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13. ABSTRACT *(Maximum 200 Words)*
Physiological stress conditions associated with solid tumors play a role in chemotherapeutic resistance. Treatment with hypoxia or chemical stress agents causes EMT6 mouse mammary tumor cells to develop resistance to teniposide and etoposide, a topoisomerase II inhibitor. We have shown that prostaglandin A1 can fully reverse stress-induced resistance to teniposide or etoposide and the PGA1 can reverse this resistance when given either prior to or after the stress. PGA1 could also block activation of the transcription factor, NF-kB, as measured by gel shift assays or a luciferase reporter gene. To test whether NF-kB was directly involved in stress-induced resistance, an inducible promoter plasmid system containing a mutant IkB gene (which was non-phosphorylatable) was introduced into EMT6 cells as a dominant negative mutant. Expression of the dominant negative mutant prevented the stress activation of NF-kB and reverted the resistant phenotype to a drug sensitive phenotype. These results imply that NF-kB directly mediates both chemical and physiological stress-induced drug resistance in cancer cells and suggest that agents like PGA1 which prevent NF-kB activation may improve the efficacy of topoisomerase II inhibitors.

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Katherine Kennedy 7-28-00

PI - Signature Date

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Introduction

Successful breast cancer treatment is often limited by the development of drug resistance. A unique form of drug resistance has been described that is induced by the physiological conditions associated with solid tumors. EMT6 mouse mammary tumor cells treated with hypoxia or the chemical stress agent brefeldin A (BFA) induce resistance to agents that inhibit topoisomerase II (Lin *et al.*, 1998). Our data have shown that hypoxia- and BFA-induced resistance to topoisomerase II inhibitors is not mediated by alterations in p-glycoprotein, drug concentration, or topoisomerase II activity. Our recent data suggest that chemically-induced resistance is mediated by the nuclear transcription factor NF-κB (Lin *et al.*, 1998). The overall scope of the work is to determine if NF-κB is the mediator of physiologic induced resistance and whether agents which alter NF-κB activation can alter the effectiveness of topoisomerase II type drugs.

Body

Six tasks were proposed for this first year of the award. **Tasks 1, 2 and 3** were to insert the NFκBp65 plasmid and then isolate, verify expression and determine drug resistance in a high and low expressor cell line. While we were able to obtain cell lines transfected with the NF-κB plasmid for p65 or the alternative subunit, p50, the actual expression of p65 or p50 was not elevated and in fact appeared to be lower. Upon treatment with stress, the activation of NF-κB was actually lower not higher as measured by the κB dependant luciferase reporter gene. This anomalous result was puzzling. However, at the AACR meetings, another group reported a similar result in that overexpression of p65 resulted in down regulation of a NF-κB dependant gene. To circumvent this phenomenon, we will insert the p65 and p50 subunits into the ecdysone inducible system and then create EMT6 cell lines in which p65 and p50 expression are induced by ponasterone A. As will be seen below, we have successfully used this system to create an EMT6 cell line in which the dominant negative mutant of IκB is induced by ponasterone treatment.

Task 4 was to transfect into EMT6 cells the non-phosphorylatable mutant of IκB to produce a dominant negative mutant EMT6 cell line. **Task 5** was to evaluate the responses of the cell lines to stress. We were successful again in putting the IκBαM sequence under a hygromycin selectable CMV promoter and transfecting it into cells. However, the clones that were selected did not contain the IκBαM gene. We carried out the transfection protocols 3 times; and were unsuccessful all three times. In fact, close observation of the transfected cells as well as experiments with transient tranfection studies suggested that EMT6 cells were highly dependent upon the NFκB response in order to survive the stress of transfection and selection. Therefore, we reasoned that a more profitable route to pursue was that of using an inducible vector. Many of the inducible vectors that are available use inducing agents that actually activate NFκB. Therefore, we did initial experiments to demonstrate that the ecdysone

inducible system, which is commercially available, did not alter the activation of NFκB. These experiments showed that the inducing ligand, ponasterone A, did not activate NFκB as measured by changes in gel shift assays and κB dependent luciferase reporter assays. Furthermore, we also determined that ponasterone A did not interfere with stress induction of NFκB and that ponasterone A was also not toxic to EMT6 cells as measured by colony forming cytotoxicity assays.

We then constructed the pIND-IκBαM plasmid and co-transfected the pIND-IκBαM plasmid with the pVgRXR (containing the ecdysone receptor) at a ratio of 1:5 into EMT6 mouse mammary tumor cells. Empty vector cells were also made and used as controls. Cells were selected with hygromycin and several colonies were obtained and isolated. Briefly, we verified that ponasterone induced expression of IκBαM in the cells; that expression of the IκBαM protein prevented NF-κB activation by brefeldin A (BFA), hypoxia (H), or okadaic acid(OA); that the presence of the IκBαM vector or the expression of IκBαM protein in the cells did not alter the cytotoxic effects of etoposide in EMT6 cells in the *absence* of stress; that hypoxia and brefeldin A both induced resistance in EMT6 cells containing either the vector only construct or the uninduced IκBαM construct; and that induction of IκBαM expression with ponasterone A prevented the development of stress-induced resistance (induced by hypoxia or brefeldin A) to etoposide. In fact, ponasterone A induced cells were not statistically different from non-transfected EMT6 cells in their susceptibility to etoposide ($p>0.05$). We are currently investigating the cytotoxic effects of doxorubicin in our unique cell lines. Detailed results of these experiments are found in the reprint of the poster presented on this work at the 91st Annual meeting of the American Association for Cancer Research and can be found in the Appendix. These data give further support to our hypothesis that NFκB mediates stress induced drug resistance and show the power of using the inducible expression system for studying stress induced drug resistance. It should be noted that with these data we have mostly completed Task 2 from year 2 as well.

Furthermore, we have continued to confirm and enhance the preliminary data we had with prostaglandin A_1 and have demonstrated that this compound which reverses drug resistance does interfere with NFκB activation in EMT 6 cells. In addition, the data show that prostaglandin A_1 can reverse chemical or physiological induced resistance to teniposide and etoposide; can prevent or partially prevent the stress resistant phenotype even if given prior to or after the stress; does not interfere with the activity of topoisomerase II, and that stress does not interfere with or alter the uptake of etoposide by the cells. These results were also reported at the 91st Annual meeting of the American Association for Cancer Research. Detailed results can be found in the reprint of the poster presentation in the Appendix. They further strengthen our understanding of the effects of prostaglandin A_1 in our system and prepare us better to determine *in vivo* activity of prostaglandin A_1 as a reversal agent.

Both of these presentations are being prepared for submission for publication.

Task 6 was to determine the maximal dose of prostaglandin A_1 for Balb c mice and to begin *in vivo* studies. We have not begun these studies. The animal quarters has been plagued by mouse hepatitis virus and as a consequence, we have not had animals shipped in. This issue is resolving and other investigators with mice in the facility have found that the mice are no longer testing positive for elaboration of virus. Further sero testing is necessary to ensure that the subsequent mice are negative. We will probably be able to commence these studies in the Fall of 2000. We anticipate that these studies should be successful. In support of our hypothesis and the appropriateness of pursuing our *in vivo* studies, Ogiso et al. (Cancer Res. 60: 2429-2434, 2000) showed that the use of lactacystin, a proteosome inhibitor, prevented stress induced etoposide resistance *in vivo* and *in vitro*. These data *in vitro* are similar to our previously published work with the proteosome inhibitor, MG132.

Recommended Changes to the Statement of Work for Year 2:

1. Use the inducible expression system to express p65 and p50 in EMT6 cells and then compare cytotoxicity responses to etoposide in vector and ponasterone induced cells.

2. Perform the tumor sensitivity studies with prostaglandin A_1.

Key Research Accomplishments

- Determined that prostaglandin A_1 inhibited both brefeldin A and hypoxia induced activation of NFκB
- Determined the prostaglandin A_1 reversed both hypoxia and brefeldin A induced toxicity to etoposide and teniposide. Prostaglandin A_1 can cause partial or complete reversal when given prior to or up to 7 hours after stress.
- Prostaglandin A_1 does not alter topoisomerase II activity directly.
- The stress agent, brefeldin A does not directly effect topoisomerase II activity directly nor does it alter drug uptake in cells.
- Constructed the pIND-IκBαM plasmid which contained the nonphosphorylatable form of IκB.
- Transfected pIND-IκBαM and pVgRXR plasmids into EMT6 cells and isolated hygromycin resistant clones.
- Showed that ponasterone A, the ecdysome receptor ligand, does not alter NFκB activation and does not effect long term survival of EMT6 cells.
- Showed that ponasterone A induction of IκBαM cells resulted in the expression

of IκBαM protein and that ponasterone A induction of IκBαM cells resulted in the inhibition of stress-induced activation of NF-κB.

- Showed that induction of IκBαM cells resulted the diminution of measurable NFκB complexes in gel shift assays with brefeldin A, hypoxia or okadaic acid as the stress agent.

- Induction of IκBαM in nonstressed cells did not alter cellular sensitivity ot etoposide per se.

- Induction of IκBαM in stressed cells reversed stress induced resistance to etoposide.

REPORTABLE OUTCOMES

Presentations:

L. M. Brandes, Y. C. Boller, Z-P. Lin, S. R. Patierno, and K.A. Kennedy, NF-κB mediates stress induced resistance to etoposide. Proc. Amer. Assoc. for Cancer Res. 41: 751, 2000.

Y. C. Boller, R. L. Russell, L. M. Brandes, Z-P. Lin, S . R. Patierno, and K. A. Kennedy, Prostaglandin A_1 reverses stress-induced resistance to teniposide, Proc. Amer. Assoc. Cancer Res. 41: 867, 2000.

Cell Lines:

Stable ecdysone inducible expression of IκBαM in EMT6 mouse mammary tumor cell line –EMT6-IκBM.

Funding applied for based upon work supported by this award:

Lori M. Brandes, Predoctoral Award, US Army BCRP, " Alterations in gene transcription by physiological stress: a mechanism for drug resistance through NFκB activation", Submitted June, 2000.

Reserach opportunities applied for and/or received on experiences/training supported by this award:

Lori M. Brandes, Fellowship Award from the Washington Metropolitan Chapter of the Achievement Rewards for College Scientists (ARCS), 2000-2001.

CONCLUSIONS

Our results so far show strong and convincing pharmacologic and genetic evidence that NFκB mediates physiologic induced drug resistance. Because cancer cells in solid breast tumors exist in abnormal, physiological-based stress environments, our results imply that one can take these intrinsically resistant cells and sensitize them to topoisomerase II directed drugs like etoposide by interfering with the activation of NFκB. Our studies demonstrate that one can even sensitize cells that have already been exposed to stressful environments. These data suggest improved therapies for patients if a topoisomerase II dependent agent is combined with an anti- NFκB type agent.

References

Z-P. Lin, Y.C. Boller, S.M. Amer, R. L. Russell, K.A. Pacelli, S. R. Patierno, and K. A. Kennedy, Prevention of brefeldin A-induced resistance to teniposide by the proteasome inhibitor, MG-132: involvement of NF-κB activation in drug resistance, Cancer Res. 58: 3059-3065, 1998.

Y. Ogiso, A. Tomida, S. Lei, S. Omura and T. Tsuruo, Proteasome inhibition circumvents solid tumor resistance to topoisomerase II- directed drugs, Cancer Res. 60: 2429-2434, 2000.

L. M. Brandes, Y. C. Boller, Z-P. Lin, S. R. Patierno, and K.A. Kennedy, NF-κB mediates stress induced resistance to etoposide. Proc. Amer. Assoc. for Cancer Res. 41: 751, 2000.

Y. C. Boller, R. L. Russell, L. M. Brandes, Z-P. Lin, S . R. Patierno, and K. A. Kennedy, Prostaglandin A_1 reverses stress-induced resistance to teniposide, Proc. Amer. Assoc. Cancer Res. 41: 867, 2000.

Appendix

L. M. Brandes, Y. C. Boller, Z-P. Lin, S. R. Patierno, and K.A. Kennedy, NF-κB mediates stress induced resistance to etoposide. Proc. Amer. Assoc. for Cancer Res. 41: 751, 2000.

Y. C. Boller, R. L. Russell, L. M. Brandes, Z-P. Lin, S . R. Patierno, and K. A. Kennedy, Prostaglandin A_1 reverses stress-induced resistance to teniposide, Proc. Amer. Assoc. Cancer Res. 41: 867, 2000.

Poster from 2000 American Association for Cancer Research Meeting

LM Brandes, YC Boller, ZP Lin, SR Patierno, KA Kennedy (2000) NF-kB mediaes stress-induced resistance to etoposide. Proc. Amer. Assoc. Can. Res. 41: 751.

Abstract

Physiological stress conditions associated with solid tumors play a role in chemotherapeutic resistance. Treatment with hypoxia or the chemical stress agents brefeldin A (BFA) and okadaic acid (OA) causes EMT6 mouse mammary tumor cells to develop resistance to teniposide, a topoisomerase II inhibitor. We have also shown that prostaglandin A_1 (PGA_1) can fully reverse hypoxia- and BFA-induced resistance to teniposide. Here we show that BFA induces resistance to etoposide, another topoisomerase II inhibitor, and that this resistance can be reversed by PGA_1. Since PGA_1 is known to inhibit NF-kB activation, we tested whether PGA_1 could block stress-induced NF-kB activation in EMT6. Using a luciferase reporter gene and electrophoretic mobility shift assays, we show PGA_1 inhibits hypoxia-, OA-, and BFA-induced NF-kB activation. To test whether NF-kB was directly involved in stress-induced resistance, we introduced an inducible promoter plasmid system containing a mutant IkB gene (IkBM) into EMT6 cells. Our results suggest inducible IkBM expression inhibits stress-induced NF-kB activation and reverses BFA-induced resistance to etoposide. Taken together, these results imply NF-kB may mediate both chemical and physiological stress-induced drug resistance in cancer cells. Furthermore, they suggest that agents which prevent NF-kB activation may improve the efficacy of topoisomerase II inhibitors in anticancer therapy.

Introduction

The success of breast cancer treatment is often limited by the development of drug resistance. Our laboratory has characterized a unique form of drug resistance that is induced by the physiological conditions associated with solid tumors. We have previously shown that EMT6 mouse mammary tumor cells treated with hypoxia or the chemical stress agent brefeldin A (BFA) induce resistance to agents that inhibit topoisomerase II (Lin *et al.*, 1998).

The mechanism of this stress-induced resistance is unknown but remains an exciting therapeutic target for the treatment of cancer. We have shown that hypoxia- and BFA-induced resistance to topoisomerase II inhibitors is not mediated by alterations in p-glycoprotein, drug concentration, or topoisomerase II activity (abstract #5509). Furthermore, the stress protein GRP78 is induced by BFA and hypoxia, but is not necessary for the development of resistance.

Our recent data suggest that chemical- and physiological-induced resistance is mediated by the nuclear transcription factor NF-κB. We have shown that treatment with BFA or hypoxia causes activation of NF-κB in EMT6 cells. Importantly, inhibition of NF-κB with either prostaglandin A_1 (PGA_1) or MG-132 reverses both BFA- and hypoxia-induced resistance to topoisomerase II inhibitors (abstract #5509).

To determine if NF-κB mediates stress-induced resistance, we introduced an inducible expression system containing a mutant NF-κB inhibitory protein (IκBαM-S32,36A) into EMT6 cells.

we have tested the hypothesis that IκBαM expression can inhibit stress-induced activation of NF-κB and reverse stress-induced resistance to the topoisomerase II inhibitor etoposide.

Reagents and Treatments. Brefeldin A (Sigma, St. Louis, MO) was dissolved in 70% ethanol to a concentration of 10mg/ml and stored at 4°C. For gel shift assays, cells were exposed to 10 μg/ ml BFA for 2 hours and then incubated 2 hours in BFA-free media. For all other assays, cells were exposed to BFA for 2 hours and then in BFA-free media for 6 hours. Okadaic acid (Calbiochem, La Jolla, CA) was kept at a concentration of 100mM in DMSO and stored at -20°C. Cells were treated with 60nM OA for 8 hours for all experiments. Prostaglandin A_1 (Biomol, Plymouth Meeting, PA) was kept at a stock concentration of 30mM in acetone and stored at -20°C. Cells were treated with 30μM PGA_1 one half-hour prior to stress treatment. Etoposide (Sigma, St. Louis, MO) at 100μM in DMSO was stored at -20°C. Cells were treated with 10-50μM etoposide one hour prior to clonogenicity assay. Ponasterone A (Promega, Madison WI) was rehydrated in 70% ethanol to a concentration of 10mM and stored at -20°C. Transfected cells were treated with 10μM ponasterone A for 24 hours to allow for maximal IκBαM expression. For hypoxia, cells were grown in 75cm^2 glass flasks and fitted with a rubber septum with a 18G, 1" needle inlet and a 18G, 1.5" needle outlet. Cells were exposed to a humidified mixture of 95% N2, 5% CO_2 (O_2<0.05ppm) for 8 hours at 37°C.

Cell Culture. EMT6 mouse mammary tumor cells were provided by Dr. S. Rockwell from Yale University. Cells were grown in Waymouth MB 752/1 medium (GibcoBRL, Grand Island, NY) supplemented with L-glutamine (Life Technology, Gaithersburg, MD), 15% fetal bovine serum (Sigma, St. Louis, MO) and 1X antibiotic/antimycotic solution (Biofluids, Rockville, MD). They were split every 3-4 days and maintained at a density of 5 X 10^4 cells/ml in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Stable Transfection. Cells were seeded at a density of 3-4 X 10^4 cells/ml in 25cm^2 flasks and grown for 20 hours and transfected for 1 hour with 1μg of pIND-IκBαM (IκBαM provided by Dr. M. Karin), 5μg pVgRXR, and 36μl TransFast lipid transfection reagent (Promega, Madison, WI) in 2.5ml of serum-free Waymouth's media. Transfected cells were trypsinized and seeded in 100mm dishes at a density of 1 X 10^4 cells/ml. Cells are exposed to 400μg/ml hygromycin 24 hours to select for stable transfectants. Selective media was replenished every three days for a total of 10 days of growth. Antibiotic-resistant colonies were isolated using cloning cylinders and trypsin and expanded in Waymouth's media containing hygromycin.

Western Blot Analysis. Cells were seeded in 100mm dishes or 75cm^2 glass flasks and grown for 18-40 hours. After drug treatments, cells were washed and collected in cold PBS. Cell pellets were lysed in 100μl of 1X SDS sample buffer (125mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 0.006% bromophenol blue) and then boiled for 5 minutes. Protein concentration is determined using the BCA method. 1ml of diluted protein (1:250-1:1000) was incubated with 1mL BCA reagents for 1 hour at 60°C. The OD at 562nm was measured and protein concentration determined by using OD values for a standard albumin curve. 20-100μg protein was mixed with 2X SDS (250mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.012% bromophenol blue, 2% β-mercaptoethanol) and boiled for 5 minutes. Protein samples were separated on a 10% SDS-polyacrylamide gel (4% stacking gel, pH 6.8, 10% resolving gel, pH 8.8, 30: 0.8 acrylamide:bisacrylamide) at 125 Volts for 2 hours in a running buffer containing 25mM Tris base, 192mM glycine, and 0.1% SDS. The gel was transferred to a nitrocellulose membrane electrophoretically for 30 minutes at 55V in 1X CAPS buffer (10mM CAPS, pH 11). Following

transfer, the membrane was stained with Ponceau S solution (0.5% Ponceau S and 1% acetic acid) and then incubated in 1X TBST. The primary antibody, diluted 1000-4000 fold in 1X TBST with 1% BSA is added to the blot overnight at 4°C. The membrane was then washed with 1X TBST twelve times over one hour. HRP-conjugated antibody was added at a 1:4000-1:10000 dilution in 1X TBST with 1% BSA for 1 hour at room temperature. The membrane was then washed with

1X TBST 12 times over 1 hour. Antibody binding was visualized with an enhanced chemiluminescent reagent containing luminol substrate for HRP. The membrane was exposed to X-ray film for 30 seconds-10 minutes at room temperature. Membranes were stripped for reprobing by incubation in stripping buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 0.67% β-mercaptoethanol) at 50°C for 30 minutes followed by three washes in 1X TBST over 1 hour.

Transient Transfection and Luciferase Reporter Gene Assay. Cells were seeded at a density of 3-4 X 10^4 cells/ml in 60mm dishes or 25cm^2 flasks and grown for 20 hours. 2.5ml of serum-free Waymouth's media containing 3ug of luciferase reporter gene [pTk-$(kB)_6$-Luc] (provided by Dr. H. Pahl), 1ug of pcDNA-LacZ, 12ul TransFast reagent (Promega, Madison, WI) was incubated at room temperature for 20 minutes and then added to EMT6 cells for 2 hours. Following transfection, 2.5ml of Waymouth's media containing 30% serum was added. Transfected cells were grown for 18 hours before drug treatment and harvested by the 48th hour after transfection. To harvest, cells were washed in cold PBS and lysed for 15 minutes at room temperature in 400ul of Reporter Lysis Buffer (Promega, Madison, WI). Cells were collected and centrifuged at 10,000rpm for 2 minutes at 4°C. For the luciferase assay, 100μl of Luciferase Assay Reagent (Promega, Madison, WI) was added to 20μl of cell lysate. Light emission was measured 10 seconds after addition of the assay reagent using a scintillation counter at the single photon monitor mode over a 1-minute interval. Cells were also assayed for *lacZ* expression by mixing 150μl of cell lysate with 150μl of assay buffer containing o-nitrophenyl-β-D-galactopyranoside. After an incubation for 1.5 hours at 37°C, OD at 420nm was measured using a spectrophotometer. The relative β-galactosidase activity for each sample was used to normalize luciferase activites.

Electrophoretic Mobility Shift Assay. Cells were seeded at a density of 3-4 X 10^4 cells/ml in 150mm dishes or 150cm^2 glass flasks. Nuclear extraction was performed by the methods previously published by Stein et al. (1989). Following drug treatment, cells were washed in cold PBS and lysed in 100μl lysis buffer (10mM HEPES, pH 7.9, 1mM EDTA, 60mM KCl, 1mM DTT, 0.5% NP-40, 0.5mM sodium orthovanadate, 1mM PMSF) on ice for 5 minutes. Cell nuclei were separated by centrifugation at 5,000rpm for 5 minutes and then washed with 500μl washing buffer (10mM HEPES, pH 7.9, 1mM EDTA, 60mM KCl, 1mM DTT, 0.5mM sodium orthovanadate, 1mM PMSF). Nuclei were then broken by three freeze-thaw cycles in an ethanol-dry ice bath. Protein concentration was determined using the micro BCA method. 3.5pmole of oligonucleotide containing the NF-κB consensus sequence was incubated with 1μl of [γ-^{32}P]ATP (10mCi/ml, 6,000 Ci/mmol), 5 units of T4 polynuceotide kinase and 10μl of end-labeling buffer at 37°C for one hour. Following incubation, 90μl 1X STE was added and the reaction passed through a G-25 spin column. 20μg of nuclear protein extract was incubated with 3μg poly dI·dC and 0.035pmole of radiolabeled oligonucleotide (100,000-200,000cpm) and brought up to a 20μl final volume in binding buffer (10mM Tris-HCl, pH 7.5, 50mM NaCl, 0.5mM EDTA, 1mM $MgCl_2$, 0.5mM dithiothreitol, 4% glycerol). After a 20-minute incubation at room temperature, samples were loaded onto a non-denaturating 6% polyacrylamide gel (30:1 acrylamide: bisacrylamide, 0.5X TBE, 2.5% glycerol) with 0.5X TBE and separated at 110 Volts for 3 hours. The gel was then transferred to filter paper, dried under vacuum pressure, and exposed to X-ray film.

Colony Forming Assay. Cells were seeded in 25cm^2 tissue culture flasks or in 75cm^2 glass flasks at a density of 3-4 X 10^4 cells/ml and grown for 18-40 hours prior to treatment. Following drug exposure, cells were harvested by the 48th hour of

growth with trypsin and serially diluted in Waymouth's medium. For each flask, cells were seeded at two different concentrations in triplicate 60mm tissue culture dishes. Colony dishes were incubated for 7 days under maintenance conditions above and then stained with 0.25% crystal violet. Percent cell survival was determined by dividing drug-treated colony counts by control counts. The data presented here are average control survival percentages from at least three independent experiments. For transfected cell lines, cells were grown for 24 hours in non-selective media to ensure stress did not occur from the antibiotic agent.

Results

Figure 1. Effects of PGA_1 on BFA-induced resistance to etoposide. EMT6 cells were treated with 30μM prostaglandin A_1 (PGA_1) one-half hour prior to treatment with 10μg/mL brefeldin A (BFA). Cells were exposed to BFA 2 hours and then allowed to recover in BFA-free media for 6 hours. Etoposide was added one hour prior to clonogenicity assay. Results are percent control cell survival averages of triplicate plates ± SEM from at least three independent experiments.

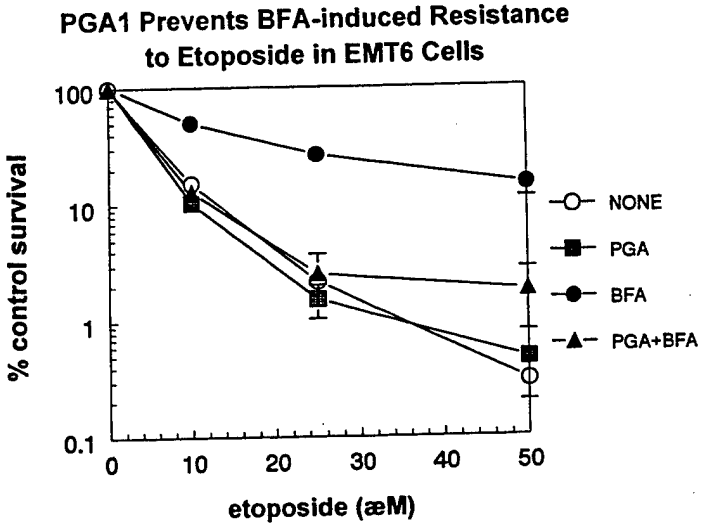
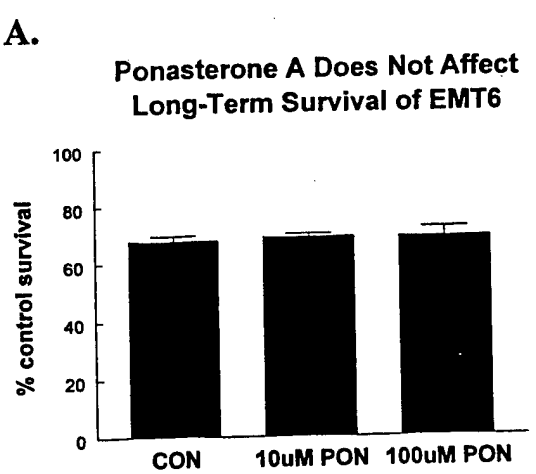


Figure 2. Effects of ponasterone A on EMT6 cells. **A.** Cells were treated with ponasterone A (PON) for 24 hours prior to colony forming assay. Results are reported as percent control cell survival averages of triplicate plates ± SEM from at least three independent experiments. **B.** Cells were transiently transfected with an NF-κB-sensitive luciferase reporter gene. After transfection, cells were treated with PON for 24 hours or 60nM okadaic acid (OA) for 8 hours. Lysates from transfected cells were assayed for luciferase expression by measuring light intensity in the presence of luciferase assay substrate. The light intensity from drug-treated cells was divided by non-treated or solvent-treated cells to determine relative fold changes in luciferase activity. Results are the average fold control luciferase activity ± SEM from at least three independent experiments.



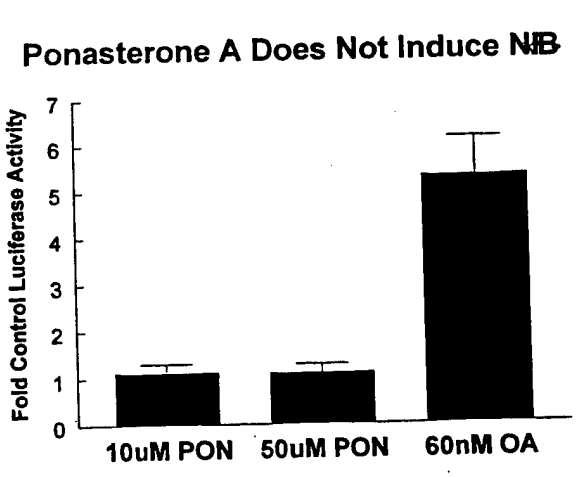


Figure 3. EMT6 cells were transfected with a 1:5 ratio of pIND: pVgRXR (vector transfected cells -VCT) or 1:5 ratio of pIND-IκBαM: pVgRXR (IκBαM). Following antibiotic selection, cells were seeded for protein collection and treated with 10μM ponasterone A for 24 hours. Whole cell lysates were collected and analyzed by western blot with anti-IκBα, anti-HA, and anti-actin primary antibodies. Results shown are a representative blot from one of at least three independent experiments.

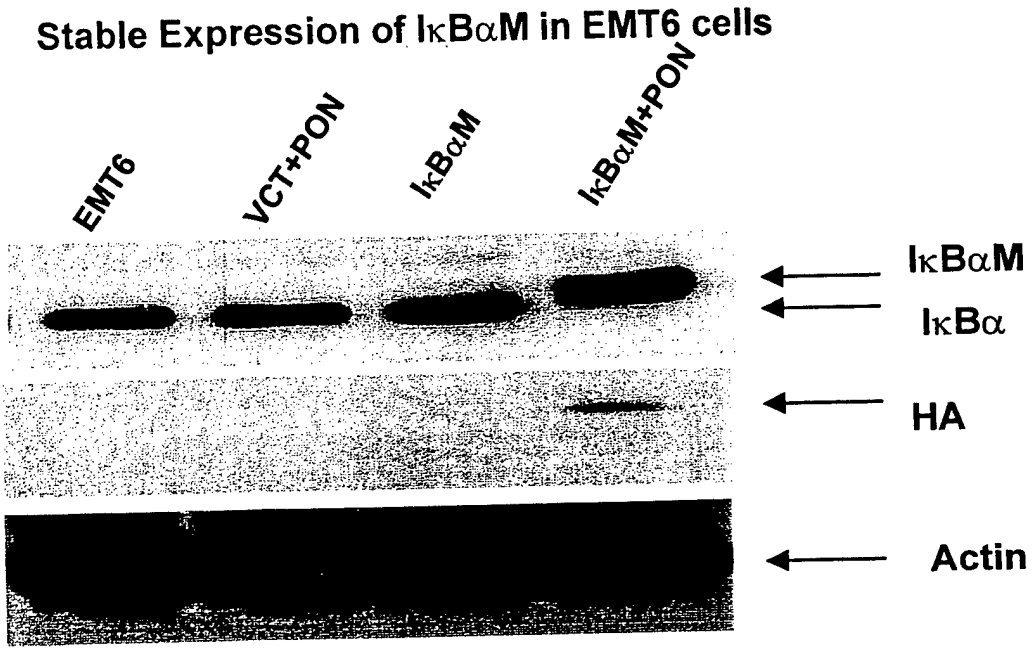


Figure 4. EMT6 cells, vector-transfected cells (VCT), and IκBαM cells were transiently transfected with an NF-κB-sensitive luciferase reporter gene. After transfection, cells were treated with 10μM ponasterone A (PON) for 24 hours and a stress treatment of either 10μg/mL brefeldin A (BFA) for 4 hours, hypoxia (HYP) for 2 hours, or 60nM okadaic acid (OA) for 8 hours. The light intensity from drug-treated cells was divided by non-treated or solvent-treated cells to determine relative fold changes in luciferase activity. Results are the average fold control luciferase activity ± SEM from at least three independent experiments. * - a statistically significant decrease in BFA-induced luciferase activity was seen in IκBαM treated with PON compared to IκBαM not treated with PON. # - a statistically significant decrease in HYP-induced luciferase activity was seen in IκBαM treated with PON compared to IκBαM not treated with PON. + - a statistically significant decrease in OA-induced luciferase activity was seen in IκBαM treated with PON when compared to IκBαM not treated with PON. ($p<0.05$, t-test)

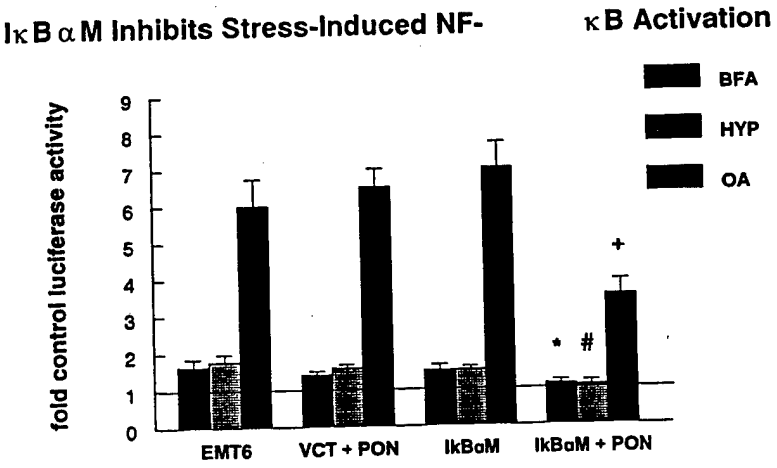


Figure 5. Effects of IκBαM expression on stress-induced NF-κB activation. Vector-transfected (VCT) and IκBαM cells were treated with 10μM ponasterone A (PON) for 24 hours followed by stress treatment of either 60nM okadaic acid (OA) for 8 hours, 10μg/mL brefeldin A (BFA) for 4 hours, or hypoxia (HYP) for 2 hours. Nuclear extracts were collected and analyzed by gel shift assay.

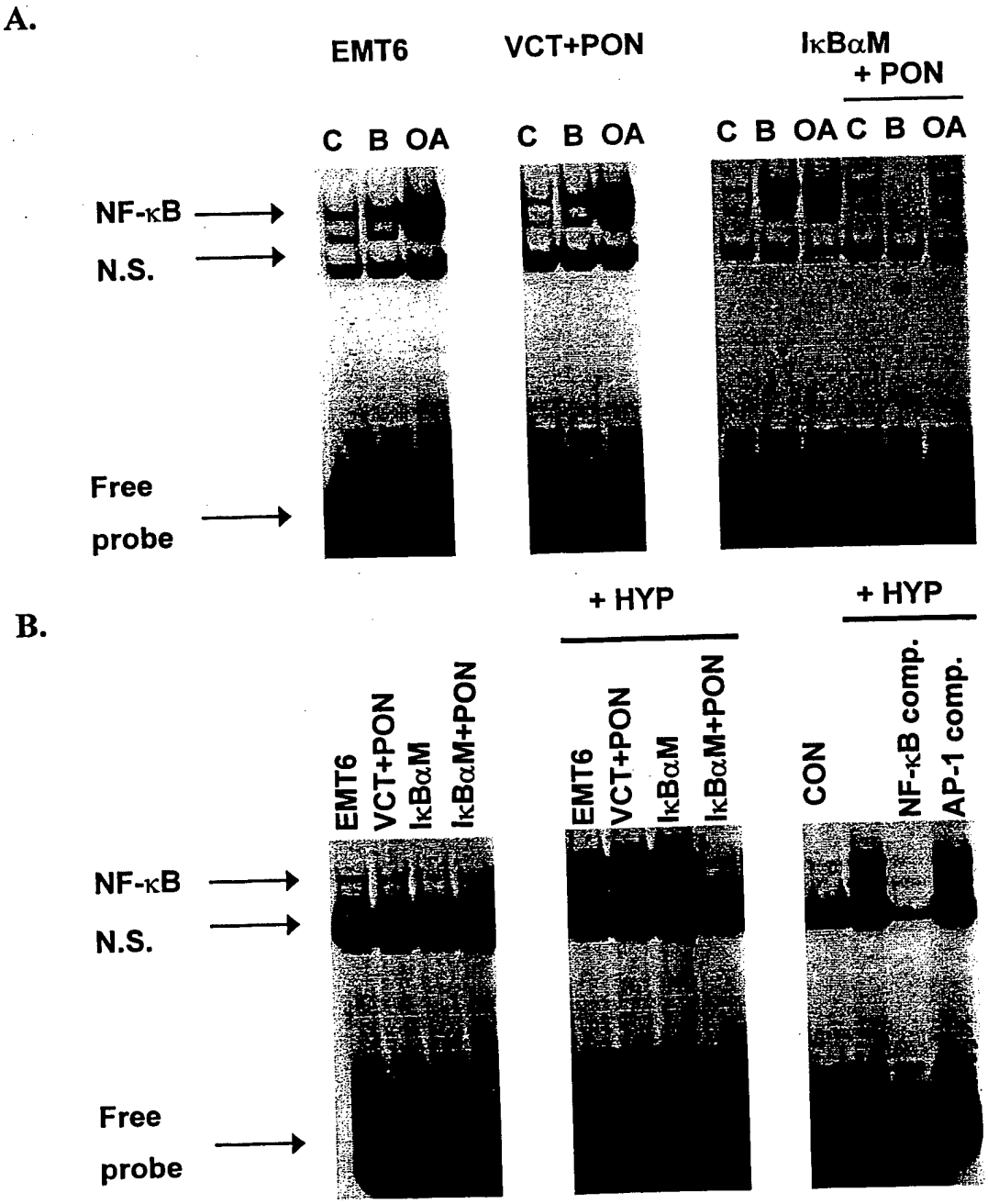


Figure 6. Toxicity profile of stably-transfected EMT6 cells. EMT6 cells, vector-transfected cells (VCT), and IκBαM cells were treated with ponasterone A (PON) for 24 hours and then etoposide one hour prior to clonogenicity assay. Results are percent control cell survival averages of triplicate plates ± SEM from at least three independent experiments. Survival of transfected cells with BFA, HYP or PON treatment was also not significantly different from that of EMT6 cells.

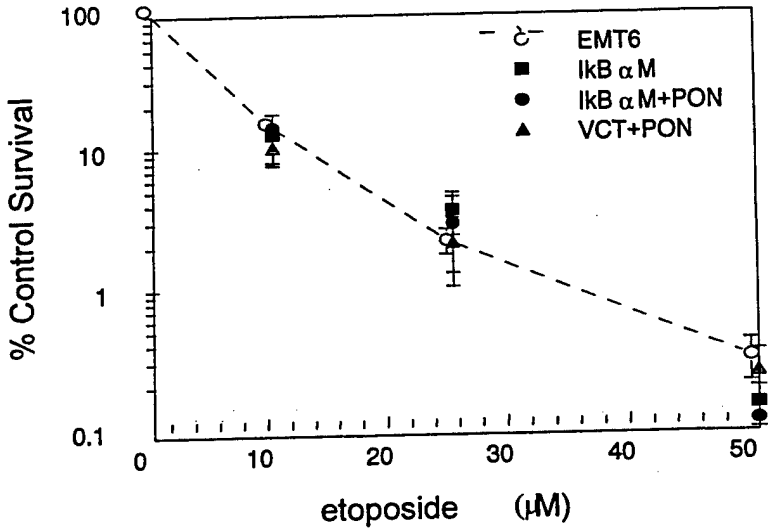
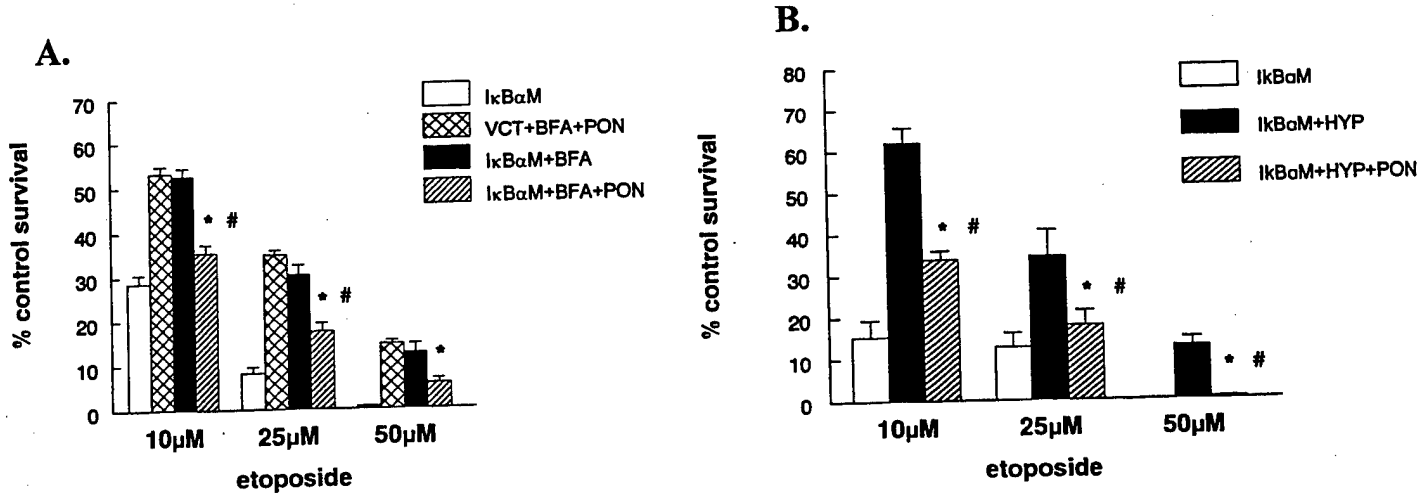


Figure 7. Effects of IκBαM expression on stress-induced resistance. Vector-transfected cells (VCT) and IκBαM cells were treated with 10μM ponasterone A (PON) for 24 hours and a stress treatment of either **A.** 10μg/mL brefeldin A (BFA) or **B.** hypoxia (HYP) for 8 hours. Etoposide was added one hour prior to clonogenicity assay. Results are percent control cell survival averages of triplicate plates ± SEM from at least three independent experiments. * - a statistically significant decrease in cell survival was seen in stress-treated IκBαM cells treated with PON compared to IκBαM not treated with PON ($p<0.05$, t-test). # - a statistically significant difference was NOT seen in the cell survival of stress-treated IκBαM cells treated with PON when compared to nonstressed-IκBαM treated only with etoposide.



Conclusions

- Stress-induced NF-kB activation is inhibited by IκBαM expression.
- Expression of IkBαM reverses stress-induced resistance to etoposide.
- NF-kB mediates BFA- and hypoxia-induced resistance to etoposide, suggesting agents which inhibit NF-κB activation may enhance the clinical efficacy of topoisomerase II inhibitors.

Poster from 2000 American Association for Cancer Research Meeting

YC Boller, RL Russell, LM Brandes, ZP Lin, SR Patierno, KA Kennedy (2000) Prostaglandin A_1 reverses stress-induced resistance to teniposide. Proc. Amer. Assoc. Can. Res., 41: 867.

Abstract

The presence of stress microenvironments within solid tumors has a significant effect on responses to chemotherapeutic agents. EMT6 mouse mammary tumor cells treated with hypoxia or the stress-inducing agent, brefeldin A (BFA), developed resistance to teniposide, a topoisomerase II inhibtor. This resistance was not due to decreased drug uptake or altered topoisomerase II activity during stress. Because hypoxia and BFA activate the nuclear transcription factor NF-kB, we tested the hypothesis that pharmacological inhibition of NF-kB reverses stress-induced resistance to teniposide. Prostaglandin A_1 (PGA_1), which is known to inhibit NF-kB activation, completely reversed hypoxia- or BFA-induced resistance to teniposide in EMT6 cells when added prior to the initiation of stress. Detailed time course studies showed that PGA_1 could partially reverse the resistant state when added up to seven hours after stress treatment (BFA for 0.5 hours). These results suggest PGA_1 may be effective in reversing chemotherapeutic resistance induced by the physiological stresses of the tumor microenvironment. Moreover, the time course study has clinical significance because it implies that resistance can be reversed after it has been induced by the tumor microenvironment.

Introduction

Clinical drug resistance to chemotherapeutic agents is a major obstacle to curative therapy of cancer. Stress microenvironments such as hypoxia, low pH, or nutrient deprivation lead to the formation of heterogeneous tumor cell subpopulations due to poor vascularization within a tumor. The presence of stressful microenvironments in solid tumors have significant effects on responses to chemotherapeutic agents. Stress can be induced *in vitro* by a number of physical and chemical agents including hypoxia, low pH, glucose deprivation, brefeldin A, glucosamine, tunicamycin, and thapsigargin. These agents induce changes in protein expression which enables cells to survive the cytotoxic effects of anticancer drugs.

Recent studies have shown that at least two distinct stress responses exist in cells: the unfolded protein response (UPR) and the ER overload response (EOR). The UPR results in the induction of GRP78, a stress responsive protein of the heat shock protein family and the EOR response results in the activation of NF-κB, and early response nuclear transcription factor. In addition tumor cells become resistant to the topoisomerase II-directed drugs. Our previous data suggest that activation of NF-κB rather than the induction of GRP78 resulted in resistance to teniposide, a topoisomerase II inhibitor. Our objective in the present study was to test the hypothesis that stress-induced drug resistance was the result of NF-κB activation in EMT6 mouse mammary tumor cells. We have tested whether pharmacological inhibition of NF-κB with prostaglandin A_1 (PGA_1) inhibits stress-induced activation of NF-κB and reverses stress-induced resistance to teniposide.

Materials and Methods

Cell Culture. EMT6 mouse mammary tumor cells provided by Dr. Sara Rockwell (Yale University) are grown in a monolayer in Waymouth's MB 752/1 medium (Life Technology, Gaithersburg, MD) with L-glutamine (Life Technology, Gaithersburg, MD) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO), 100 units/ml streptomycin, and 25μg/ml gentamicin sulfate (Biofluids, Rockville, MD). These cells were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C and passaged every 3-4 days.

Chemicals and Reagents. Teniposide from Bristol Meyers (Syracuse, NY); brefeldin A (BFA) from Sigma (St. Louis, MO); prostaglandin A1 (PGA1) from Biomol (Plymouth Meeting, PA); okadaic acid (OA) from Calbiochem (San Diego, CA);

Hypoxia. EMT6 cells were seeded at a density of 3-4 X 10^4 cells/ml in gas-impermeable glass flasks. Cells were allowed to grow for 36-40 hours prior to treatment in normoxic conditions at 37°C in humidified atmosphere of 5% CO_2/ 95% air. PGA_1 was added to cells 30 minutes before exposure to hypoxic conditions. A hypoxic environment was created by exposing EMT6 cells to a constant flow of humidified 5% CO_2/ 95% nitrogen (oxygen content <10ppm). Glass flasks were fitted with sub seal rubber septa (Aldrich, Milwaukee, WI) to ensure an air-tight seal. The flasks were then placed in a 37°C incubator and gassed continuously using 18 gauge 1.5" needles for inflow and 18 gauge 1" needles for outflow. Cells were exposed to hypoxic conditions for 8 hours and at the last hour, teniposide was added to the flasks using a Hamilton syringe (Hamilton, Rena, VA) without disturbing the hypoxic environment. Percent survival was determined by clonogenicity assay.

Clonogenicity Assay. Cells were seeded in 25cm^2 tissue culture flasks at a density of 3 X 10^4 cells/ml or in 75cm^2 glass flasks and grown for 18-40 hours prior to treatment. Following drug exposure, cells were harvested with trypsin and serially diluted in Waymouth's medium. For each flask, cells were seeded at two different concentrations in triplicate 60mm tissue culture dishes. Colony dishes were incubated for 7 days under maintenance conditions above and stained with 0.25% crystal violet.

Electrophoretic Mobility Shift Assay (Gel Shift Assay). Cells were seeded at a density of 3-4 X 10^4 cells/ml in 150mm dishes or 150cm^2 glass flasks. Following drug treatment, cells were washed in cold PBS and scraped into PBS with a plastic spatula. After centrifugation, cell pellets were lysed in 100μl lysis buffer (10mM HEPES, pH 7.9, 1mM EDTA, 60mM KCl, 1mM DTT, 0.5% NP-40, 0.5mM sodium orthovanadate, 1mM PMSF) on ice for 5 minutes. Cell nuclei were separated by centrifugation at 5,000rpm for 5 minutes and then washed with 500μl washing buffer (10mM HEPES, pH 7.9, 1mM EDTA, 60mM KCl, 1mM DTT, 0.5mM sodium orthovanadate, 1mM PMSF). Nuclei were then subjected to three freeze-thaw cycles in an ethanol-dry ice bath. Protein concentration of these samples was determined using the micro BCA method (Pierce, Rockford, IL). 3.5pmole of oligonucleotide containing the NF-κB consensus sequence was incubated with 1μl of [γ-^{32}P]ATP (10mCi/ml, 6,000 Ci/mmol, Amersham, Arlington Heights, IL), 5 units of T4 polynuceotide kinase (Promega, Madison, WI) and 10μl of end-labeling buffer (Promega, Madison, WI) at 37°C for one hour. Following incubation, 90μl 1X STE was added and the reaction was passed through a G-25 spin column (Worthington, Plymouth Meeting, PA). 20μg of nuclear protein extract was incubated with 3μg poly dI·dC (Sigma, St. Louis, MO) and 0.035pmole of radiolabeled oligonucleotide (100,000-200,000cpm) and brought up to a 20μl final volume in binding buffer (10mM Tris-HCl, pH 7.5, 50mM NaCl, 0.5mM EDTA, 1mM $MgCl_2$, 0.5mM dithiothreitol, 4% glycerol). This reaction was incubated at room temperature for 20 minutes, loaded onto a non-denaturating 6% polyacrylamide gel (30:1 acrylamide:bisacrylamide, 0.5X TBE, 2.5% glycerol) and separated in 0.5X TBE buffer at 110 Volts for 3 hours. The resulting gel was transferred to filter paper (BioRad, Hercules, CA) and dried under vacuum pressure for 1 hour using a Model 583 gel dryer (BioRad, Hercules, CA). After drying, the gel was exposed to X-ray film at -70°C in a cassette with intensifying screens.

Transient Transfection and Luciferase Assay Cells were seeded at a density of 3-4 X 10^4 cells/ml in 60mm dishes or 25cm^2 flasks and grown for 20 hours. For each dish, 2.5ml of serum-free Waymouth's media containing 3ug of

luciferase reporter gene [pTk-$(kB)_6$-Luc], 1ug of pcDNA3.1-LacZ, 12ul TransFast reagent (Promega, Madison, WI) was added following a 20 minute incubation to allow for lipid/DNA complexes to form. After a 2 hour incubation, 2.5ml of Waymouth's media containing 30% serum was added. Transfected cells were grown for 18 hours before drug treatment and harvested by the 48th hour after transfection. To harvest, cells were first washed twice in cold PBS and then lysed for 15 minutes at room temperature in 400ul of Reporter Lysis Buffer (Promega, Madison, WI). Cells were scraped with a plastic spatula and centrifuged at 10,000rpm for 2 minutes at 4°C. For the luciferase assay, 100ul of Luciferase Assay Reagent (Promega, Madison, WI) was added to 20μl of cell lysate. Light emission was measured 10 seconds after addition of the assay reagent using a model LS-6500 scintillation counter (Beckman, Fullerton, CA) using the single photon monitor mode over a 1 minute interval. Cells were also assayed for lacZ expression to correct for differences in transfection efficiency. 150μl of cell lysate was mixed with 150μl of assay buffer containing o-nitrophenyl-β-D-galactopyranoside (Promega, Madison, WI) and incubated 1.5 hourd at 37°C. OD at 420nm was measured using a spectrophotometer (Spectronic, Rochester, NY). The relative β-galactosidase activity for each sample was then used to normalize luciferase activites.

Figure 1. Effects of prostaglandin A_1 (PGA_1) on brefeldin A (BFA)-induced resistance to teniposide in EMT6 cells. Cells were exposed to PGA_1 (30μM) 30 minutes before the introduction of BFA. Cells were then treated with BFA (10μg/ml) for 2 hours after which medium was replaced with medium containing PGA_1 but not BFA. At the last hour of the experiment, teniposide was added. Percent survival was determined by comparing percent plating efficiency of BFA-treated cells with the percent plating efficiency of non-stressed cells. Each point represents the mean ± SEM of at least three independent experiments.

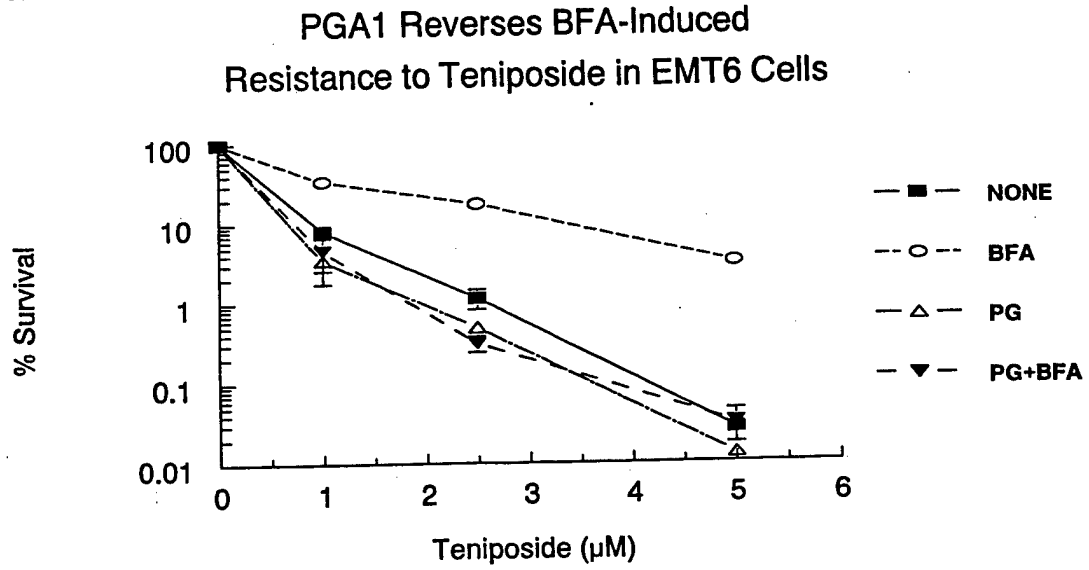


Figure 2. Effects of prostaglandin A_1 (PGA_1) on hypoxia-induced resistance to teniposide in EMT6 cells. Cells were exposed to PGA_1 (30μM) 30 minutes before the introduction of hypoxia for 8 hours. At the last hour of the experiment, teniposide was added. Percent survival was determined by comparing percent plating efficiency of BFA-treated cells with the percent plating efficiency of non-stressed cells. Each point represents the mean ± SEM of at least three independent experiments.

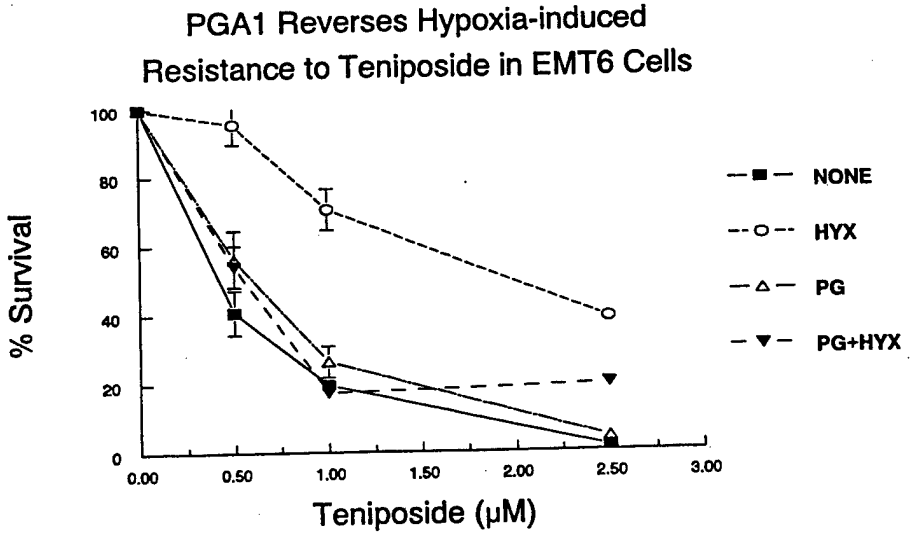


Figure 3A. Reaction mixtures of 2 units human topoisomerase II enzyme, 200ng kinetoplast DNA, 4μl assay buffer (0.5M Tris-Cl, pH 8, 1.2M KCl, 100mM $MgCl_2$, 5mM ATP, 5mM dithiothreitol, 300μg/ml bovine serum albumin) and either prostaglandin A_1 (PGA_1) or BFA were incubated for 1 hour at 37°C. Decatenation products were visualized on a 1% agarose gel containing 1μg/ml ethidium bromide in 0.5X TBE buffer. Gel bands were quantitated using a Stratagene Eagle Eye transilluminator. Percentage of DNA decatenated was determined by dividing fluorescence intensity of decatenated bands by total lane intensity. Data represent mean decatenation ± SEM from at least three independent experiments. * - significantly less $p < 0.05$ than control by Dunnett's test.

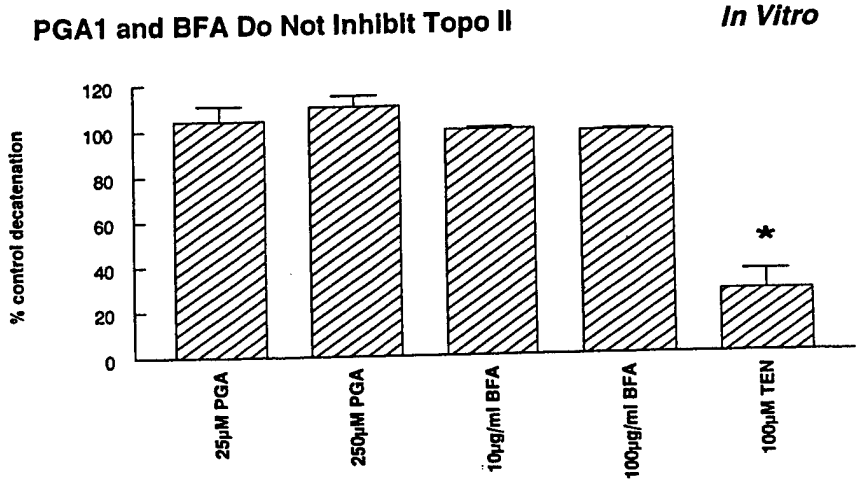


Figure 3B. The uptake of ^3H etoposide was measured in EMT6 exposed to stress-inducing conditions. During the final hour of stress-induction, ^3H etoposide (0.5μCi/ml, 5.5nM) and 0.1μCi ^{14}C inulin in 50μM etoposide was added to the cells. Following drug exposure, cells were washed, lysed, and analyzed by scintillation spectrometry. The thoroughness of the washes was monitored by the removal of ^{14}C inulin in the washes. Drug uptake was determined from the number of counts (^3H etoposide) incorporated in EMT6 cells and converted to picomoles of etoposide per 10^6 cells.

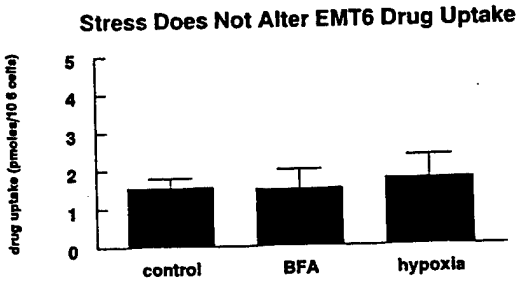


Figure 4. Effects of prostaglandin A_1 (PGA_1) on NF-κB transactivation. EMT6 cells were first transfected with a NF-κB-sensitive luciferase reporter gene and then pretreated with 30μM PGA_1 one-half hour prior to stress treatment. Cells were then treated with either 10μg/ml BFA for 4 hours, 60nM okadaic acid (OA), or hypoxia for 8 hours (time points of maximal NF-κB activation). Following drug treatments, cells were collected and assayed for luciferase expression by measuring light intensity in the presence of luciferase assay substrate. The light intensity from drug-treated cells was divided by non-treated or solvent-treated cells to determine relative fold changes in luciferase activity. Results are the average fold control luciferase activity ± SEM from at least three independent experiments.

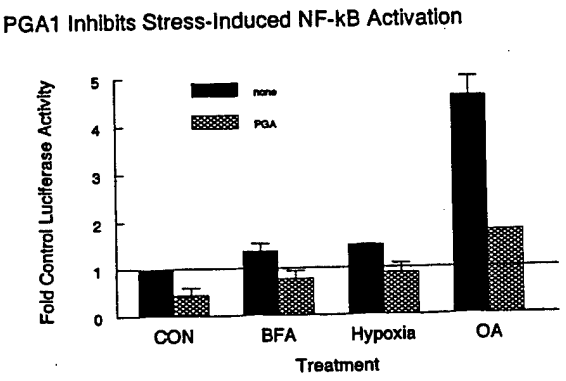


Figure 5. Prostaglandin A_1 (PGA_1) inhibits stress-induced NF-κB activation. EMT6 cells were treated with PGA_1 one-half hour prior to stress treatment. Cells were then treated with 10μg/ml brefeldin A (BFA) for 4 hours, 60nM okadaic acid (OA) for 8 hours, or 8 hours of hypoxia (time points of maximal NF-κB activation. Nuclear extracts of drug-treated cells were incubated with a ^{32}P-labeled probe of the κB consensus sequence and then analyzed by gel shift assay. Shown are representative gels from at least 3-4 independent experiments for each stress.

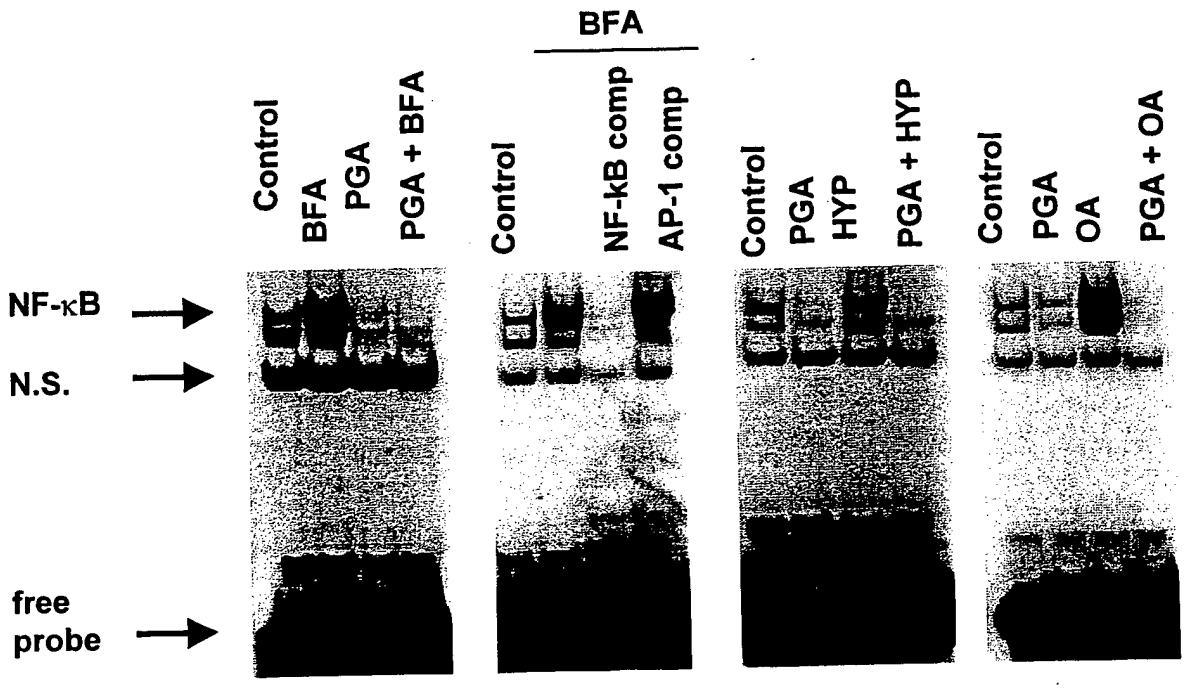


Figure 6. Effects of half-hour brefeldin A (BFA) treatment on drug resistance in EMT6 cells. Cells were exposed to 10μg/ml BFA for 30 minutes and then BFA-free medium for seven and a half hours of for 2 hours and then in BFA-free medium for 6 hours. During the last hour of the experiment, 2.5μM teniposide (T) was added. Percent survival was determined by comparing percent plating efficiency of BFA-treated cells with percent plating efficiency of non-stressed cells. Each point represents the mean ± SEM from at least three independent experiments. * - statistically different from alone, but not statistically different from 2 hour BFA treatment by Dunnett's test ($p<0.05$).

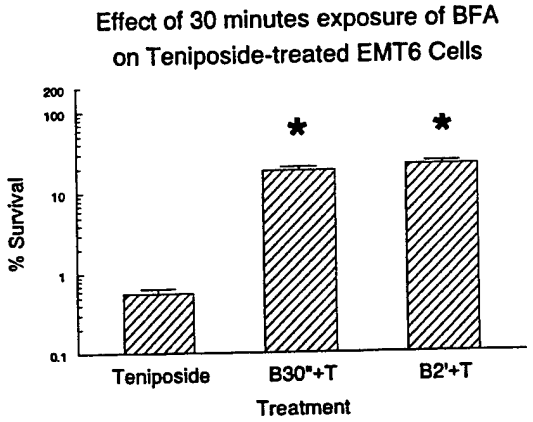
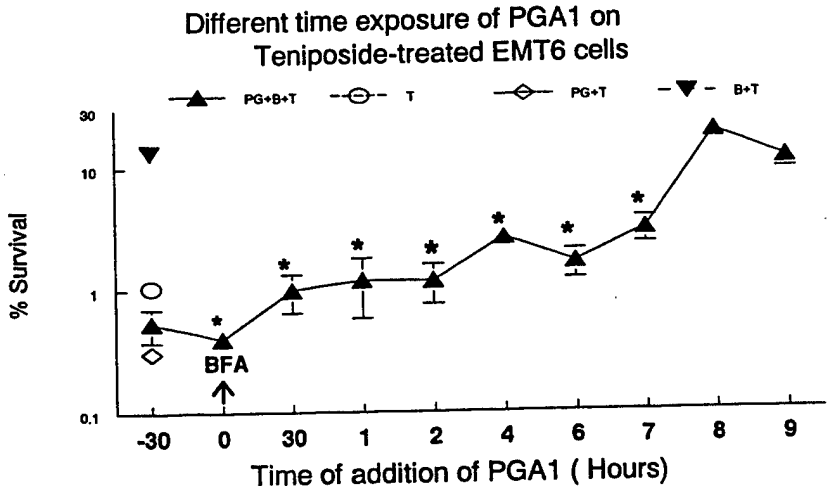


Figure 7. Time-dependence of prostaglandin A_1 (PGA_1) addition to reversal of brefeldin A (BFA)-induced resistance. Cells were exposed to 10μg/ml for 30 minutes (0 time) and then BFA-free medium for 7.5 hours. PGA_1 was added either 7 hours after BFA treatment [at the same time as teniposide (T)], 8 hours after BFA treatment (1 hour after T, immediately before colony assay), or 9 hours after BFA treatment (T) was removed and PGA_1 was added with the colony assay 1 hour after). Percent survival was determined by comparing percent plating efficiency of BFA-treated cells with the percent plating efficiency of non-stressed cells. Each point represents the mean ± SEM of three independent experiments. * - statistically significant ($p<0.05$) from BFA-treated cells, Dunnett's test.



Conclusions

PGA_1 inhibits BFA- and hypoxia-induced activation of NF-kB

PGA_1 reverses BFA- and hypoxia-induced resistance to teniposide in EMT6 cells. These data suggest that NF-kB activation is associated with stress-induced drug resistance.

Pharmacologic reversal of stress induced drug resistance does not need to precede the stress but can occur during or after has occurred. (Fig. 7) The data suggest that pharmacological inhibition of stress response pathways could enhance the therapeutic efficacy of cancer chemotherapeutics

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