubicin and daunomycin are photosensitive, all manipulations were carried out under subdued light.

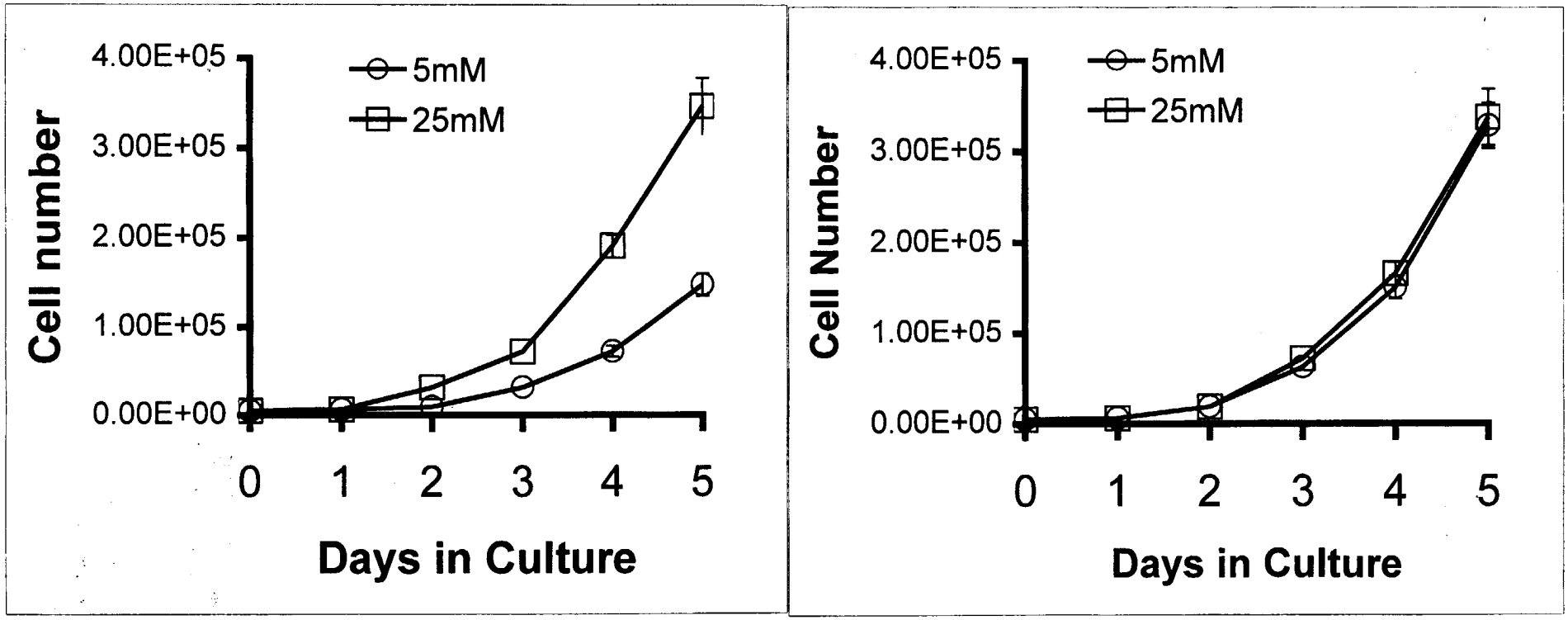

Figure 2: The influence of glucose on proliferation of MCF-7 and MCF-7/ADR cells in culture

The effects of glucose concentration on cell proliferation were investigated in MCF-7 (left panel) and MCF-7/ADR (right panel) cells. Single cell suspensions were prepared from exponentially growing monolayer cultures. Cells were seeded in several 6 well plates at approximately 1 x 10^4 cells/well in 3 ml of RPMI 1640 medium containing 5% fetal bovine serum and normmal (5 mM) (open circle) or high (25 mM) (open square) glucose. Total viable cell counts were determined daily using 0.4% trypan blue dye exclusion and the number of cells per dish calculated. the proliferation rate of MCF-7/ADR cells was higher than that of MCF-7 cells at normal glucose concentration. The proliferation of MCF-7 cells was stimulated by high glucose (left panel) but there was no no difference in the proliferation rates of MCF-7/ADR cells exposed to normal and high glucose (right panel). Data are mean ± SD for triplicate determinations which were repeated in three separate experiments.

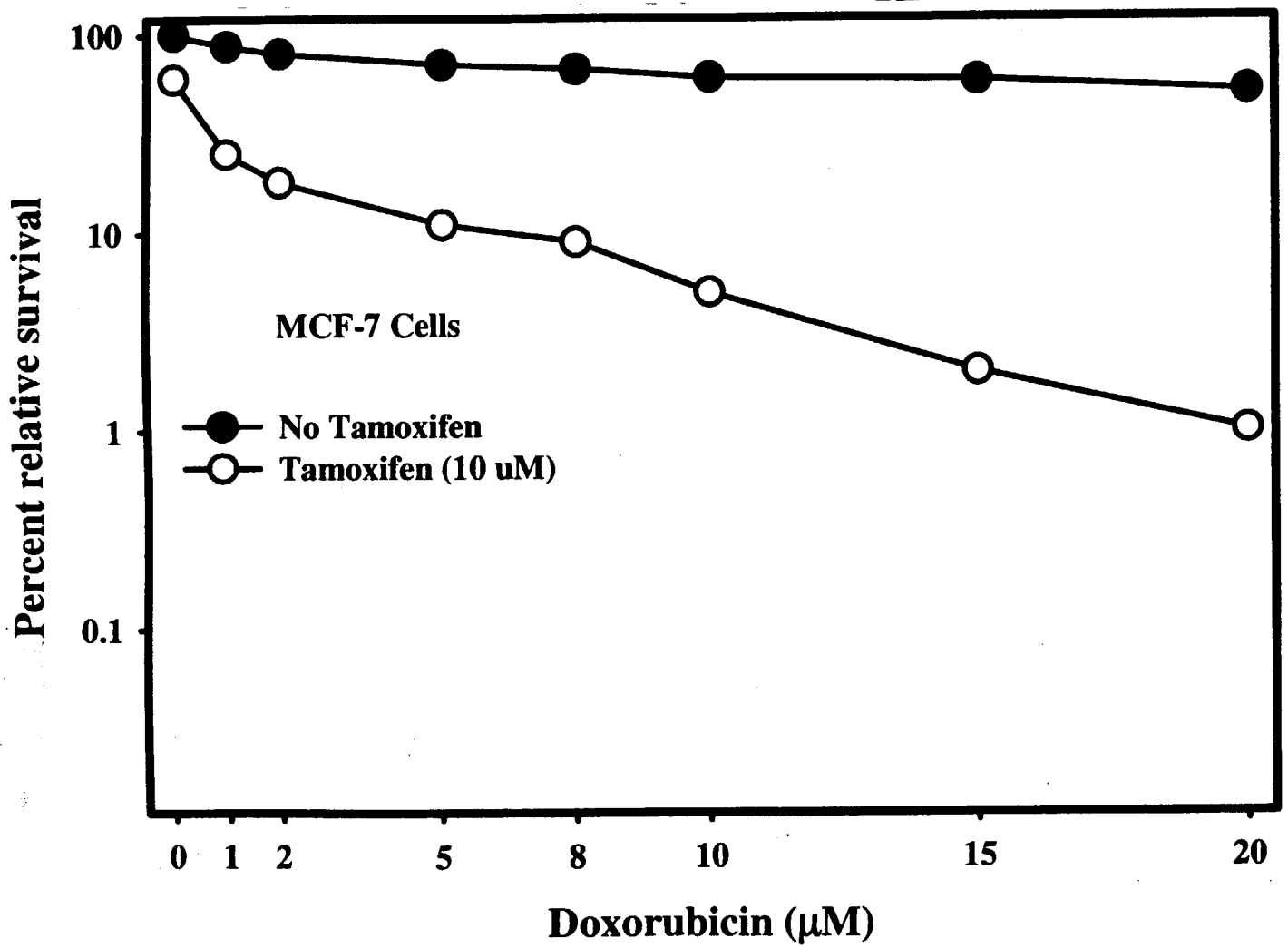

Figure 3: Cell survival curves for MCF-7 cells treated with graded concentrations of doxorubicin with or without tamoxifen

Cell viability was assessed using clonogenicity measurements. Approporiate numbers of exponentially growing MCF-7 cells were plated in triplicate onto 60 x 15 mm tissue culture dishes containing 5 ml complete medium. Cultures were treated the next day with doxorubicin (0 to 20 μM) alone (closed circle) or with tamoxifen (10 μM) (open circle) for 6 hours. At the end of the 6 hour drug treatment, the medium containing the drug was removed, and the cultures were washed with phosphate buffered saline and replenished with fresh medium. The cultures were incubated for 10 to 12 days and then stained with 1% methylene blue in 50% ethanol. Colonies containing 50 or more cells were considered to be reproductively viable. Survival of cells with respect to untreated controls was plotted against doxorubicin concentration.

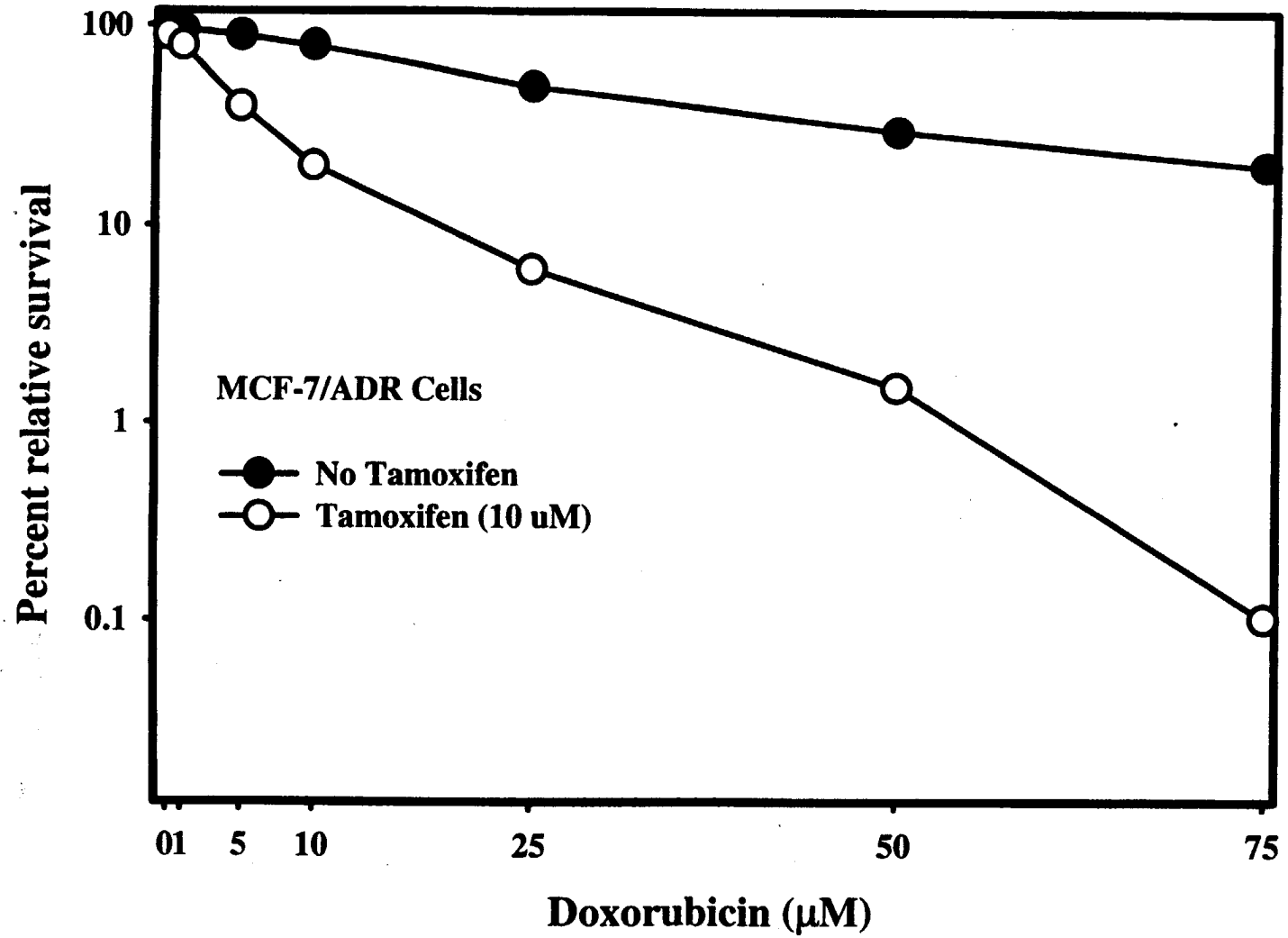

Figure 4: Cell survival curves for MCF-7/ADR cells treated with graded concentrations of doxorubicin with and without tamoxifen.

Cell viability was assessed using clonogenicity measurements. Approporiate numbers of exponentially growing MCF-7/ADR cells were plated in triplicate onto 60 x 15 mm tissue culture dishes containing 5 ml complete medium. Cultures were treated the next day with doxorubicin (0 to 75 μM) alone (closed circle) or with tamoxifen (10 μM) (open circle) for 6 hours. At the end of the 6 hour drug treatment, the medium containing the drug was removed, and the cultures were washed with phosphate buffered saline and replenished with fresh medium. The cultures were incubated for 10 to 12 days and then stained with 1% methylene blue in 50% ethanol. Colonies containing 50 or more cells were considered to be reproductively viable. Survival of cells with respect to untreated controls was plotted against doxorubicinb concentration.

Tamoxifen (μM)	MCF-7 CELLS		MCF-7/ADR CELLS	
	Dox(μM) IC_{50}	Enhancement Index (EI)	Dox(μM) IC_{50}	Enhancement Index (EI)
0.0	0.96	-	25.0	-
1.0	0.91	1.1	20.5	1.2
2.0	0.76	1.3	15.9	1.6
5.0	0.47	2.0	5.1	4.9
10.0	0.06	16.0	0.7	35.7

Table 3: Enhancement of doxorubicin cytotoxicity by tamoxifen

Appropriate numbers of exponentially growing MCF-7 and MCF-7/ADR cells were plated in triplicate onto 60 x 15 mm tissue culture dishes containing 5 ml of complete RPMI 1640 medium. Doxorubicin (0 to 20 μ M in case of MCF-7 cells; 0 to 75 μM in the case of MCF-7/ADR cells) and / or tamoxifen (0 to 10 μM) were added the next day. After 6 hours of drug treatment, the medium was removed from the cultures which were subsequently washed with phosphate buffered saline, and then refed with complete medium. The cultures were incubated for another 10 to 12 days and then stained with methylene blue (1% solution in 50 % ethanol). Colonies containing 50 or more cells were considered to represent a viable cell. The number of colonies arising from viable cells was determined and the survival was calculated relative to untreated control cultures. and the dose response curves were plotted and analyzed by the method of Chou and Talalay (4) to determine IC_{50} values. The enhancement index (EI) was calculated as the ratio

$$\frac{(IC_{50} \text{ of Doxorubicin in the absence of Tamoxifen})}{(IC_{50} \text{ of Doxorubicin in the presence of Tamoxifen})}$$

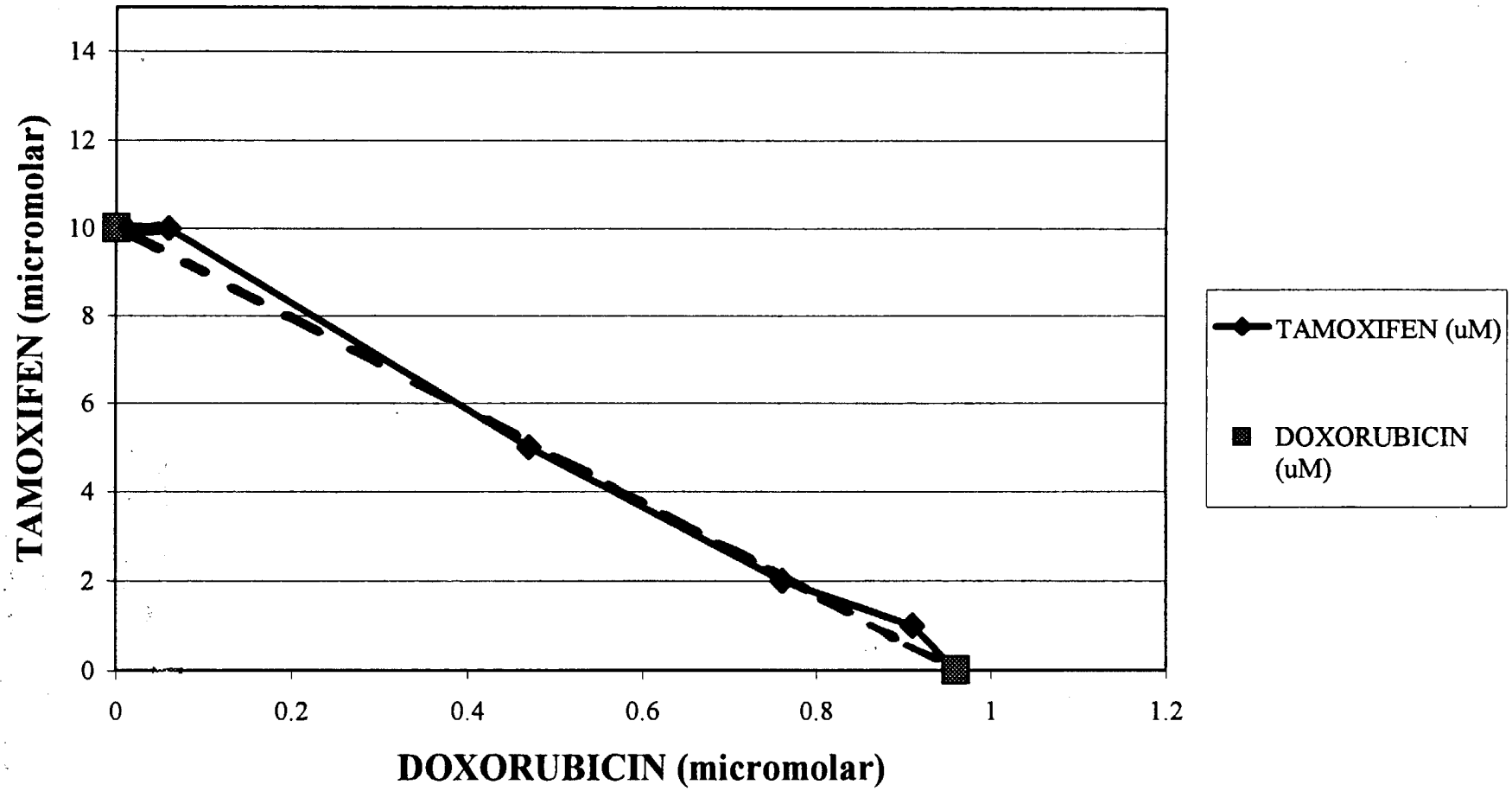

Figure 5: Isobologram analysis of cell survival data on MCF-7 cells

The nature of the interaction of doxorubicin and tamoxifen was evaluated according to the method of Berenbaum (5). Since each drug was cytotoxic when used alone, the combination of doxorubicin and tamoxifen was considered homoergic. Cells were treated with tamoxifen alone or doxorubicin alone or combinations of the two drugs over a range of concentrations and IC_{50} values were determined as indicated in table 3. The IC_{50} isobole was constructed and the combination of tamoxifen and doxorubicin was found to act additively.

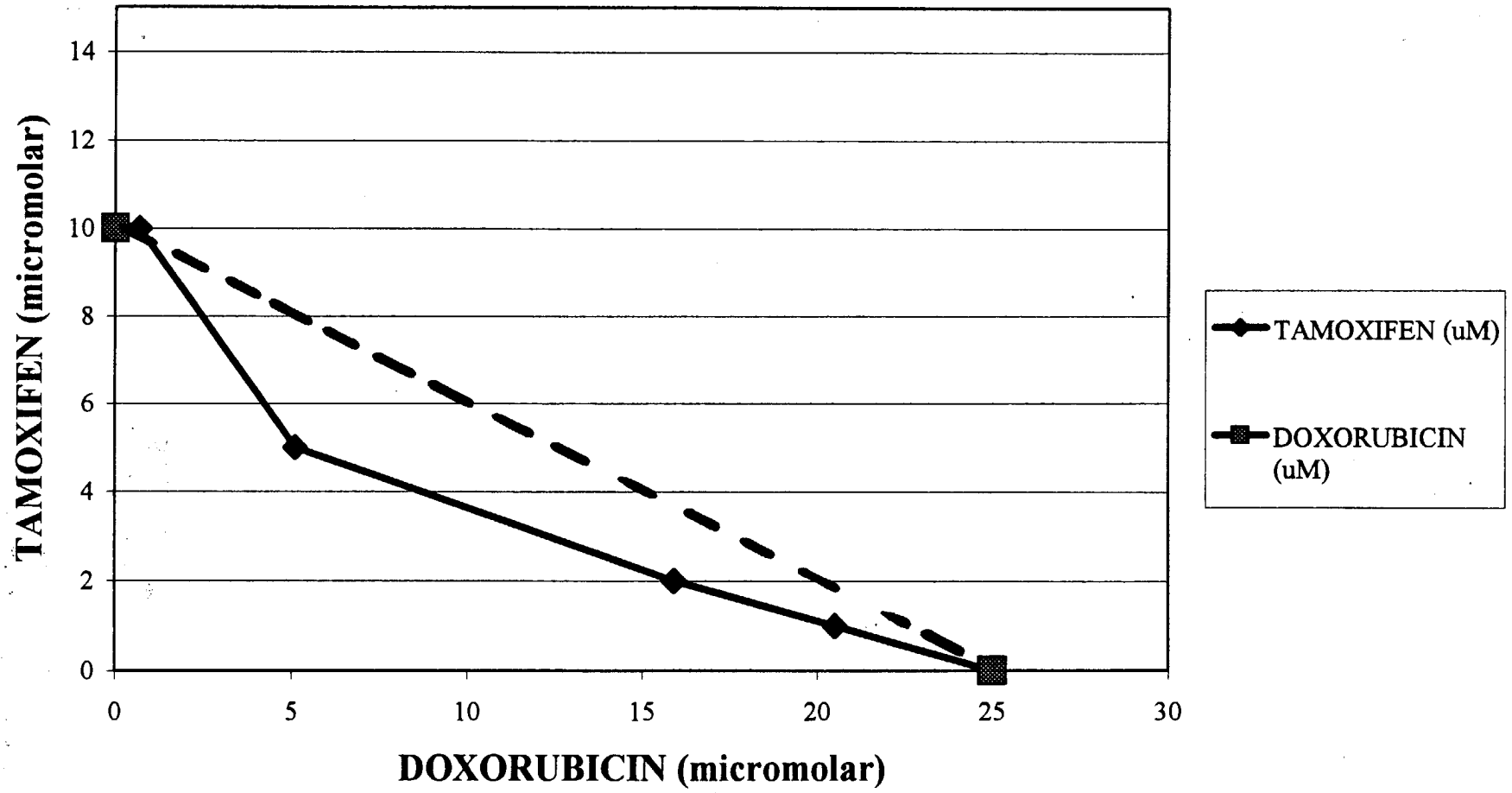

Figure 6: Isobologram analysis of cell survival data on MCF-7/ADR cells

The nature of the interaction of doxorubicin and tamoxifen was evaluated according to the method of Berenbaum (5). Since each drug was cytotoxic when used alone, the combination of doxorubicin and tamoxifen was considered homoergic. Cells were treated with tamoxifen alone or doxorubicin alone or combinations of the two drugs over a range of concentrations and IC_{50} values were determined as indicated in table 3. The IC_{50} isobole was constructed and the combination of tamoxifen and doxorubicin was found to act synergistically.

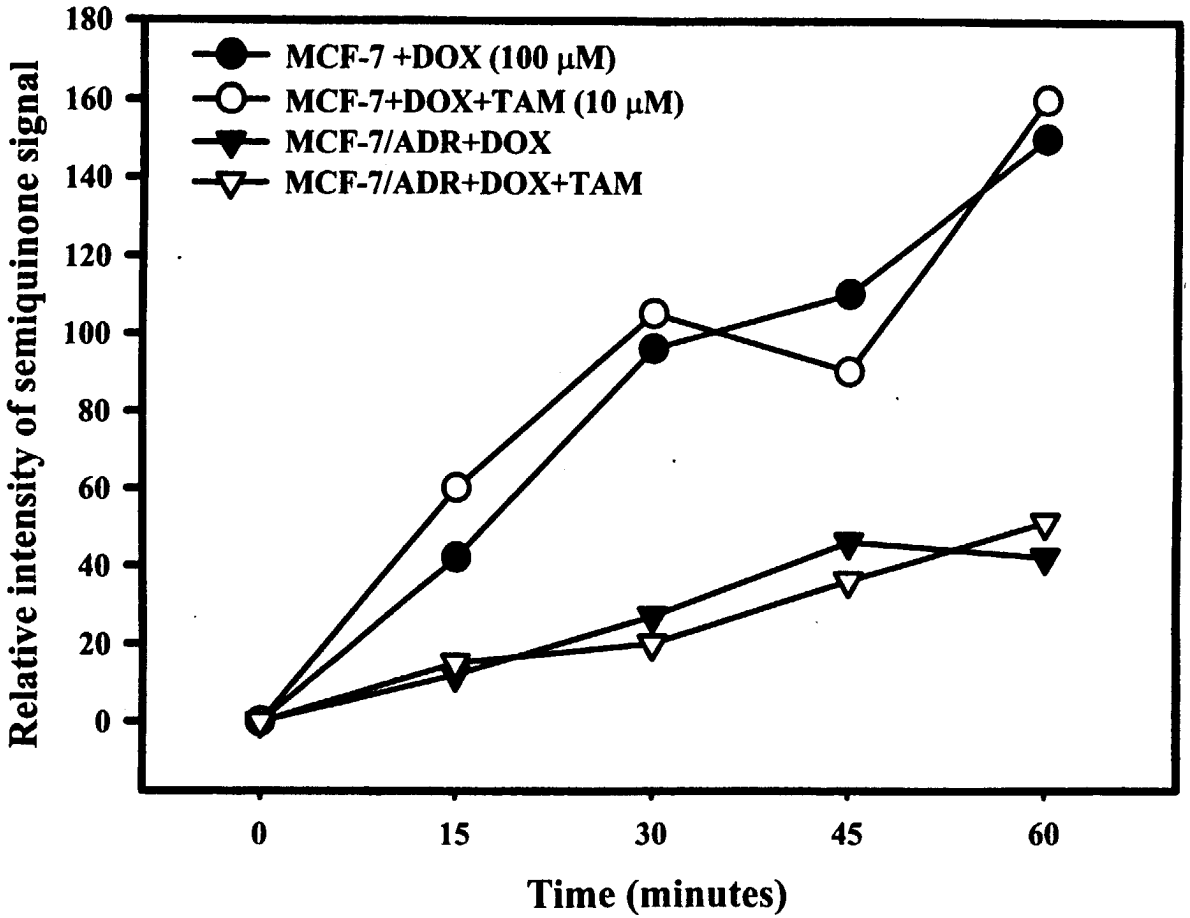

Figure 7: Comparison of doxorubicin semiquinone free radical production during anaerobic metabolism of doxorubicin by MCF-7 and MCF-7/ADR cells

Single cell suspensions were prepared from exponentially growing monolayer cultures. Anaerobic incubations were carried out in closed sample vials (2 ml capacity) containing (10^7 cells/ml)in phosphate buffered saline (pH 7.4), glucose (0.2 mM) and doxorubicin (0.1 mM). The samples were transferred anaerobically into an ESR sample cell after incubation at 37°C and the spectra recorded over a 60 minute period in order to assess the kinetics of semiquinone formation. The relative intensity of the adriamycin semiquinone signal was plotted against the duration of incubation. Inclusion of tamoxifen (10 μM) did not affect the formation of semiquinone free radical. The signal due to doxorubicin semiquinone was higher in the case of MCF-7 cells compared to MCF-7/ADR cells. It is important to mention that the concentration of doxorubicin used in these experiments cause reproductive death of almost all the cellls. The two cell lines differ in their tolerance or sensitivity to doxorubicin.

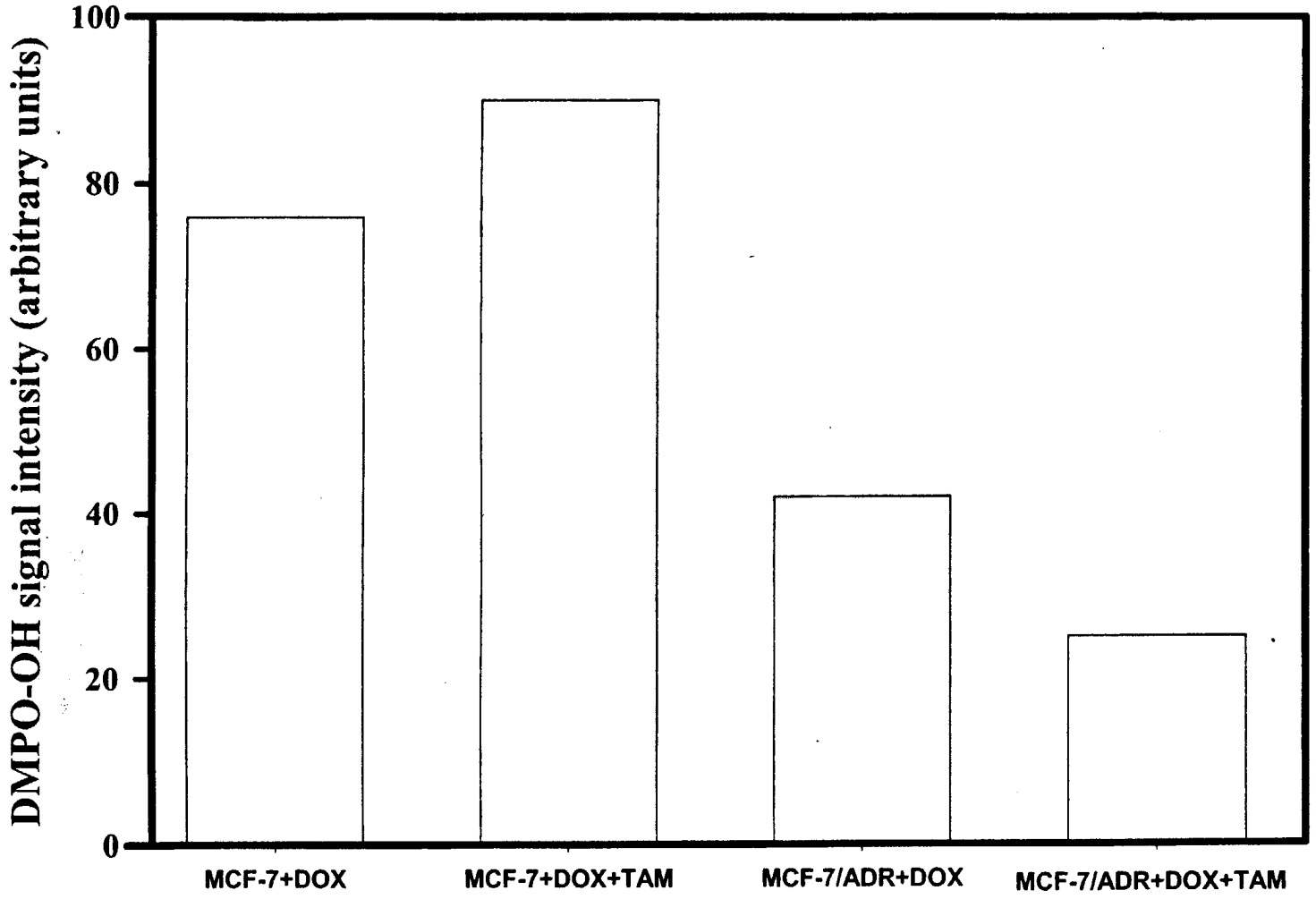

Figure 8: Comparison of hydroxyl free radical production in aerobic incubations of MCF-7 and MCF-7/ADR cells containing the spin trap (DMPO) and doxorubicin with or without tamoxifen

For these experiments, aerobic incubation mixtures contained 10^7 cell per ml in phosphate buffer (pH 7.4), glucose (2 mM), doxorubicin (100 μM) and DMPO (100 μM) with or without tamoxifen (10 μM). Aeration was ensured by bubbling air through the incubation mixture maintained at 37°C. Electron spin resonance spectra were acquired at different times over a period of 1 hour. The intensity of the second peak of the 1:2:2:1 quartet of the DMPO-OH signal was plotted against duration of incubation.

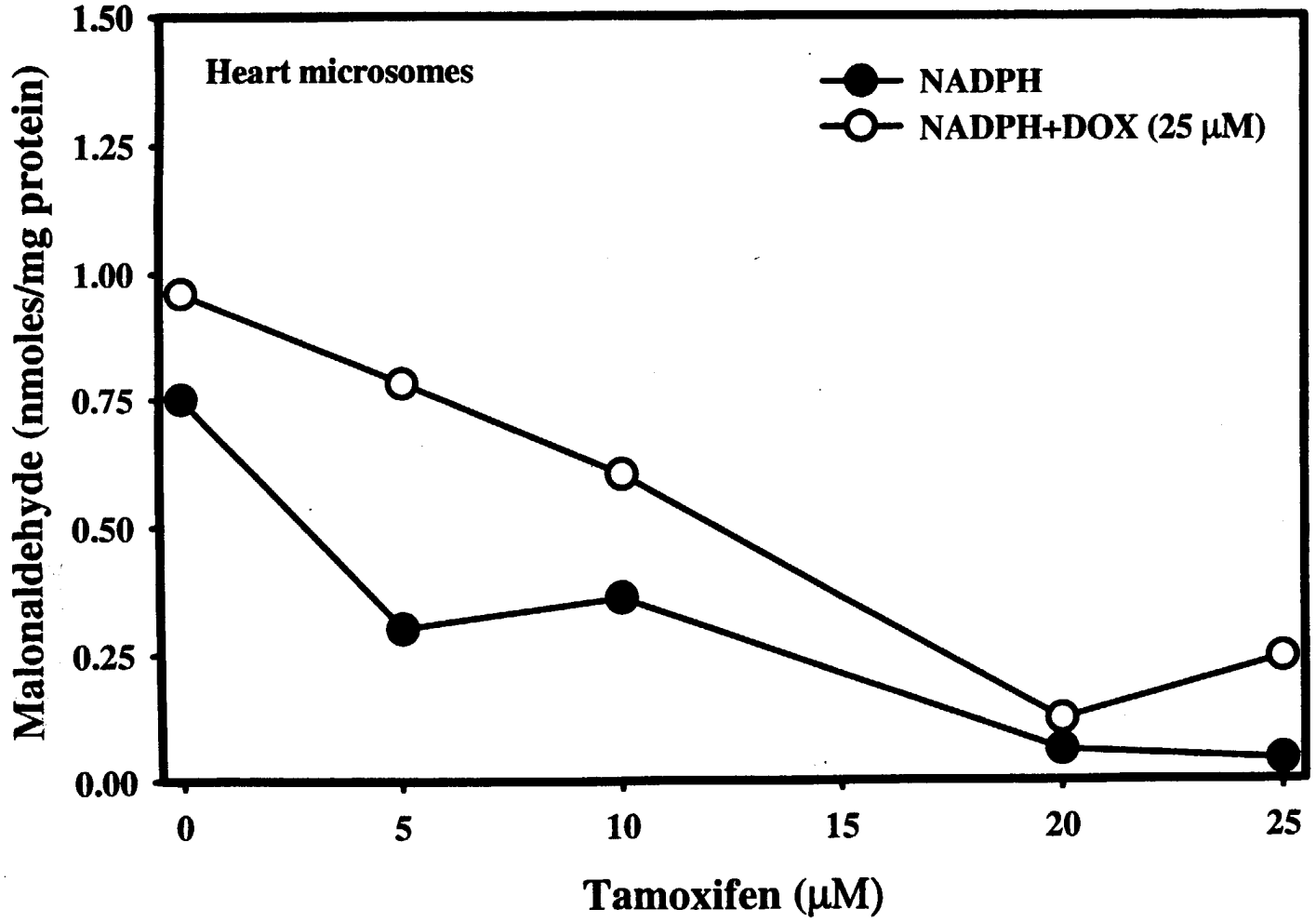

Figure 9: Inhibition of cardiac microsomal lipid peroxidation by tamoxifen.

Incubation mixtures contained 3 mg microsomal protein per ml, 2.5 mM NADPH with or without doxorubicin (25 μM) and tamoxifen (0 to 25 μM). Malonaldehyde was determined by measuring the absorbance at 532 nm of the thiobarbituric acid conjugation product after processing with thiobarbituric acid reagent under acidic conditions (6). Lipid peroxidation was allowed to proceed for 60 minutes. No effort was made to exclude adventitious iron in the buffers or reagents used.

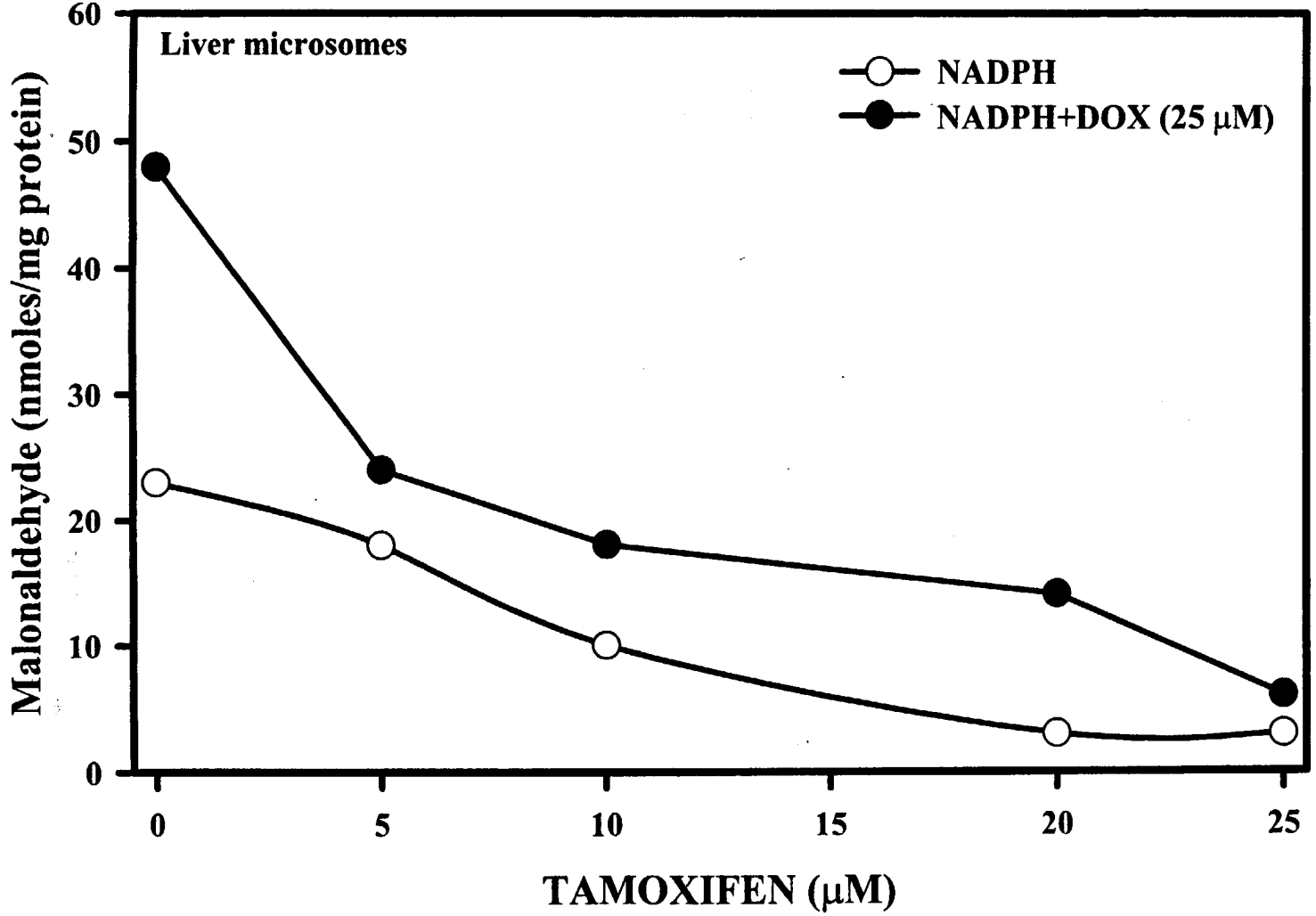

Figure 10: Influence of tamoxifen on doxorubicin induced lipid peroxidation in liver microsomes

Incubation mixtures contained 1 mg of microsomal protein per ml and an NADPH generating system composed of glucose -6-phosphate (5 mM), NADP (0.3 mM0 and 0.5 units of D-glucose-6-phosphate dehydrogenase. Lipid peroxidation was determined on the basis of thiobarbituric acid reactive substances which have a strong absorbance at 532 nm (6). Lipid peroxidation was allowed to proceed at 37°C for 60 minutes in the presence or absence of doxorubicin (25 μM) with or without tamoxifen (10 μM)

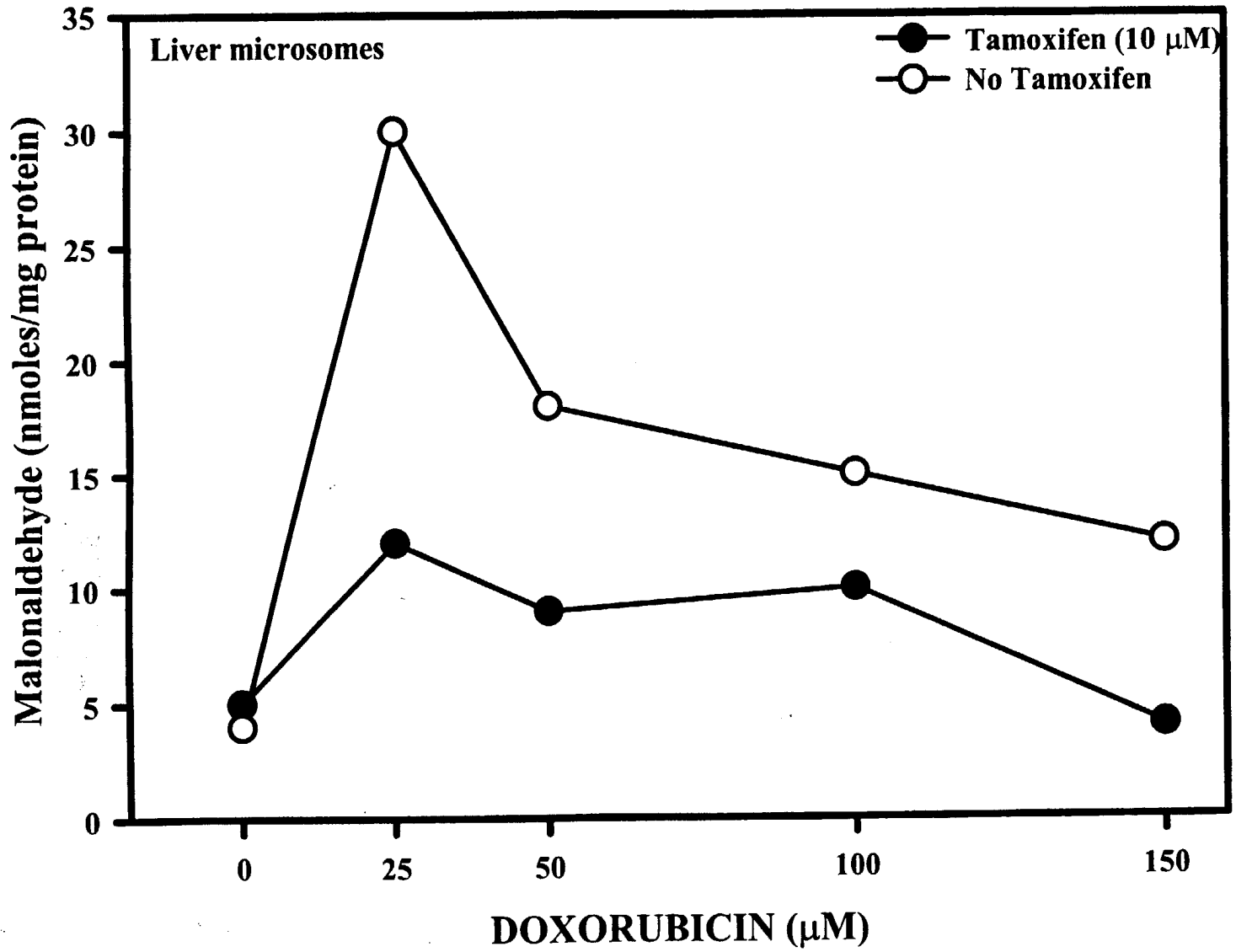

Figure 11: Effect of doxorubicin concentration on microsomal lipid peroxidation.

Incubation mixtures contained 1 mg of microsomal protein per ml and an NADPH generating system and graded concentrations of doxorubicin (0 to 150 μM) with or without tamoxifen (10 μM). Lipid peroxidation was allowed proceed for 60 minutes at 37°C and the extent of peroxidation was assayed by measuring thiobarbituric acid reactive substances (6).

APPENDIX # 15

FASEB J. 13: A920, 1999. Abstract #682.14 presented at "EXPERIMENTAL BIOLOGY 99", Washington, DC April 17-21, 1999.

682.14

MCF-7 AND MCF-7/ADR CELLS RESPOND DIFFERENTLY TO CHANGES IN GLUCOSE CONCENTRATIONS. R. Sridhar, M. Yamamoto, N.A. Patel and D.R. Cooper. Howard Univ. Hospital and Cancer Center, Washington, DC 20060 and Univ. of South Florida College of Medicine and J.A. Haley Veterans Hospital, Tampa, FL 33612.

Glucose is a major source of energy in tumor cells and diabetes may be a risk factor for breast cancer and endometrial cancer. The human breast cancer cell line MCF-7 and its multidrug resistant variant MCF-7/ADR differ in their response to changes in glucose concentrations in the culture medium. A shift of glucose concentration from normal (5.5 mM) to high (25.0 mM) levels caused an increase in DNA synthesis and proliferation of MCF-7 cells but not MCF-7/ADR cells in monolayer cultures. High glucose induced decreases in PKC-βII protein and mRNA levels during DNA synthesis phase in MCF-7 cells but not in MCF-7/ADR cells. The levels of protein kinase C–βII (PKC-βII) protein and the corresponding mRNA levels were 3 to 4 fold higher in MCF-7/ADR cells compared to MCF-7 cells, but were not down regulated by high glucose. These results indicate a possible role of PKC-βII isozyme down regulation in the acceleration of the cell cycle in MCF-7 cells cultured in medium containing high glucose concentration.

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