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TITLE: A Biologically-Based Rationale for Combination Chemotherapy of Novel Agents with Doxorubicin in Human Breast Cancer Cell Lines

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Histone deacetylase inhibitors (HDACI) may increase breast cancer cell susceptibility to topoisomerase poisons such as doxorubicin (DOX) by increasing expression of topoisomerase IIalpha (topoII). Two HDACIs, phenylbutyrate (PB) and trichostatin A (TSA) were investigated for their ability to induce topoII in the estrogen receptor (ER) positive and ER negative human breast cancer cell lines, MCF-7 and MDA MB 231. No measurable changes in topoII protein levels were detected up to 48h after treatment with nontoxic doses of TSA or PB in either cell line. We, next, determined whether pretreatment with nontoxic doses of either TSA or PB affected the IC50 of DOX in MCF-7 or MDA MB 231 cells. DOX toxicity curves generated from HDACI pretreated plates were no different than controls. Transcription array screening for genes activated by PB identified death receptor 5 gene (DR5), a cell surface receptor that induces apoptosis when bound by TNFa related apoptosis inducing ligand (TRAIL). Similar studies were, therefore, performed with TRAIL. TRAIL toxicity curves generated from HDACI pretreated plates were no different than controls. In conclusion, nontoxic doses of HDACIs were insufficient to either induce topoII protein expression or potentiate either doxorubicin or TRAIL toxicity in MCF-7 or MDA MB 231 cells.

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Table of Contents

Introduction

It is common to treat cancer patients with a cocktail of cytotoxic chemotherapeutic agents designed to eradicate cancer cells without overwhelming healthy cells. Unfortunately, patient toxicity often necessitates reduced dosing at the expense of therapeutic success. Recently, researchers have been interested in identifying nontoxic adjunct therapies which could be used to increase the sensitivity of cancer cells to more commonly used chemotherapeutics, allowing reduced prolonged dosing for greater therapeutic success in the absence of patient toxicity. In pursuit of this goal, much attention has fallen on a group of agents called histone deacetylase inhibtors (HDACI). As suggested by their name, these compounds prevent deacetylation of histones resulting in a relaxation of the DNA near the hyperacetylated histones. This 'opening up' of DNA appears to facilitate transcription of the DNA and consequent increased expression of a number of genes [1,2]. Increased transcription from the topoisomerase II α (topoII) promoter was observed in NIH3T3 cells treated with the HDACI, trichostatin A (TSA) [3]. Topoisomerase poisons such as doxorubicin (DOX) are commonly used chemotherapeutics. The sensitivity of cells to these compounds is directly proportional the amount of intracellular topoII protein expression [4]. Transcription of the gene encoding death receptor 5 (DR5) appears to increase in response to the HDACI phenylbutyrate (PB, Kroll unpublished). When bound by its ligand, TNFα related apoptosis inducing ligand (TRAIL), DR5 initiates a signaling cascade that causes the cell to undergo apoptosis. Although DR5 is widely expressed, TRAIL killing occurs more readily in tumor cells, perhaps due to a lack of expression of the inhibitory decoy receptor, TRID [5,6]. HDACIs may, thus, sensitize cells to doxorubicin and/or TRAIL, providing a nontoxic adjunct therapy, which would be expected to work even in advanced breast cancer disease. Increased sensitivity to chemotherapeutics would allow reduced dosing, potentially preventing dose-limiting patient toxicity without jeopardizing therapeutic success.

Body

1. Optimization studies for induction of topoisomerase II α and death receptor 5 by histone deacetylase inhibitors.

In order to address the hypothesis that histone deacetylase inhibitors (HDACI) induce expression of topoisomerase IIα (topoII) protein in human breast cancer cells lines, we first determined the toxicity of two HDACIs, phenyl butyrate (PB) and trichostatin A (TSA) in the estrogen receptor positive human breast cancer cell line, MCF-7 as well as in the estrogen receptor negative human breast cancer cell line, MDA MB 231. Zhou et al (2000) [7] recently reported differentiation of both MCF-7 and MDA MB 231 cells in which histone deacetylase activity was lost. Growth inhibition of normal human breast epithelial cells, MCF-7 cells, and MDA MB 231 cells, probably due to differentiation, was also observed in response to PB by Davis et al (2000) [8]. Sodium butyrate, another HDACI, induced apoptosis in MCF-7 cells and G2/M arrest in MDA MB 231 cells [9]. TSA has also been reported to reduce colony formation by MDA MB 231 cells [10] and cause cell death in MCF-7 cells [11]. It was, therefore, necessary to determine PB and TSA toxicity in both MCF-7 and MDA MB 231 cells so that topoII and DR5 induction could be evaluated in the absence of confounding toxicity.

Methods

MCF-7 cells were cultured in Eagles minimum essential media supplemented with 1 mm sodium pyruvate, 0.01 mg/ml bovine insulin, 10% fetal bovine serum, and 1% penicillin/streptomycin/L-glutamine at 37°C under a humidified atmosphere (5% CO_2/air). MDA MB 231 cells were cultured in minimum essential media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/L-glutamine at 37°C under a humidified atmosphere (5% CO_2/air).

Toxicity of either PB or TSA was determined as follows. Briefly, cells were plated in 96 well plates at 20,000 cells/ml in complete media. 18h later, media was replaced with complete media containing 0 - 50 mM PB or 0 - 1 μM TSA. Cells were exposed to treatments for 24h @37°C under a humidified atmoshpere (5% CO2/air). After the treatment period, treatment-containing media was replaced with fresh media and cells were allowed to recover for 3-5 days. Cells were then exposed to 1 mg/ml 3-[4,5-domethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT reagent) in complete media for 4 h @37°C under a humidified atmoshpere (5% CO2/air), after which time media was removed and formazan crystals were dissolved in dimethylsulfoxide and quantitated at 550 nm on a Thermomax microplate reader.

Cells were plated in complete media for induction studies. 18h later, media was replaced with complete media containing 0, 1, 2 mM PB (MDA MB 231), 0, 0.5, 1 mM PB (MCF-7), or 0, 1, 10 nM TSA (both cell lines). Monolayers were collected at various time points after treatment as follows. Each 100 mm plate was rinsed twice with ice cold PBS and then scraped into 0.5 ml 2X SDS Laemmli Sample Buffer (Sigma). Samples were sheared with 18 gauge needle to break up DNA until viscosity was reduced enough to allow accurate pipetting. Samples were then boiled for 3-5 min and 20 µl/lane separated on a 4-20% gradient polyacrylamide gel electrophoresis. Protein was transferred to PVDF membranes and probed with an anti topoII antibody. In order to control for loading, membranes were also probed with anti actin antibody. Membranes were developed using enhanced chemiluminescence.

Data

Toxicity curves are shown in Figure 1 (Appendix 1). Based on these toxicity curves, nontoxic concentrations of PB and TSA were selected to use in induction studies.

No change in topoII alpha protein levels were detected in either cell line with any of the treatments 12, 24, or 48h after treatment (Figure 2, Appendix 1). Protein levels were also assessed 18 h after TSA treatment in both cell lines. As at other time points, no change in topoII protein was observed (data not shown).

Summary of Results

In summary, we were unable to detect any measurable induction of topoisomerase II alpha protein by nontoxic doses of either PB or TSA in either human breast cancer cell line. The toxicity of topoisomerase poisons is directly correlated with the amount of topoII protein in the cell [4]. The protein induction experiments described above were the critical experiments and the lack of change in intracellular topoII protein levels precluded further investigation of mechanisms of protein induction.

2. Determination of HDACI induced changes in sensitivity to doxorubicin and TRAIL

Recently, researchers reported that inhibition of histone deacetylase increased the sensitivity of two human cell lines to topoisomerase II poisons despite a lack of detectable topoII induction [12]. We therefore decided to proceed with the second portion of the work regardless of the negative results obtained in the work described above.

Methods

In order to determine whether HDACI exposure sensitized human breast cancer cell lines to doxorubicin (topoisomerase poison) or TRAIL (DR5 ligand), we used MTT assay described in section one. Briefly, cells were plated in 96 well plates at 40,000 cells/ml (MCF-7) or 30,000 cells/ml (MDA MB 231) in complete media. 18h later, media was supplemented with 0.4 mM PB (MCF-7), 0.8 mM PB (MDA MB 231), PBS (vehicle control for PB), 1 nM TSA (MCF-7), 10 nM TSA (MDA MB 231), or EtOH (TSA vehicle control). Cells were exposed to pretreatments for 24h @37°C under a humidified atmoshpere (5% CO2/air). After the pretreatment period, pretreatment-containing media was replaced with fresh incomplete media containing 0 - 200 μM doxorubicin or 0 - 10 μg/ml TRAIL for 2h. Cells were allowed to recover in treatment-free complete media for 3-5 days. Cells were then exposed to 1 mg/ml MTT reagent in complete media for 4 h @37°C under a humidified atmoshpere (5% CO2/air), after which time media was removed and formazan crystals were dissolved in dimethylsulfoxide and quantitated at 550 nm on a Thermomax microplate reader.

Data

Doxorubicin toxicity curves generated from plates of either MCF-7 or MDA MB 231 cells pretreated with nontoxic doses of PB or TSA were no different than those generated from plates pretreated with vehicle controls (Figure 3, Appendix 1). TSA pretreatment also failed to affect the sensitivity of MCF-7 or MDA MB 231 cells to TRAIL (Figure 4, Appendix 1). Experiments shown in Figure 4 revealed no difference between TSA pretreated and vehicle pretreated cells with regard to TRAIL susceptibility.

Summary of Results

In summary, both PB and TSA failed to affect the sensitivity of either MCF-7 or MDA MB 231 cells to doxorubicin. This is consistent with the lack of induction of topoII observed in the first phase of experiments. Experiments using TRAIL showed no increased toxicity in cells pretreated with TSA.

Potential Problems

Unfortunately, TRAIL was received in solution and, despite efforts to minimize freeze thawing, the stability of the preparation of TRAIL is undocumented. It remains a possibility that experiments using other more stable formulations of TRAIL may yield more promising results.

Key Research Accomplishments

- Phenylbutyrate failed to induce topoisomerase IIα protein in MCF-7 cells.
- Phenylbutyrate failed to induce topoisomerase IIα protein in MDA MB 231 cells.
- Trichostatin A failed to induce topoisomerase IIα protein in MCF-7 cells.
- Trichostatin A failed to induce topoisomerase IIα protein in MDA MB 231 cells.
- MCF-7 cells pretreated with phenylbutyrate were no more sensitive to the topoisomerase IIα inhibitor, doxorubicin, than MCF-7 cells pretreated with vehicle control.
- MDA MB 231 cells pretreated with phenylbutyrate were no more sensitive to doxorubicin than MDA MB 231 cells pretreated with vehicle control.
- MCF-7 cells pretreated with trichostatin A were no more sensitive to doxorubicin than MCF-7 cells pretreated with vehicle control.
- MDA MB 231 cells pretreated with trichostatin A were no more sensitive to doxorubicin than MDA MB 231 cells pretreated with vehicle control.
- MCF-7 cells pretreated with trichostatin A were no more sensitive to TRAIL than MCF-7 cells pretreated with vehicle control.
- MDA MB 231 cells pretreated with trichostatin A were no more sensitive to TRAIL than MDA MB 231 cells pretreated with vehicle control.

Reportable Outcomes

This data will be submitted for presentation at the 2003 American Association for Cancer Research Meeting.

Conclusions

We were unable to see any HDACI induced changes in sensitivity of MCF-7 or MDA MB 231 cells to doxorubicin or TRAIL. Despite these negative results, HDACI should not be abandoned as a potential adjunct therapeutic for cancer therapy. Future work may be more successful using other cellular systems. Also, topoisomerase IIα protein expression varies significantly with cell cycle and is induced to a much greater extent in G1/G0 cells [3]. HDACI induction of topoII protein may, therefore, be more easily detected in synchronized cultures. Keane et al (1999)[13] reported that doxorubicin increased TRAIL-induced apoptosis and others have observed that DR5 is induced by DNA damage [14]. It would be interesting to determine whether this synergistic toxicity between doxorubicin and TRAIL was further enhanced by HDACI pretreatment. Combinatorial chemotherapy including HDACI remains a potentially interesting area for study, although we were unable to provide evidence supporting its use in two human breast cancer cell lines.

References

1. Vidali, G., Boffa, L. C., Bradbury, E. M. & Allfrey, V. G. Butyrate suppression of histone deacetylation leads to accumulation of multiacetylated forms of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences. *Proc Natl Acad Sci U S A* **75**, 2239-2243. (1978).

2. Boffa, L. C., Vidali, G., Mann, R. S. & Allfrey, V. G. Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. *J Biol Chem* **253**, 3364-3366. (1978).

3. Adachi, N., Nomoto, M., Kohno, K. & Koyama, H. Cell-cycle regulation of the DNA topoisomerase IIalpha promoter is mediated by proximal CCAAT boxes: possible involvement of acetylation. *Gene* **245**, 49-57.

4. Davies, S. M., Robson, C. N., Davies, S. L. & Hickson, I. D. Nuclear topoisomerase II levels correlate with the sensitivity of mammalian cells to intercalating agents and epipodophyllotoxins. *J Biol Chem* **263**, 17724-17729. (1988).

5. Sheridan, J. P. *et al.* Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* **277**, 818-821. (1997).

6. Pan, G. *et al.* An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277**, 815-818. (1997).

7. Zhou, Q. *et al.* Rapid induction of histone hyperacetylation and cellular differentiation in human breast tumor cell lines following degradation of histone deacetylase-1. *J Biol Chem* **275**, 35256-35263. (2000).

8. Davis, T., Kennedy, C., Chiew, Y. E., Clarke, C. L. & deFazio, A. Histone deacetylase inhibitors decrease proliferation and modulate cell cycle gene expression in normal mammary epithelial cells. *Clin Cancer Res* **6**, 4334-4342. (2000).

9. Chopin, V., Toillon, R. A., Jouy, N. & Le Bourhis, X. Sodium butyrate induces P53-independent, Fas-mediated apoptosis in MCF- 7 human breast cancer cells. *Br J Pharmacol* **135**, 79-86. (2002).

10. Bovenzi, V. & Momparler, R. L. Antineoplastic action of 5-aza-2'-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor beta and estrogen receptor alpha genes in breast carcinoma cells. *Cancer Chemother Pharmacol* **48**, 71-76. (2001).

11. Schmidt, K., Gust, R. & Jung, M. Inhibitors of histone deacetylase suppress the growth of MCF-7 breast cancer cells. *Arch Pharm (Weinheim)* **332**, 353-357. (1999).

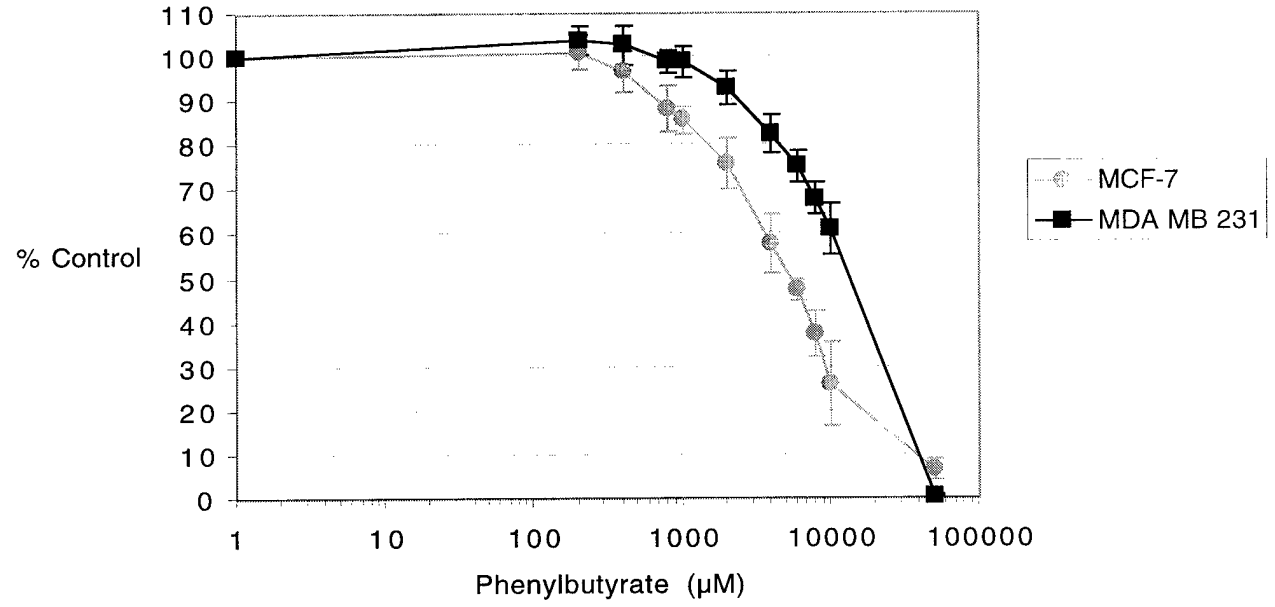
12. Kim, M., Blake, M., Baek, J. & Carrier, F. Inhibition of histone deacetylase increases topoisomerase inhibitors efficiency in cells clinically resistant to Top2 inhibitors. *Proceedings for the American Association for Cancer Research* **43**, 2533 (2002).

13. Keane, M. M., Ettenberg, S. A., Nau, M. M., Russell, E. K. & Lipkowitz, S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. *Cancer Res* **59**, 734-741. (1999).

14. Wang, T. T. & Jeng, J. Coordinated regulation of two TRAIL-R2/KILLER/DR5 mRNA isoforms by DNA damaging agents, serum and 17beta-estradiol in human breast cancer cells. *Breast Cancer Res Treat* **61**, 87-96. (2000).

Appendix 1: Figures

A.



B.

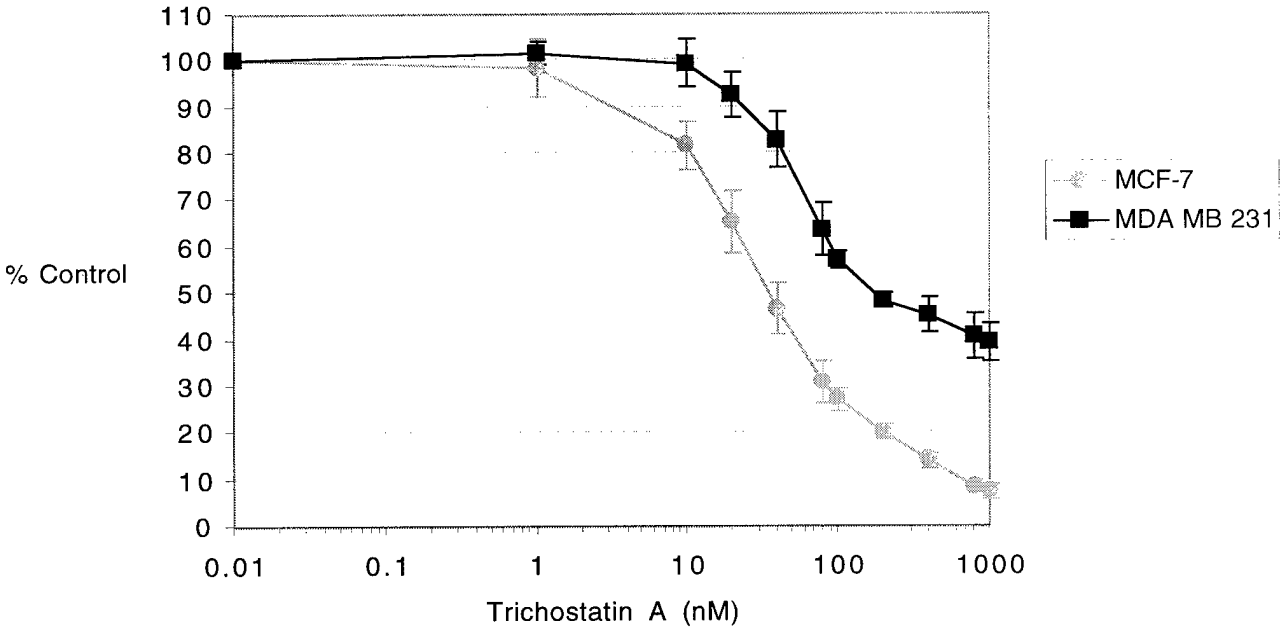


Figure 1 **Phenylbutyrate (PB) and trichostatin A (TSA) toxicity.** MCF-7 (gray circles) and MDA MB 231 (black squares) cells were exposed to PB (A) or TSA (B) for 24 h in complete media. Cytotoxicity was determined by MTT assay as described in ***Methods*** section of **Body**. Data represent mean ± standard deviation of n = 2 in triplicate.

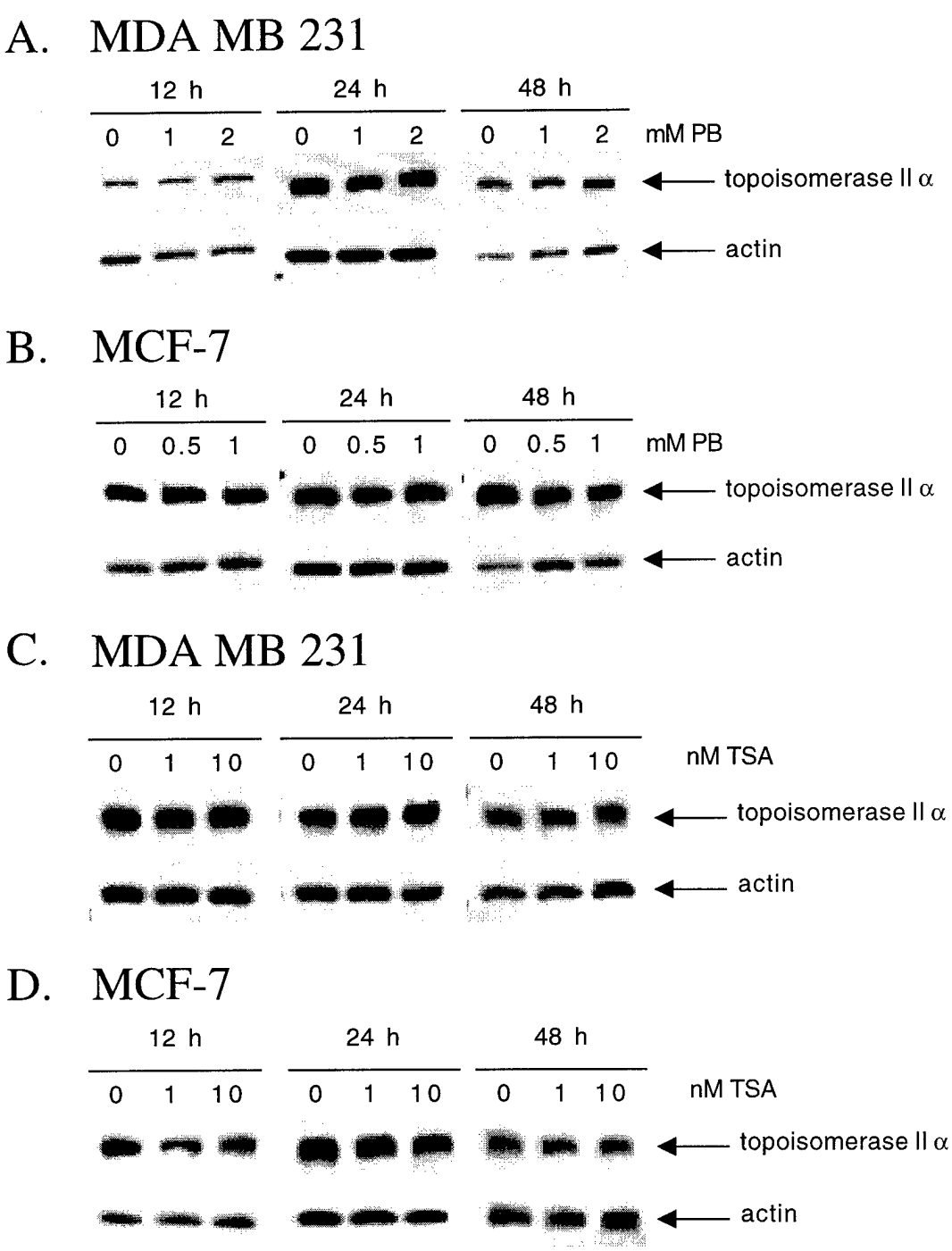
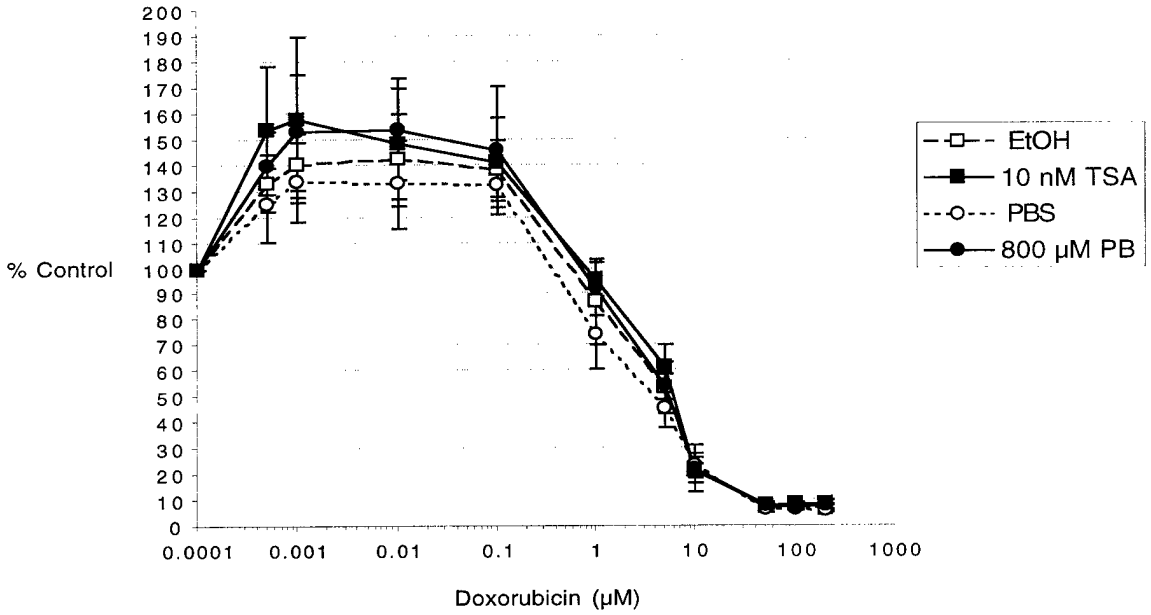


Figure 2 Phenylbutyrate (PB) and trichostatin A (TSA) failed to induce topoisomerase II α protein. MDA MB 231 (A, C) and MCF-7 (B, D) cells were exposed to various concentrations PB (A, B) or TSA (C, D) for 12, 24, or 48 h in complete media. Cells were harvested and protein detected by immunoblot analysis as described in ***Methods*** section of **Body**. Membranes were probed for actin as a loading control.

A. MDA MB 231



B. MCF-7

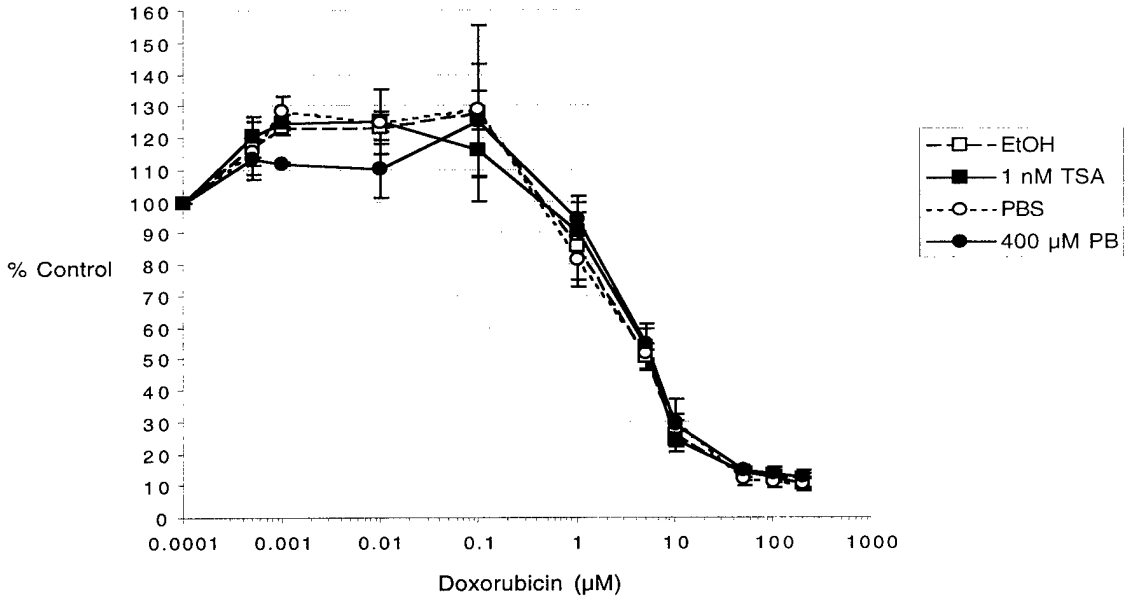


Figure 3 Pretreatment with either phenylbutyrate (PB) or trichostatin A (TSA) failed to affect doxorubicin toxicity. MDA MB 231 (A) and MCF-7 (B) cells were pretreated with nontoxic concentrations of PB or TSA or vehicle controls for 24 h in complete media. Cells were then exposed to doxorubicin for 2 h in incomplete media. Cytotoxicity was assessed by MTT assay as described in ***Methods*** section of **Body**. Data are mean ± standard deviation of n = 1 in triplicate.

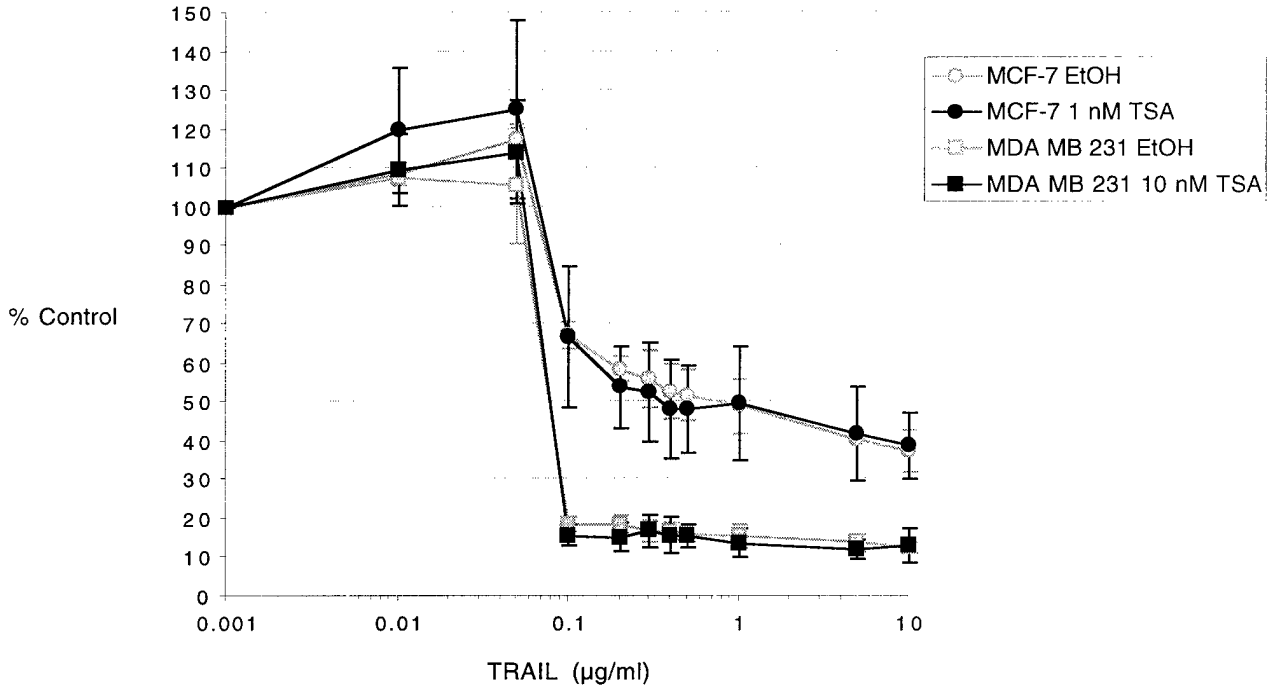


Figure 4 Pretreatment with trichostatin A (TSA) failed to affect TRAIL toxicity. MCF-7 (circles) and MDA MB 231 (squares) cells were pretreated with nontoxic concentrations of TSA (black symbols) or EtOH (vehicle control; gray symbols) for 24 h in complete media. Cells were then exposed to TRAIL for 2 h in incomplete media. Cytotoxicity was assessed by MTT assay as described in ***Methods*** section of **Body**. Data are mean ± standard deviation of n = 1 in triplicate.

Appendix 2: Personnel Receiving Pay from the Research Effort

David Ross, Ph. D.....6 %
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